

## The Ubiquitin-like Protein FAT10 Forms Covalent Conjugates and Induces Apoptosis\*

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**FAT10 is a ubiquitin-like protein that is encoded in the major histocompatibility complex class I locus and is synergistically inducible with interferon- $\gamma$  and tumor necrosis factor  $\alpha$ . The molecule consists of two ubiquitin-like domains in tandem arrangement and bears a conserved diglycine motif at its carboxyl terminus commonly used in ubiquitin-like proteins for isopeptide linkage to conjugated proteins. We investigated the function of FAT10 by expressing murine FAT10 in a hemagglutinin-tagged wild type form as well as a diglycine-deficient mutant form in mouse fibroblasts in a tetracycline-repressible manner. FAT10 expression did not affect major histocompatibility complex class I cell surface expression or antigen presentation. However, we found that wild type but not mutant FAT10 caused apoptosis within 24 h of induction in a caspase-dependent manner as indicated by annexin V cell surface staining and DNA fragmentation. Wild type FAT10, but not its diglycine mutant, was covalently conjugated to thus far unidentified proteins, indicating that specific FAT10 activating and conjugating enzymes must be operative in unstimulated fibroblasts. Because FAT10 expression causes apoptosis and is inducible with tumor necrosis factor  $\alpha$ , it may be functionally involved in the programmed cell death mediated by this cytokine.**

The covalent posttranslational modification of proteins is a versatile principle of determining the half-life, intracellular localization, and activity of proteins. In addition to modification by small molecules like orthophosphate, acetic acid, lipids, or sugars, the attachment of protein tags via isopeptide linkage to lysine residues in target proteins has recently been recognized as a frequently used and very diverse theme in cell biology. The prototype of such a protein tag is ubiquitin, which, when assembled as a polyubiquitin chain onto substrate proteins, can target these proteins to the 26S proteasome for degradation (1). Recently, it has become clear that the manner in which ubiquitin is linked in polyubiquitin chains defines the fate of the modified protein. The specific targeting signal for the proteasome pathway is a polyubiquitin chain that uses the K48 residue of the proximal ubiquitin as a target for further rounds of ubiquitination. The formation of K63-linked ubiquitin polymers, in contrast, does not lead to degradation but seems to

play a role in DNA repair (2) and endocytosis (3). The conjugation of receptors with a single ubiquitin moiety can also serve as a signal for endocytosis (4), whereas in the case of histone H2B, monoubiquitination appears to regulate nucleosome function and cell division (5).

The specificity of substrate selection and the mode of ubiquitin conjugation are determined by an enzymatic cascade required for the activation and specific transfer of ubiquitin. It consists of ubiquitin-activating enzyme (E1)<sup>1</sup>, which uses the energy of ATP to form a thioester linkage between a cysteine residue of the E1 enzyme and the carboxyl terminus of ubiquitin consisting of two conserved glycine residues. The activated ubiquitin is then transferred onto a ubiquitin-conjugating enzyme (ubiquitin carrier protein; E2), which, in turn, can pass it directly onto the substrate protein or first to ubiquitin-protein isopeptide ligase (E3), which then will catalyze the formation of the isopeptide linkage between ubiquitin and the specific substrate (6).

Over the past 5 years, the proteinacious tag theme has been extended by the discovery of numerous "ubiquitin-like proteins" (7, 8). As their family name implies, these proteins contain domains with homology to ubiquitin ranging from 15% to 60% identity on the amino acid level. They can be grouped in two classes: (a) the ubiquitin-domain proteins, which contain ubiquitin-like domains but are not conjugated to other proteins; and (b) the ubiquitin-like modifiers (UBLs), which can be covalently conjugated to target proteins by specific enzymatic cascades similar to those used for ubiquitin conjugation (8, 9). A conserved feature of ubiquitin and UBLs that is required for conjugate formation is the diglycine motif situated either directly at the carboxyl terminus of the UBL or within the sequence of a UBL precursor, thus requiring carboxyl-terminal limited proteolysis for the liberation of the diglycine motif. A ubiquitin-like protein that, based on its primary structure, would qualify as a member of the UBL family is the FAT10 protein (10) (formerly called diubiquitin (11–13)). FAT10 is an 18-kDa protein consisting of two ubiquitin-like domains combined by a short linker. The amino-terminal domain of FAT10 is 29% identical to ubiquitin, whereas the carboxyl-terminal domain displays a 36% identity to ubiquitin. Lysine residues corresponding to position 29, 48, and 63 of ubiquitin that could potentially serve as sites for conjugation are conserved in both domains and the carboxyl-terminal domain ends with a free diglycine motif, which is free for isopeptide linkage to target proteins. However, no experimental evidence for the formation

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<sup>1</sup> The abbreviations used are: E1, ubiquitin-activating enzyme; IFN, interferon; LCMV, lymphocytic choriomeningitis virus; MHC, major histocompatibility complex; MCMV, mouse cytomegalovirus; tet, tetracycline; NEPHGE, non-equilibrium pH gradient gel electrophoresis; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; UBL, ubiquitin-like modifier; wt, wild type; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; HA, hemagglutinin; UBL, ubiquitin-like modifier; PCR, polymerase chain reaction; mAb, monoclonal antibody; TNF-R, tumor necrosis factor receptor.

of covalent conjugates of FAT10 has been obtained to date (10).

Thus far, very little is known about the function of FAT10, although its expression pattern and genomic localization may provide some hints with regard to the pathways in which it may be functionally involved. The FAT10 gene was originally discovered by genomic sequencing and found to be localized in the MHC class I locus of the human and the mouse (11). Initially, expression of FAT10 was found to be restricted to mature B cells and dendritic cells, suggesting that FAT10 may play a role in antigen presentation in professional antigen-presenting cells (12). Subsequently, it was found that FAT10 could be detected in a wide range of human cell lines when the cells were treated with the cytokines interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$ , but not with IFN- $\alpha$  (10, 13). Interestingly, IFN- $\gamma$  and TNF- $\alpha$  displayed a strong synergism in their ability to induce FAT10 expression, possibly indicating that FAT10 may play a role in the signal transduction or effector functions of these two pro-inflammatory cytokines. In a two-hybrid screen, Liu *et al.* (10) found an interaction of FAT10 with spindle assembly checkpoint protein MAD2. The interaction of FAT10 and MAD2, which appears to be noncovalent, could be confirmed in glutathione *S*-transferase pull-down assays and immunoprecipitation experiments, but a functional implication of FAT10 in anaphase arrest has not been reported to date. Thus far, there are no cell lines or mice available that lack or overexpress mutant or wild type forms of FAT10.

We previously tried to overexpress the human FAT10 protein in stable transfectants of HeLa cells; however, of 15 clones that had integrated the FAT10 expression construct into their genomic DNA, only 1 clone expressed FAT10 mRNA at a level sufficient for detection in Northern analysis. Remarkably, this clone suffered from continuous cell death and poor proliferation (13). The viability only improved when cells emerged in culture that had lost FAT10 expression. This observation suggested that stable overexpression of FAT10 was incompatible with the survival of cells. Therefore, we decided to express a wild type form of murine FAT10 as well as a mutant form that lacked the carboxyl-terminal diglycine motif in a tetracycline (tet)-repressible manner in mouse fibroblasts. Overexpression of FAT10 but not the carboxyl-terminal mutant induced apoptosis in transfectants within 24 h after removal of tet. The induction of FAT10 expression resulted in the appearance of a monomeric FAT10 protein as well as several proteins of higher molecular weight that were not formed in the  $\Delta$ GG mutant, strongly suggesting that FAT10 can become covalently conjugated to thus far unidentified target proteins. Therefore, FAT10 is a functional member of the family of ubiquitin-like modifiers.

#### EXPERIMENTAL PROCEDURES

**DNA Constructs**—The expression vectors pTET-tTA (which contains the tet-regulated tTA transcription activator) and pTet-Splice have been described previously (14). An influenza virus hemagglutinin (HA) tag was cloned in frame at the 5'-end of murine FAT10 cDNA. This construct was cloned into the pTet-Splice plasmid via *Hind*III and *Spe*I restriction sites using standard methods. pLXSP and pLXSH are plasmids containing puromycin and hygromycin resistance genes, respectively. The  $\Delta$ GG mutant of FAT10 was generated with site-directed mutagenesis using PCR with the 3'-oligonucleotide 5'-GCCCTCTAGACTCAGGCCGAGTGCAGTGTGTT-3', in which the nucleotides encoding for two glycine residues are replaced by the amino acids AACV. The 5'-oligonucleotide was the same as that used for wild type cDNA and is described later. The sequences were verified via dideoxy sequencing.

**Cell Lines and Transfectants**—B8-E11, a subclone from the B8 murine fibroblast cell line expressing murine cytomegalovirus (MCMV) IE1 protein pp89 (15), was transfected with pTet-tTak and pLXSH constructs by the standard calcium phosphate precipitation method. Clones were selected with hygromycin B (400  $\mu$ g/ml) in medium containing 1  $\mu$ g/ml tetracycline. The presence of tTak was confirmed by genomic PCR analysis using the oligonucleotides 5'-ATGCTAGATTA-

GATAAAAGTAAAG-3' and 5'-CTACCCACCGTACTCGTCAA-3', which were specific for the coding sequence of the transactivator gene. The tet-dependent repression of tTak expression was verified by transient transfection of these cells with a luciferase reporter gene, which was cloned into the pTet-Splice vector (14). B8tTA.F4, a clone that showed high tTak expression in the absence of tet but very low tTak expression in the presence of tet, was selected as a recipient for further experiments. B8tTA.F4 cells were transfected with either pTet-Splice/HA-FAT10 (wt) and pLXSP or pTet-Splice/HA-FAT10- $\Delta$ GG (mutant) and pLXSP as described above. Puromycin-resistant clones were isolated and cultured in Iscove's modified Dulbecco's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 100 units/ml penicillin/streptomycin, 400  $\mu$ g/ml hygromycin (Calbiochem), 5  $\mu$ g/ml puromycin (Calbiochem), and 1  $\mu$ g/ml tetracycline (Sigma). The integration of the HA-FAT10-wt and HA-FAT10- $\Delta$ GG cDNAs into the genome was confirmed by genomic PCR analysis using the described oligonucleotides. The expression of the HA-FAT10 proteins was induced by removing tetracycline from the medium with at least three washes with phosphate-buffered saline and examined by immunoblot analysis. One clone expressing wild type HA-FAT10 (TB1N) and one clone expressing HA-FAT10- $\Delta$ GG (mTB14) were chosen for most of the experiments because they showed a high tet-dependent expression of either wt HA-FAT10 or HA-FAT10- $\Delta$ GG.

**Antibodies**—The following antibodies were used: anti-HA (HA probe F-7, sc-7392; Santa Cruz Biotechnology), anti-ubiquitin (DAKO), anti-human actin (carboxyl-terminal peptide; Santa Cruz Biotechnology), goat anti-mouse IgG POD (DAKO), anti-CD95 (Immunotech), anti-CD29 (clone HM $\beta$ 1-1; PharMingen), goat anti-mouse IgG secondary antibody (Alexa 546; Molecular Probes), anti-H2-L<sup>d</sup> (mAb clone 28-14-81), anti-H2-K<sup>d</sup> (mAb clone 15-5-5S), anti-H2-D<sup>d</sup> (mAb clone 19/191), and anti-LCMV glycoprotein (mAb clone KL25).

**Electrophoresis and Western Blot Analysis**—For one-dimensional Laemmli SDS-PAGE, total cell lysates were prepared using the following lysis buffer: 20 mM Tris-HCl, pH 7.8, 1 mM EDTA, 0.01% SDS, and 0.85 mM phenylmethylsulfonyl fluoride. The solubilized cell pellet was freeze-thawed four times and centrifuged for 15 min at 14,000  $\times$  *g*. The protein concentration of the supernatant was determined by absorption at 280 nm, assuming that 1 OD = 1 mg/ml. 50  $\mu$ g of total protein was loaded per lane. For non-equilibrium pH gradient gel electrophoresis (NEPHGE)/SDS-PAGE, about 3  $\times$  10<sup>6</sup> cells were lysed in 100  $\mu$ l of NEPHGE sample buffer, incubated for 3 h at room temperature under agitation, and centrifuged for 15 min at 14,000  $\times$  *g*. The supernatant was applied to gels, and NEPHGE/SDS-PAGE was performed as described previously (16). Subsequently, Western blotting was performed as described elsewhere (17).

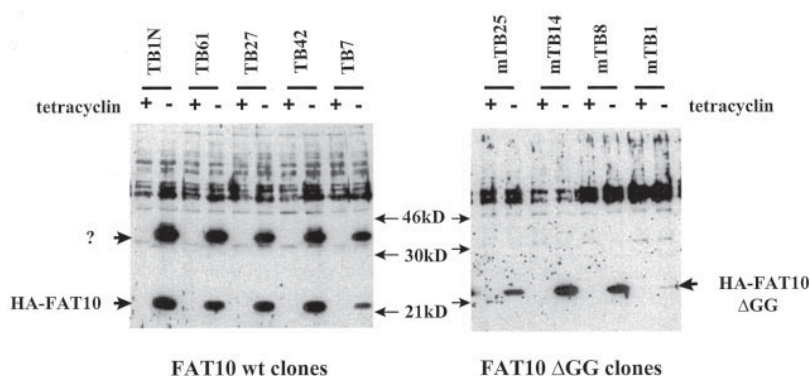
**Immunofluorescence Microscopy**—A wild type HA-FAT10 transfectant (TB1N) and a diglycine mutant (mTB14) were prepared for microscopy in the presence and absence of tetracycline. 24 h after induction, cells were fixed in freshly prepared 4% paraformaldehyde for 10 min and permeabilized in PB buffer (phosphate-buffered saline with 2% fetal calf serum, 5 mM EDTA, 1% saponin, and 0.1% NaN<sub>3</sub>) for 30 min. Incubations with primary antibody (anti-HA diluted 1:100 in PB buffer) and secondary antibody (Alexa 546 diluted 1:1000 in PB buffer) were each for 1 h at room temperature. Cells were washed twice after each incubation with PB buffer. At the end, cells were washed with MACS buffer (PB buffer without saponin) and embedded in Mowiol (Sigma). To test the specificity of the anti-HA antibody, this antibody was omitted in control stainings.

**T-cell Hybridoma Assays**—For quantification of H-2L<sup>d</sup>-restricted presentation of the T-cell epitopes MCMV pp89/168–176 and LCMV NP118, specific T-cell hybridomas that express a  $\beta$ -galactosidase reporter gene under the control of the interleukin 2 promoter were used. T-cell activation can therefore be monitored using a colorimetric lacZ assay as described previously (17). For determining pp89 presentation, nonviable cells were washed off the plate, and adherent TB1N cells were collected with calcium/magnesium-free medium 0, 24, and 48 h after the induction of FAT10 expression and used directly as antigen-presenting cells in the assay. To measure NP118 presentation, we infected TB1N cells with 0.01 plaque-forming unit of LCMV strain WE for 1 day, and then FAT10 expression was induced for 0, 24, or 48 h. Induced and uninduced cells were used in parallel as antigen-presenting cells in the hybridoma assay. No significant differences were recorded between induced and uninduced cells in two independent experiments for either the MCMV pp89 epitope or the LCMV NP118 epitope.

**Annexin V Staining and Flow Cytometry**—The expression of FAT10 was induced in TB1N and mTB14 cells. Uninduced and induced cells were harvested with calcium/magnesium-free medium at the indicated time points, washed once with phosphate-buffered saline, and stained



**FIG. 1. Western blot analysis of tetracycline-regulated HA-FAT10 protein expression in several wt and  $\Delta$ GG mutant transfectants.** Total cell lysate of uninduced (+) and induced (-) cells was loaded on 12% SDS-PAGE and analyzed by Western blotting using anti-HA antibody. The 35-kDa band labeled with a question mark in the FAT10-wt clones is not detected in the FAT10- $\Delta$ GG clones and may therefore represent a FAT10 conjugate.



for annexin V using the ApoAlert Annexin V Apoptosis Kit (CLONTECH). Jurkat cells incubated with anti-CD95 antibody (7C11; Immunotech) at a concentration of 100 ng/ml for 6 h were used as positive control. For determination of MHC class I cell surface expression, TB1N cells were stained 0, 24, and 48 h after the removal of tet from the growth medium with mAbs anti-H2-L<sup>d</sup>, anti-H2-K<sup>d</sup>, anti-H2-D<sup>d</sup>, and anti-CD29 (as control) and subsequently stained with goat anti-mouse Ig-Fab-fluorescein isothiocyanate secondary antibody (Silenus, Victoria, Australia). For cell cycle analysis, nuclei were prepared from TB1N cells 0, 24, and 48 h after the induction of FAT10 expression, and nuclei were stained with propidium iodide exactly as described previously (18). Fluorescence of cells or nuclei was analyzed using a FACScan flow cytometer and LYSIS II software (Becton Dickinson).

**DNA Fragmentation Assay**—TB1N and mTB14N clones were induced to express FAT10 for 24, 48, and 72 h. To determine caspase sensitivity, cells were cultivated without tet for 24 h in the presence or absence of 25  $\mu$ M of the caspase inhibitor Z-VAD-fmk (Bachem, Bubendorf, Switzerland). A pellet of  $10^7$  cells was resuspended in 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM EDTA, 1% SDS, and 100 mg/ml proteinase K) and digested for 2 h at 37 °C. After standard phenol/chloroform extraction, nucleic acids were precipitated in 70% ethanol/500 mM NaCl, washed in 70% ethanol, and resuspended in Tris-EDTA buffer containing 100 mg/ml RNase A. After incubation for 1 h at 37 °C, about 5  $\mu$ g of DNA was analyzed for fragmentation on a 1.5% agarose/ethidium bromide gel. As a positive control, we used Jurkat cells incubated for 6 h with anti-CD95 antibody as described above in the presence or absence of 25  $\mu$ M Z-VAD-fmk.

**Real-time PCR**—TB1N cells were induced to express FAT10. The uninduced and induced cells were harvested after 24, 48, and 72 h, and total RNA was isolated according to the acid guanidinium thiocyanate-phenol-chloroform procedure (19). Reverse transcription of 5  $\mu$ g of total RNA was performed at 42 °C for 1 h using Moloney murine leukemia virus reverse transcriptase (Promega) and oligo(dT)<sub>15</sub> (Promega). The synthesized cDNA was precipitated with ethanol and washed with 70% ethanol, and the cDNA was resuspended in 20  $\mu$ l of H<sub>2</sub>O. Real-time reverse transcription-PCR was performed with the Light Cycler (Roche) using DNA Master SYBR Green I (Roche) according to the protocols provided by the manufacturer. Sense and antisense primers used for PCR amplification were as follows: 5'-GATAAGGTACCTATGGCTTC-TGTCCGCACCTGTG-3' and 5'-GCCCTCTAGACTCATCCCCAGTG-CAGTGTGTTG-3' (used for FAT10 cDNA at an annealing temperature of 57 °C) and 5'-TCCTGACCCTGAAGTACC-3' and 5'-TTGCCAATG-TGATGACC-3' (used for mouse  $\beta$ -actin cDNA at an annealing temperature of 60 °C). The amount of cDNA was normalized using  $\beta$ -actin as the standard. Different dilutions of TB1N cDNA with the highest expression of FAT10 were prepared and used to obtain a calibration curve. The coefficient after linear regression was  $r = 1$ .

**Viability Assay**—Cells were collected 0, 24, and 48 h after the induction of FAT10 expression and stained with 1% trypan blue in phosphate-buffered saline. Viable cells that excluded the dye and blue cells that were scored as dead cells were counted in a Neubauer chamber.

## RESULTS

**FAT10 in a Wild Type but not in a  $\Delta$ GG Mutant Form Is Covalently Conjugated to Unidentified Target Proteins**—The previous failure to stably overexpress human FAT10 in HeLa cells suggested that ectopic FAT10 expression may adversely affect cellular growth or survival (13, 10). Therefore, we decided to express the FAT10 mRNA in a cell line under the

control of a tet-repressible promoter, which would allow the generation and expansion of transfectants in the absence of FAT10 expression and enable us to study the functional consequences of inducing FAT10 expression simply by removing tet from the growth medium. The localization of the FAT10 gene in the MHC class I locus and the finding that FAT10 expression was synergistically inducible with IFN- $\gamma$  and TNF- $\alpha$  suggested that FAT10 may play a role in MHC class I-restricted antigen presentation. Because we have established murine T-cell hybridomas that allow us to monitor the MHC class I-restricted presentation of several viral antigens by murine cell lines (20), we decided to examine the consequence of FAT10 expression for antigen presentation in the murine system. The mouse fibroblast cell line B8 (15) was first stably transfected with the expression construct pTet-tTak, which encodes a tet-responsive transcriptional inducer (14). The transfectants were tested by transient transfection of tet-regulated  $\beta$ -galactosidase or luciferase reporter constructs, and a clone designated B8tTA.F4 was selected that showed a strong expression of  $\beta$ -galactosidase and luciferase in the absence of tet but very little expression of  $\beta$ -galactosidase and luciferase in the presence of tet (data not shown). Next, we performed two stable transfections of B8tTA.F4 with two different tet-responsive FAT10 expression constructs. The first construct encoded the full-length murine FAT10 cDNA fused to an HA tag at the amino terminus. The second construct encoded an HA-tagged mutant form of murine FAT10 (FAT10- $\Delta$ GG), in which the carboxyl-terminal diglycine motif was replaced by the amino acids AACV. During transfection of B8tTA.F4 cells, 76 and 36 clones were scored positive by genomic PCR for the wt and mutant FAT10, respectively. Western analysis of all clones with  $\alpha$ HA antibodies revealed FAT10 expression in the absence of tet but not in the presence of tet in about half of the clones, of which a few representative clones are shown in Fig. 1. All clones of the wt and  $\Delta$ GG series showed an HA-reactive band at about 21 kDa, corresponding to the expected molecular mass of the HA-FAT10 proteins. Interestingly, a very prominent band of about 35 kDa appeared in all FAT10 wt clones in the absence of tet but not in the presence of tet. None of the  $\Delta$ GG clones showed this band, suggesting that the appearance of this band apparently depended on the two carboxyl-terminal glycine residues of FAT10. Because this protein resisted boiling in the presence of mercaptoethanol and SDS, we conclude that the 35-kDa band is not due to thioester linkage of FAT10 to putative E1 or E2, but that it most likely represents an isopeptide-linked conjugate between the FAT10 protein and a thus far unidentified target protein. In parallel to the stable transfection of the B8tTA.F4 clone, we had transfected another mouse fibroblast line named Mec29, which also expressed the tet-responsive transcriptional inducer (21) with the same FAT10 expression constructs. The result obtained was the same as

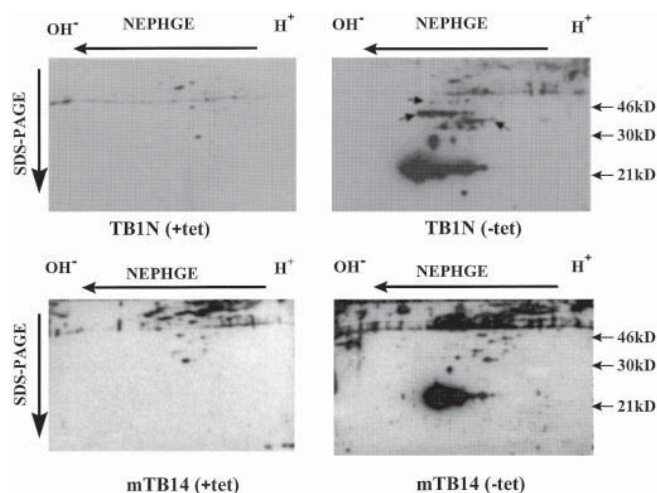


FIG. 2. Anti-HA Western blot of NEPHGE/SDS-PAGE for the analysis of HA-FAT10 protein expression in a wild type transfectant (TB1N) compared with a  $\Delta$ GG transfectant (mTB14). The right panels (-tet) show the analysis of cells expressing the respective FAT10 proteins, whereas the left panels (+tet) show the analysis of cells lacking FAT10 expression. The wt and  $\Delta$ GG form of FAT10 appear at 22 kDa in multiple differently charged forms that are separated by NEPHGE. The arrows in the top right panel indicate putative conjugates of FAT10.

that obtained in the B8tTA.F4 transfectants, namely, the appearance of FAT10 as well as an additional 35-kDa band in the wt transfectants but not in the  $\Delta$ GG transfectants (data not shown).

We wondered whether the 35-kDa band could be a dimer of FAT10, and we decided to investigate this possibility with help of  $\alpha$ HA Western analysis of two-dimensional NEPHGE/SDS-PAGE, with the prediction that a FAT10 dimer should migrate with the same charge in the first dimension as the monomer. For further analysis, we selected the wt FAT10 clone TB1N and the  $\Delta$ GG mutant clone mTB14, which were representative in the expression of the respective forms of FAT10 proteins. Due to the better resolution of the two-dimensional gels shown in Fig. 2, it became apparent that the fairly broad HA-reactive band at around 35 kDa was in fact composed from a number of different proteins (Fig. 2, top right panel) that appeared in the wt FAT10 transfectant after the induction of FAT10 expression (-tet) but did not appear in the  $\Delta$ GG mutant or in the absence of FAT10 expression (+tet). Interestingly, some of these putative FAT10 conjugates migrated as a series of differently charged spots, as was observed for the FAT10 monomer itself. The appearance and distance of the differently charged FAT10 spots would be consistent with a covalent modification of FAT10 by phosphorylation, but we have not yet addressed this issue experimentally. The molecular weight and charge of the putative conjugates would be compatible with one of them being a FAT10 dimer. However, none of them (labeled with arrows in Fig. 2) occurred in the  $\Delta$ GG mutant, even after overexposure. The latter finding would argue against FAT10 dimer formation because the transfectants endogenously express murine FAT10 (data not shown), which should be available for isopeptide linkage to the ectopically expressed FAT10. Nevertheless, at present, we cannot rule out dimer formation, and identification of the conjugated proteins will be required to resolve this issue.

**FAT10 Immunofluorescence Is More Prominent in the Cytoplasm than in the Nucleus**—Covalent conjugation of the well-characterized ubiquitin-like modifier SUMO-1 to the PML protein has recently been found to cause a localization of the PML-SUMO-1 conjugate in the so-called PML bodies in the

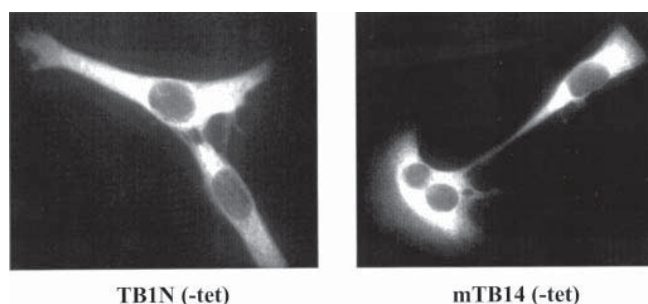


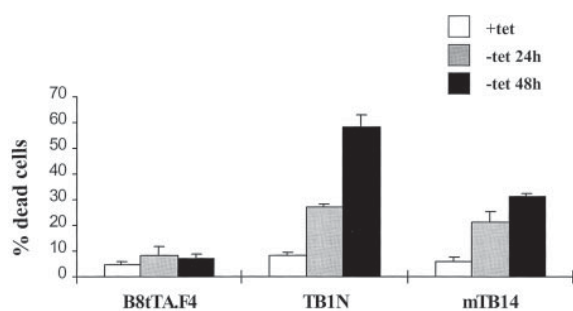
FIG. 3. Intracellular localization of wt and  $\Delta$ GG forms of HA-FAT10 as determined by HA immunofluorescence microscopy. TB1N and mTB14 cells were cultivated in the absence of tet to induce the expression wt HA-FAT10 and the  $\Delta$ GG mutant HA-FAT10, respectively. The same cells grown in the presence of tet did not show any fluorescence (data not shown), indicating that the signal is specific for HA-FAT10.

nucleus (22). This finding was an incentive for us to examine using fluorescence microscopy where FAT10 is localized in mouse fibroblast transfectants and whether the formation of FAT10 conjugates may induce a new localization of FAT10. As shown in Fig. 3, staining of the FAT10 wt transfectant TB1N with  $\alpha$ HA antibodies indicated that the HA-tagged FAT10 protein was predominantly localized in the cytoplasm and only weakly stained the nucleus. No evidence for a preferential staining of cytoplasmic or nuclear substructures could be obtained. The intracellular localization of the  $\Delta$ GG mutant of FAT10 in the mTB14 clone was very similar to the intracellular distribution of wt FAT10 in the TB1N clone, indicating that the putative formation of FAT10 conjugates does not result in a major intracellular relocation of FAT10. Apparently, the carboxyl-terminal glycine residues do not influence the intracellular localization of FAT10.

**The Induction of FAT10 Leads to Cell Death and the Down-regulation of FAT10 Expression**—A major goal for the generation of tet-regulated FAT10 transfectants was to test whether ectopic FAT10 expression would have a negative effect on cellular survival and proliferation, as suggested from our failure to generate stable human FAT10 transfectants of HeLa cells. Intriguingly, this notion was unambiguously confirmed in the tet-regulated FAT10 transfectants. As shown in the trypan blue staining assay presented in Fig. 4, cultivation of the FAT10 wt transfectant TB1N in the absence of tet, which results in the induction of FAT10 biosynthesis, caused extensive cell death of more than 50% of the TB1N cells and all other tested FAT10 wt transfectants within 2 days. In contrast, the viability of B8tTA.F4 recipient cells expressing the tetracycline-responsive transcriptional inducer alone or of B8tTA.F4 transfectants expressing  $\beta$ -galactosidase in a tet-regulated manner was not affected by the withdrawal of tet. A certain decrease in viability was observed in some of the FAT10- $\Delta$ GG transfectants, as shown for the mTB14 clone in Fig. 4, but cell death in the mutant clones was 2-fold lower or less when compared with the wt FAT10 transfectants. A very similar result was obtained in thymidine incorporation assays, where the proliferation arrest after tet removal was always much more prominent in wt FAT10 transfectants than in FAT10- $\Delta$ GG-expressing cells but was not apparent in the B8tTA.F4 transfectants (data not shown). Because the expression levels of wt FAT10 in TB1N cells and FAT10- $\Delta$ GG in mTB14 cells were comparable, these results suggest that the ability of FAT10 to form conjugates may be responsible at least in part for the induction of cell death but that a certain level of toxicity was also induced by the expression of the diglycine-deficient FAT10.

Although most of the clonal TB1N cells died upon the induc-





**FIG. 4. The induction of FAT10 expression leads to cell death.** Trypan blue exclusion was determined for B8tTA.F4 recipient cells expressing the tetracycline-regulated transcriptional inducer alone, clone TB1N expressing FAT10-wt protein, and clone mTB14 expressing the FAT10- $\Delta$ GG mutant in a tetracycline-repressible manner. The assay was performed 0, 24, and 48 h after the removal of tet. The data represent the means and S.E. of three independent experiments. Clones TB1N and mTB14 were representative for a whole series of transfectants.

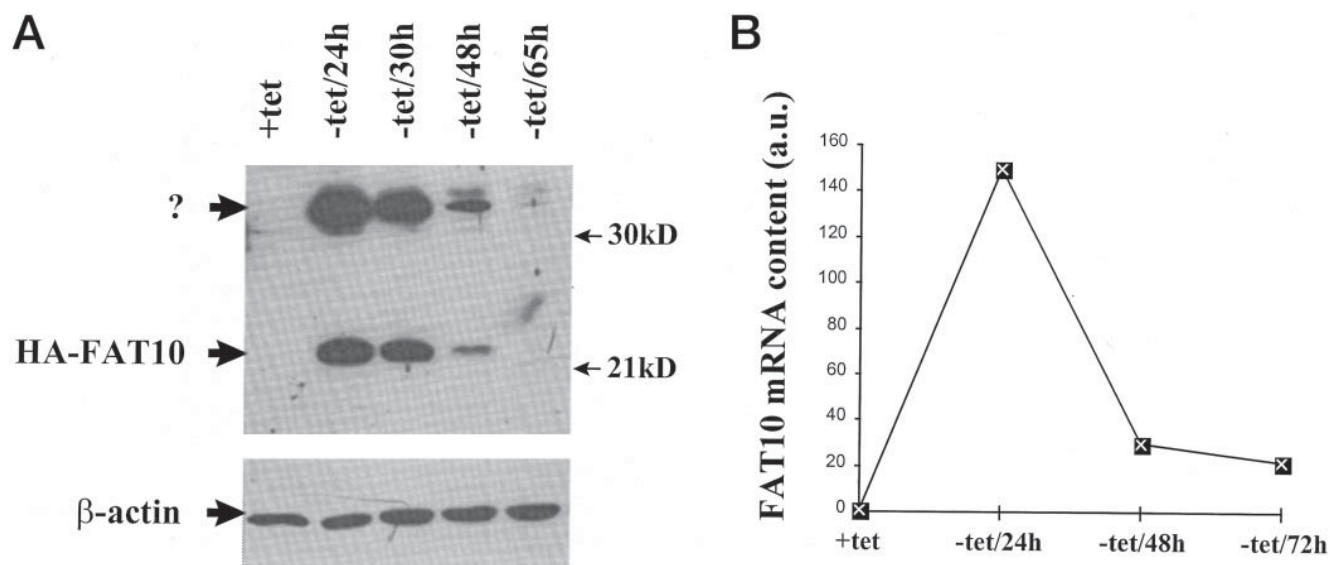
tion of FAT10 expression, 10–30% of the cells survived and continued to grow normally in the absence of tet. One reason that could account for their survival would be the down-regulation of FAT10 expression in these clones. We decided to test this possibility by following the expression levels of the FAT10 protein in  $\alpha$ HA Western blots for several days after the removal of tet from the growth medium of TB1N cells (Fig. 5A). Clearly, the FAT10 expression (which was maximal 24 h after tet removal) declined rapidly until 65 h after induction, when the levels of FAT10 and its putative conjugates were virtually undetectable. The loss of FAT10 protein was specific because the same amount of total cellular proteins was loaded for each time point, as evidenced by probing the Western blot with a  $\beta$ -actin-specific antibody. Furthermore, no down-regulation of  $\beta$ -galactosidase or PA28 $\gamma$  was apparent when tet-regulated transfectants expressing these proteins were grown without tet for several days (data not shown). To test whether the loss of FAT10 protein was due to a loss of FAT10 mRNA, we performed a real-time reverse transcription-PCR analysis with the same cells that had been used for the Western analysis. For this analysis, the amount of cDNA in the different samples was normalized to  $\beta$ -actin expression, and the FAT10 cDNA content in the different samples was quantified in the linear range of detection. From this quantitative analysis (Fig. 5B), it is obvious that the loss of FAT10 protein is paralleled by a rapid loss of FAT10 mRNA, despite the continued absence of tet. Based on the FAT10 expression level in the presence of tet, the quantity of FAT10 mRNA was 150-fold higher on day 1 after the removal of tet but declined by a factor of 7 on day 2 after tet withdrawal. We suspected that the loss of FAT10 mRNA and protein was due to a selective death of all cells that expressed FAT10 and that the surviving cells had lost the gene from their DNA despite maintaining drug resistance to puromycin, which was used as a selection marker during cotransfection and remained in the growth medium during the generation, selection, and cultivation of transfectants. To our surprise, a real-time PCR quantification of the transfected FAT10 expression construct in the genomic DNA of the same TB1N cells used previously for Western (Fig. 5A) and real-time reverse transcription-PCR (Fig. 5B) analysis indicated that the quantity of the FAT10 expression construct did not change significantly after the removal of tet and outgrowth of cells that had lost FAT10 mRNA and protein expression (data not shown). A stringent interpretation of these experiments would be that the surviving TB1N cells had managed to down-regulate FAT10 mRNA expression despite the presence of the FAT10 expression construct in their genomic DNA. The mechanism of FAT10 mRNA down-regula-

tion is rather elusive and requires further investigation.

**FAT10 Is Rapidly Degraded by the Proteasome but Does Not Interfere with Ubiquitin Conjugate Formation**—The dramatic decline of the FAT10 protein level within the time interval of 30 to 48 h after the induction of FAT10 expression suggested that FAT10 may be a short-lived protein. Because the most short-lived proteins are selectively degraded by the ubiquitin-proteasome pathway, we decided to investigate whether the degradation of FAT10 in our transfectants was mediated by the proteasome. Both the FAT10 wt-expressing TB1N cells and the FAT10- $\Delta$ GG-expressing mTB14 cells were induced by the removal of tet, and the expression level of FAT10 was monitored 24 and 28 h later on HA Western blots. A further aliquot of the cells was treated with 50  $\mu$ M of the proteasome inhibitor lactacystin 24 h after tet removal, and the cells were harvested after an additional 4 h of cultivation in the presence of lactacystin. As shown on the  $\alpha$ HA Western blot in Fig. 6A, the treatment of TB1N cells with lactacystin led to a strong accumulation of FAT10 protein and its putative conjugates within only 4 h of lactacystin treatment. The  $\Delta$ GG mutant FAT10 protein also accumulated dramatically within 4 h in the presence of lactacystin, indicating that the degradation signal in FAT10 is not dependent on the two carboxyl-terminal glycine residues. These experiments strongly suggest that FAT10 is degraded by the proteasome and that it is a short-lived and highly regulated protein with an estimated half-life of less than 2 h.

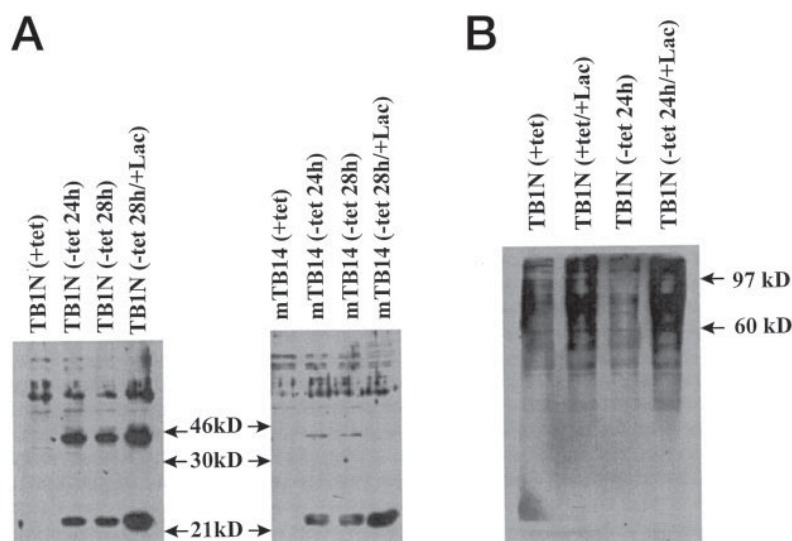
Given that FAT10 overexpression was quite strong in induced TB1N cells and that FAT10 induction resulted in sudden cell death, we were concerned that under these conditions, the ubiquitin homologue FAT10 might interfere with the ubiquitin pathway itself, which could be a cause for the observed cell death. Therefore, we monitored the effect of a 4-h treatment with 50  $\mu$ M lactacystin on the formation of polyubiquitin conjugates in TB1N cells in both the presence and absence of tetracycline. However, as shown in the ubiquitin Western blot in Fig. 6B, we found no evidence for an increase or a decrease of polyubiquitinated conjugates as a consequence of FAT10 induction, indicating that ectopic FAT10 expression did not have an impact on ubiquitin conjugation or deconjugation.

**FAT10 Induction Does Not Affect Cell Surface Expression of MHC Class I Molecules or MHC Class I-restricted Antigen Presentation**—The localization of the FAT10 gene in the MHC class I locus and the synergistic induction with TNF- $\alpha$  and IFN- $\gamma$  nourished expectations that FAT10 might be involved in the MHC class I presentation pathway. We examined the expression of the MHC class I molecules H-2L<sup>d</sup>, H-2D<sup>d</sup>, and H-2K<sup>d</sup> on the cell surface of TB1N cells by cell surface staining and flow cytometric analysis in the presence or absence of tet. No significant difference could be observed in the cell surface expression of these three class I molecules between viable TB1N cells cultivated in the presence or absence of tet for 24 and 48 h (data not shown). Next, we used T-cell hybridomas (17) that were specific for the immunodominant H-2L<sup>d</sup>-restricted T-cell epitope of the MCMV immediate early gene product pp89 to examine the presentation of this epitope in TB1N cells in the absence of FAT10 or 24 h after the removal of tet, when the FAT10 expression was maximal. The activation of the T-cell hybridoma was measured in a lacZ assay as described previously (17). This analysis was facilitated by the constitutive expression of low levels of pp89 in TB1N cells, which was due to expression of the pp89 gene in the B8 cells (15) from which the TB1N cells had been derived. In these experiments, no difference was found in the presentation of the pp89 epitope between TB1N cells cultivated in the presence or absence of tet (data not shown). To test a second T-cell epitope, we infected TB1N cells with LCMV strain WE in the presence



**FIG. 5. The expression of FAT10 protein and mRNA is down-regulated after induction.** A, anti-HA Western analysis of FAT10 expression in the tetracycline-regulated HA-FAT10 transfectant TB1N before and several days after removal of tetracycline. The positions of HA-tagged FAT10 and a putative FAT10 conjugate (?) are indicated. For loading control, the same Western blot was probed with a  $\beta$ -actin antibody. B, real-time reverse transcription-PCR analysis of HA-FAT10 mRNA content in TB1N cells before and several days after the removal of tet. The analysis was normalized to  $\beta$ -actin expression levels, and the data points were in the linear range of detection; data are presented as relative quantities (absorbance units) of FAT10 mRNA related to the quantity in the + tet sample, which was set to 1.

**FIG. 6. FAT10 is rapidly degraded by the proteasome, but FAT10 expression does not affect the level of polyubiquitin conjugates.** A,  $\alpha$ HA Western blot of TB1N and mTB14 cells expressing wt HA-FAT10 and  $\Delta$ GG mutant HA-FAT10, respectively, in a tet-repressible manner. Cells were harvested 0, 24, and 28 h after induction; for the *right lane*, 50  $\mu$ M of the proteasome inhibitor lactacystin (*Lac*) was added 24 h after tet removal, and cells were incubated for an additional 4 h. B, ubiquitin Western blot of TB1N cells grown in the presence or absence of tet for 24 h and in the absence or presence of 50  $\mu$ M lactacystin for 4 h, as indicated above the lanes. The high molecular mass portion of the blot is shown where polubiquitinated proteins migrate. Note that lactacystin treatment leads to an accumulation of ubiquitin conjugates irrespective of whether FAT10 is expressed (-tet) or not (+tet).



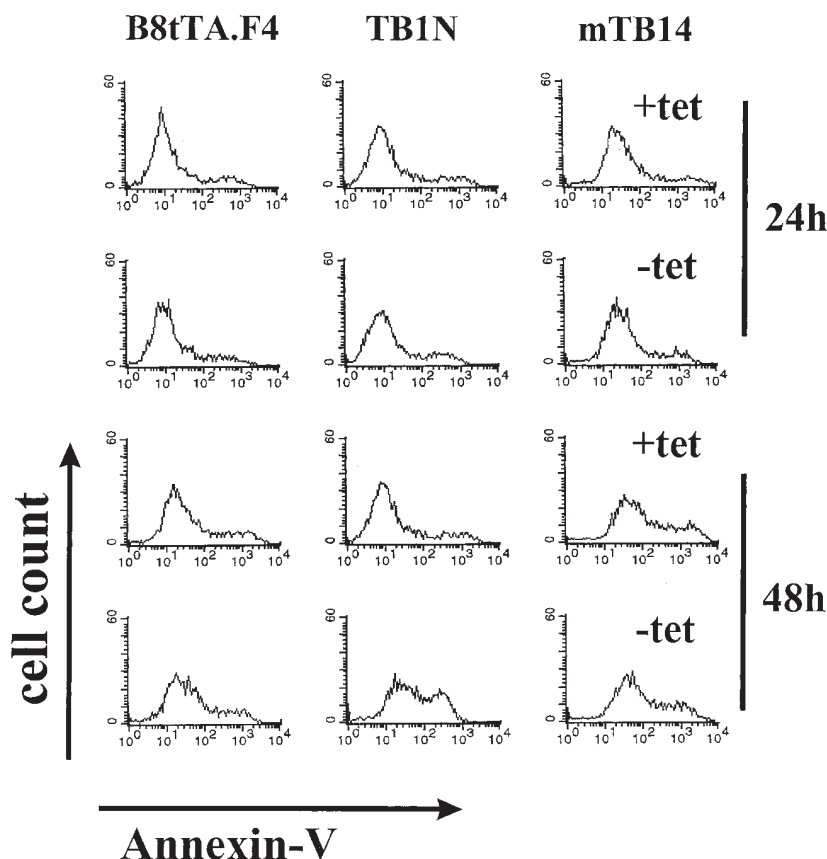
of tet in the growth medium. One day after infection, tet was removed from a portion of the cells, leading to the induction of FAT10 expression. After 24 h, the H-2L<sup>d</sup>-restricted presentation of the LCMV nucleoprotein-derived epitope NP118 was assessed with the help of a NP118-specific T-cell hybridoma in lacZ assays, but again, no effect of FAT10 overexpression on NP118 presentation could be observed (data not shown). Taken together, these experiments provided no evidence for a role of FAT10 in MHC class I-restricted antigen presentation or MHC class I cell surface expression.

**The Induction of FAT10 Expression Induces Apoptosis in a Caspase-dependent Manner**—The induction of cell death in a majority of TB1N cells within 48 h of FAT10 overexpression raised questions about the cause and mechanisms of FAT10-mediated cell death. First, we investigated whether the induction of FAT10 would lead to an arrest of TB1N cells in a certain phase of the cell cycle. This investigation was warranted, particularly because Liu *et al.* (10) had reported an association of FAT10 with MAD2, which is a protein involved in mitotic

arrest. We stained nuclei of viable TB1N cells with propidium iodide 1 or 2 days after FAT10 induction, and the DNA content was assessed by flow cytometry as described previously (18). In this analysis, no change in the distribution of DNA content in TB1N cells could be observed when cells grown in the absence or presence of tet were compared (data not shown). The treatment of TB1N cells with nocodazole or hydroxyurea, in contrast, led to a complete arrest of the cells in mitosis (4n) or S phase (2n), respectively. Therefore, we conclude that FAT10 overexpression does not induce an arrest and accumulation of cells in a specific phase of the cell cycle before the onset of cell death.

The final question addressed in this study was whether the cell death induced by FAT10 overexpression was a consequence of apoptosis or not. First, we stained B8tA.F4 recipient cells, the FAT10 wt transfectant TB1N, and the FAT10- $\Delta$ GG transfectant mTB14 with fluorescein isothiocyanate-conjugated annexin V before and after cultivation in the absence of tet for 24 and 48 h. The appearance of phosphatidylserine in the outer

**FIG. 7. Wild type FAT10 transfectants stain positive for annexin V 48 h after the induction of FAT10 expression.** B8tTA.F4 recipient cells expressing the tetracycline-regulated transcriptional inducer alone, the clone TB1N expressing FAT10 wt protein, and clone mTB14 expressing the FAT10- $\Delta$ GG mutant in a tetracycline-repressible manner were stained with annexin V-fluorescein isothiocyanate 24 and 48 h after the removal of tet from the growth medium. Annexin V binds to phosphatidylserine, which appears in the outer leaflet of the plasma membrane as a late sign of apoptosis. Annexin V binding was analyzed with a flow cytometer; the results of one of three representative experiments with similar outcome are shown.



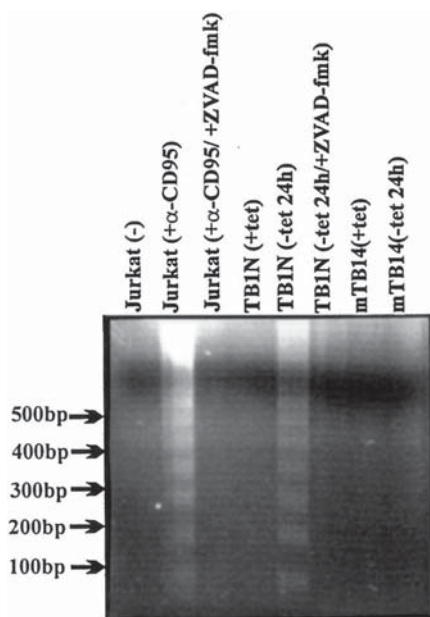
leaflet of the plasma membrane is a late sign of apoptosis that can be detected by annexin V binding to this lipid on the cell surface. We did not observe cells that stained positively with annexin V 1 day after the removal of tet, but on the second day, we observed that about half of the TB1N cells were annexin V-positive in the absence of tet, but not in the presence of tet (Fig. 7). The B8tTA.F4 and mTB14 cells, in contrast, did not show a difference in annexin V staining in the presence or absence of tet. This result suggested that apoptosis was induced by FAT10 overexpression and that the conjugation of FAT10 via the two carboxyl-terminal glycine residues was responsible at least in part for the induction of apoptosis. To confirm the induction of apoptosis as a consequence of FAT10 overexpression, we examined whether a fragmentation of DNA, which is a typical sign of apoptosis, could be detected in TB1N cells. As a positive control, we used a 6-h treatment of the human T-cell line Jurkat with activating antibodies against the Fas receptor (23) (CD95), which resulted in the appearance of DNA fragments in the form of a DNA ladder (Fig. 8). This DNA fragmentation in Jurkat cells could be prevented by a preincubation of the cells with 25  $\mu$ M of the caspase inhibitor Z-VAD-fmk. Interestingly, a DNA ladder also appeared in TB1N cells as a consequence of growing these cells for 24 h in the absence of tet, thus confirming that apoptosis was induced as a consequence of FAT10 overexpression. The fragmentation of DNA was not observed in TB1N cells cultivated in the presence of tet or preincubated with the Z-VAD-fmk inhibitor, indicating that FAT10-mediated apoptosis was dependent on caspase activity. No DNA fragmentation was apparent when the  $\Delta$ GG-FAT10 transfectant mTB14 was cultivated in the absence of tet for 24 h, which led to the induction of the mutant FAT10 protein. The latter result is in accordance with the data obtained with annexin V staining and suggests that FAT10 conjugate forma-

tion via the carboxyl-terminal glycine residues was required for the induction of apoptosis.

#### DISCUSSION

The ubiquitin-like protein FAT10 was discovered in 1996 by chromosomal sequencing of the human HLA-F locus (11). Although this protein has a number of interesting features such as expression in mature B cells and dendritic cells and synergistic induction by the pro-inflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$ , no conclusive functional data have been obtained thus far. In particular, no evidence for conjugate formation has been reported, although the primary structure of FAT10 clearly suggests that it belongs to the group of ubiquitin-like modifiers. In this study, we chose the approach of tet-regulated expression in transfectants to examine what functional consequences an ectopic expression of FAT10 may have on antigen presentation, cellular proliferation, and survival. Because both TNF- $\alpha$  and IFN- $\gamma$  strongly and synergistically up-regulate MHC class I cell surface expression (24) and because FAT10 is encoded in the class I locus, we speculated that FAT10 expression may affect the generation of MHC class I peptide ligands or other processes in the MHC class I pathway, thus leading to a change in MHC class I cell surface expression or antigen presentation. However, at least for the three MHC class I molecules examined in our transfectants, no effect of FAT10 overexpression on MHC class I cell surface expression was observed. Moreover, the H-2L<sup>d</sup>-restricted presentation of the MCMV-pp89<sub>168-176</sub> and LCMV-NP118 epitopes was not affected by FAT10 overexpression. Because these two model systems of antigen presentation were sensitive to alterations in peptide processing by the proteasome in previous investigations (17, 25-27), the utilized T-cell hybridoma-based detection systems should have indicated whether FAT10 had a positive or negative effect on





**FIG. 8. Genomic DNA fragmentation of wild type FAT10 transfectants starting 24 h after induction of FAT10 expression.** Jurkat cells, which undergo genomic DNA fragmentation upon incubation with anti-CD95 antibody for 6 h, were used as positive control. This effect was inhibited by the broad spectrum caspase inhibitor Z-VAD-fmk (+ZVAD). Genomic DNA of the wild type (TB1N) and ΔGG mutant FAT10 transfectant (mTB14) was analyzed on ethidium bromide-stained agarose gels before (+tet) or after (-tet) 24 h of FAT10 expression. Note that Z-VAD-fmk inhibits the wt-FAT10-mediated DNA fragmentation in TB1N cells, which was not detectable in mTB14 cells. The representative results of one of three independent experiments are shown.

epitope generation and/or presentation; however, this was not the case. Therefore, a role for FAT10 in MHC class I-restricted antigen presentation seems rather unlikely, although additional epitopes may have to be tested to reach a definitive conclusion.

In contrast to these negative results from antigen presentation studies, another notion about FAT10 function could be clearly confirmed in the tet-regulated FAT10 transfectants, namely, the induction of cell death. Although there are numerous reasons why the generation of stable transfectants may fail, it was noteworthy that our laboratory (13) as well as Liu *et al.* (10) failed to overexpress human FAT10 in HeLa cells. With the help of the tetracycline-regulated expression system, we were now able to elucidate the reason for this failure and to conclusively demonstrate a link between cell death and FAT10 expression. The cause of cell death was specific for FAT10 because an even stronger overexpression of  $\beta$ -galactosidase or the proteasome regulator PA28 $\gamma$  in the same recipient cells did not affect cellular viability. The tight and very reproducible control of FAT10 expression in all tested clones enabled us to determine the kinetics of FAT10-mediated cell death and to provide the first data on FAT10 function, which appears to be involved in the induction of apoptosis. This conclusion is firmly based on four observations, all of which are hallmarks of apoptosis: (a) FAT10 overexpression induces cell death in the majority of cells within 48 h (Fig. 4), (b) cells stained positive with annexin V (Fig. 7), (c) cells contained fragmented DNA in the typical form of a DNA ladder (Fig. 8), and (d) DNA fragmentation depended on caspase activity, which is known to be required to cleave an inhibitory subunit of the caspase-activated DNase (28). Given that the annexin V staining and caspase-dependent DNA fragmentation in TB1N cells after the initiation of FAT10 expression was comparable in intensity to

FAS-mediated cell death of Jurkat cells, which are frequently used as a model system for apoptosis, there remains little doubt that FAT10 is an inducer of apoptosis in this system. Interestingly, neither up-regulation of annexin V staining nor the formation of a DNA ladder occurred in cells expressing the ΔGG mutant form of FAT10, thus indicating that the carboxyl-terminal glycine residues of FAT10 are involved in the induction of apoptosis. Because the diglycine motif is required for the conjugation of ubiquitin and other ubiquitin-like modifiers (7, 8) our data suggest that it is actually the conjugation of FAT10 to thus far unidentified target proteins that is required for the induction of apoptosis. However, we should point out that in the ΔGG mutant clones, cell death also occurred in about 10–30% of cells upon the removal of tet, which was above the level of cell death obtained in recipient cells or B8tTA.F4 transfectants expressing  $\beta$ -galactosidase or PA28 $\gamma$ . Therefore, we cannot rule out the possibility that overexpression of FAT10 is toxic to some degree to cells and induces cell death that is not related to apoptosis.

In one respect, the induction of apoptosis through FAT10 overexpression meets our speculations regarding the potential function of FAT10, namely, FAT10 may mediate one or several of the diverse cellular responses to signaling through the TNF receptor (TNF-R) (13). The heterodimeric transmembrane TNF-R consists of two chains of 55 kDa and 75 kDa designated TNF-R1 and TNF-R2, respectively (29). In its cytoplasmic part, TNF-R1 contains a death domain that, if activated by the trimeric TNF- $\alpha$  ligand and subsequently bound by an adaptor protein called TNF-R-associated death domain protein, ultimately leads to the activation of procaspase-8 and the induction of apoptosis in a caspase-dependent manner (30). TNF- $\alpha$  was discovered due to its ability to induce necrosis in tumors (31) and was hence named “tumor necrosis factor,” which is a bit misleading because TNF-mediated cell death generally occurs by apoptosis rather than necrosis, although some evidence for an induction of non-apoptotic cell death has been obtained (29). Together with the Fas receptor (CD95), the TNF-R plays a role in the down-regulation of immune responses after clearance of a pathogen from the organism by inducing apoptosis of activated lymphocytes in the absence of antigen (32, 23). Moreover, TNF- $\alpha$  has been implicated in the control of self-reactive T cells because treatment with TNF- $\alpha$  prevented autoimmune diabetes in nonobese diabetic mice (33). The effect of TNF- $\alpha$  on the viability of cultured cells varies considerably with the type of cell line under inspection. The mouse fibroblast cell line B8, which served as the recipient cell line for the generation of our FAT10 transfectants, is killed by TNF- $\alpha$ , whereas the human colon carcinoma line SW620, which we used in previous studies (13), survived treatment with TNF- $\alpha$ . Triggering of the heterodimeric TNF-R also activates nuclear factor- $\kappa$ B, which has been found to antagonize the induction of apoptosis through the death domain (34); this may explain why some cells, such as SW620 cells, survive TNF- $\alpha$  treatment despite the induction of FAT10 expression. One may argue that the induction of murine FAT10 in our inducible transfectants is stronger than the induction normally reached by combined stimulation with IFN- $\gamma$  and TNF- $\alpha$  and that apoptosis may not be induced at physiological levels of FAT10 expression. In agreement with this argument, the expression of murine FAT10 in TB1N cells was higher than that in LPS matured murine dendritic cells (data not shown), and some of our transfectants expressing lower levels of murine FAT10 survived the removal of tet much better than the strong expressers. However, we would like to point out that the only stable human FAT10 transfectant that we could



rescue from a series of HeLa transfectants suffered from continuous cell death in culture and expressed lower quantities of FAT10 mRNA than SW620 cells after the stimulation with TNF- $\alpha$  and IFN- $\gamma$  (13). The latter result suggests that a nonphysiologically high level of FAT10 expression is not required to induce cell death.

An interesting observation made with our inducible FAT10 transfectants was that some 10–30% of several tested clones survived the induction of FAT10 and that the survivors returned to normal growth. We found that the surviving cells had dramatically down-regulated both mRNA and protein expression levels of FAT10. Although all transfectants were generated in separate wells under clonal conditions and were continuously kept under puromycin selection pressure, we assumed that the down-regulation of FAT10 expression was due to the selection and outgrowth of cells that had lost the transfected FAT10 expression construct. However, real-time PCR analysis indicated that this was not the case. One could argue that the surviving cells had somehow managed to suppress transcription from the FAT10 expression construct or had down-regulated the expression of the tet-responsive transcriptional inducer, but we would expect this to be a very rare event that is unlikely to occur in a 20% share of the cells. An alternative and more interesting explanation for our finding would be that the destruction of FAT10 mRNA may have been induced as a consequence of FAT10 overexpression. However, signals that regulate the half-life of a specific mRNA are usually situated in the 5'- and 3'-untranslated region of the short-lived mRNAs, and in the case of our transfectants, the 5'- and 3'-untranslated regions were encoded by the pTet-Spice vector. A specific and inducible regulation of FAT10 mRNA would therefore have to be defined by the coding region of FAT10. This is an intriguing possibility that needs to be carefully addressed in future experiments.

Like many key regulatory proteins, FAT10 is rapidly turned over. We found that the half-life of FAT10 is about 2 h and that the rapid degradation of FAT10 is sensitive to lactacystin. Although lactacystin is not an entirely monospecific inhibitor of the proteasome (35, 36), it is fair to conclude that FAT10 is most likely degraded by the proteasome. This conclusion we have reached for the HA-tagged version of mouse FAT10 was also proposed by Liu *et al.* (10), who found that IFN- $\gamma$  treatment of the B-cell lymphoma line JY with the fairly unspecific proteasome inhibitor Ac-LLN led to an accumulation of endogenously expressed human FAT10. It would be interesting to examine whether FAT10 is polyubiquitinated, but thus far, we have failed to obtain evidence for the accumulation of polyubiquitinated FAT10 in our transfectants after treatment with proteasome inhibitors (data not shown). In this respect, it is also interesting to note that murine FAT10 in our transfectants appeared in Western blots of two-dimensional gels as a series of differently charged proteins with similar molecular mass (Fig. 2). The arrangement of the FAT10 spots in these gels would be compatible with phosphorylation of FAT10, and this notion is supported by the presence of several phosphorylation sites in the FAT10 primary structure. It is intriguing to speculate that phosphorylation of FAT10 may occur as a prerequisite for ubiquitination and degradation by the proteasome, but this issue needs to be addressed experimentally.

A somewhat unexpected but important result obtained during Western analysis of FAT10 expression in our inducible transfectants was that HA-tagged FAT10 appeared not only as a single protein of the expected molecular weight but also as protein bands of higher molecular weight. Because these proteins were only apparent in the absence of tet in FAT10 wt transfectants and not in transfectants expressing a  $\Delta$ GG form

of FAT10, we consider it to be very likely that these additional bands represent covalent conjugates between FAT10 and thus far unidentified target proteins. Because these proteins were resistant to boiling in the presence of SDS and  $\beta$ -mercaptoethanol, they are unlikely to be noncovalent adducts or thioesters of FAT10 with an active site cysteine of a specific FAT10 activating (E1) or conjugating enzyme (ubiquitin carrier protein; E2) and most likely represent isopeptide-linked substrates. We were surprised to find such prominent FAT10 reactive bands of higher molecular weight in our fibroblast transfectants because Liu *et al.* (10) previously failed to detect human FAT10 conjugates by comparing IFN- $\gamma$ -treated and untreated JY cells in FAT10 Western blots (10). One reason why FAT10 conjugates may not have been detected in JY cells could be that the activity of FAT10-activating and -conjugating enzymes differs between human B cells and mouse fibroblasts. Alternatively, FAT10-deconjugating enzymes may exist that could rapidly disassemble FAT10 conjugates in JY cells but not in mouse fibroblast cells. Furthermore, the targets of FAT10 conjugation may simply be expressed in one cell type but not in the other cell type. We have examined whether TNF- $\alpha$  and IFN- $\gamma$  treatment of TB1N transfectants had an influence on the formation of putative FAT10 conjugates, but this was not the case (data not shown), suggesting that the conjugation machinery is constitutively expressed in mouse fibroblasts. A very important aim of future work will be the identification of the putative target proteins of FAT10 conjugation by microsequencing or mass spectrometry. Given that FAT10 as well as the putative conjugates appeared to be rapidly degraded by the proteasome, the ability to transiently overexpress FAT10 in our transfectants will be an invaluable tool to isolate sufficient amounts of the putative conjugates for identification. An additional task will be the identification of putative FAT10-activating and -conjugating enzymes as well as potential deconjugating enzymes, as have been described for other ubiquitin-like modifiers. Because the carboxyl-terminal diglycine motif and, as a likely consequence, FAT10 conjugation were apparently required for the FAT10-mediated induction of apoptosis, the identification of putative target proteins of FAT10 conjugation may help to elucidate the stage of apoptosis at which FAT10 takes effect.

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