ATF4 Degradation Relies on a Phosphorylation-Dependent Interaction with the SCF^{βTrCP} Ubiquitin Ligase

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The ubiquitin-proteasome pathway regulates gene expression through protein degradation. Here we show that the F-box protein βTrCP, the receptor component of the SCF E3 ubiquitin ligase responsible for IκBα and β-catenin degradation, is colocalized in the nucleus with ATF4, a member of the ATF-CREB bZIP family of transcription factors, and controls its stability. Association between the two proteins depends on ATF4 phosphorylation and on ATF4 serine residue 219 present in the context of DSGXXXS, which is similar but not identical to the motif found in other substrates of βTrCP. ATF4 ubiquitination in HeLa cells is enhanced in the presence of βTrCP. The F-box-deleted βTrCP protein behaves as a negative transdominant mutant that inhibits ATF4 ubiquitination and degradation and, subsequently, enhances its activity in cyclic AMP-mediated transcription. ATF4 represents a novel substrate for the SCF^{βTrCP} complex, which is the first mammalian E3 ubiquitin ligase identified so far for the control of the degradation of a bZIP transcription factor.

Proteasome-mediated protein degradation requires the covalent attachment of polyubiquitin to the substrate proteins (11, 25, 38). The cascade of ubiquitin transfer reactions involves the ubiquitin-activating enzyme E1, an E2 ubiquitin-conjugating enzyme that operates with specificity factor E3. The selectivity of the reaction is due to the E3 ubiquitin ligase, which interacts with both E2 and the substrate.

SCF (Skp1/Cullin/F-box protein) complexes were initially shown to function as E3 ubiquitin ligases for a variety of phosphorylated proteins involved in the yeast cell cycle (1, 15, 35, 52, 60). The core components of these complexes include Skp1, Cul-1 (Cdc53), and the newly identified protein Rbx1 (Roc1 or Hrt1), which is thought to stabilize the interaction between Cul-1 and the E2 enzyme Cdc34 (12, 14, 30, 61). The SCF complexes also contain a variable receptor subunit, an F-box-containing protein, that provides substrate specificity. The F-box motif serves to anchor the receptor subunit to the SCF complex by its interaction with Skp1 (12, 14, 52, 60). For ubiquitination, substrate proteins are recruited to the SCF E3 ubiquitin ligase complexes through interaction with the substrate binding domain (WD-40 or leucine-rich repeats) of the F-box receptor subunits. SCF complexes are the largest and most versatile class of E3 ubiquitin ligases. To date, only two human SCF complexes, SCF^{Skp2} and SCF^{βTrCP}, have been analyzed in detail and have had some of their substrates characterized (6, 7, 23, 36, 39, 41–44, 49, 62, 63, 67, 68). We made the first identification of human βTrCP (beta-transducin repeat-containing protein) as the F-box receptor component of the E3 ubiquitin ligase SCF^{βTrCP} responsible for the degradation of CD4 induced by the human immunodeficiency virus

type 1 (HIV-1) protein Vpu (43). Subsequently, we and others showed that SCF^{βTrCP} is also responsible for phosphorylation-dependent ubiquitination and then for the degradation of IκBα and β-catenin (23, 36, 39, 42, 62, 67, 68). Vpu, IκBα, and β-catenin share a common motif, DSGXXS. Phosphorylation of its serine residues is required for interaction with βTrCP. IκBα is phosphorylated on the DS₃₂GXXS₃₆ motif by IKKα and IKKβ protein kinases activated through various signaling events (31). It is commonly thought that IκBα is subsequently ubiquitinated and degraded in the cytoplasm, resulting in NF-κB nuclear translocation and transcription stimulation of target genes (31). Similarly, it is also thought that β-catenin ubiquitination and subsequent degradation take place in the cytoplasm, preventing nuclear translocation of the protein which is required for TCF/LEF transcriptional activation of target genes (53).

ATF4 is a member of the ATF/CREB proteins that include CREB (cAMP responsive element binding protein), CREM (CRE modulator), ATF1, ATF2, and ATF3 (for reviews, see references 4, 22, 46, 57 and 69). These proteins bind to DNA via their basic region and dimerize via their leucine zipper domain to form a large variety of homodimers and/or heterodimers that allow the cell to coordinate signals from different pathways (4, 22, 46, 57, 69). Thus far, two other names have been used to refer to human ATF4 (21): CREB2 (32) and TAXREB67 (65). In addition, mouse cDNAs with 85% homology to human ATF4 have been referred to as mATF4 (47), C/ATF (66), or mTR67 (8). In the rest of this report, we will refer to it as ATF4. The E2 ubiquitin-conjugating enzymes involved in the degradation of some members of the ATF/CREB family have recently been identified; they include Cdc34 and Rad6B for hICERγ and ATF5 (ATFx), respectively (51), and hUBC9 for ATF2 (16). However, the E3 ubiquitin ligases required to make mammalian ATF/CREB transcription factor

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substrates for the proteasome remain unknown. While this paper was in preparation, Meimoun et al. identified the E3 ubiquitin ligase SCF^{Cdc4} complex as responsible for degradation of the bZIP transcription factor Gcn4 in yeast (45).

There has been considerable interest in the role of ATF/CREB transcription factors and regulation of their activity by phosphorylation. In eukaryotes, cyclic (cAMP)-mediated transcription regulates multiple physiological processes, including gametogenesis, circadian rhythm, and neuroendocrine functions (13). Stimulation of this pathway is mediated via phosphorylation by protein kinase A (PKA) of a single serine in the structurally similar transcription factors CREB, CREM, or ATF1 (13). However, ATF4 lacks potential PKA phosphorylation sites and has been demonstrated to be a negative regulator of CRE-dependent transcription (2, 32). In contrast, ATF4 was proposed to be a positive regulator of transcription (40), increasing the expression of genes, such as somatostatin, serotonin, or interleukin-2 (2, 5, 66), and that of the human T-cell leukemia virus type 1 by interacting with the viral transactivator Tax (19, 54). How the versatile effects of ATF4 are regulated in these different pathways has not been investigated yet.

We found that ATF4 and βTrCP are associated in vivo and colocalized in the nucleus. Our results suggest that SCF^{βTrCP} tightly modulates the stability of the transcription factor ATF4 and therefore also modulates its transcriptional activity following activation of the cAMP pathway.

MATERIALS AND METHODS

Two-hybrid assays. Yeast two-hybrid screening was performed as described previously (3). *Saccharomyces cerevisiae* HF7 cells were transformed with the bait plasmid containing seven WD-40 repeats of human βTrCP (residues 251 to 569) fused to the Gal4 DNA binding domain in the pGBT10 plasmid and with a human Jurkat cDNA library fused to the GAL4 transactivation domain in the pGAD13-18 plasmid. Transformants were screened on plates lacking tryptophan, leucine, and histidine and were then assayed for β-galactosidase activity. For interaction assays, βTrCP, Slimb (27), or Fbw2 (9) and ATF4 or Skp1 were fused, respectively, to *Escherichia coli* LexA or the Gal4 binding domain and the Gal4 activation domain. The yeast reporter strain L40 or HF7 expressing the indicated hybrid protein pairs was analyzed for β-galactosidase expression or for histidine auxotrophy.

Plasmid construction and mutagenesis. The ATF4 cDNA found in the screen corresponds to the protein accession number P18848. ATF4ΔBZ was obtained by PCR with specific primers and was subcloned in the pGAD13-18 vector (3). ATF4 point mutations (D218N, S219N, G220A, S224N) were constructed by PCR mutagenesis. pGADSkp1 plasmid and pcDNA3 expression vectors for βTrCP-Myc and βTrCPΔF-Myc have already been described (23, 36). βTrCPΔW1, βTrCPΔW2-7, and βTrCPΔN were obtained by PCR with appropriate primers and subcloned in the pcDNA3.1A/Myc-His vector (Invitrogen). Hemagglutinin (HA)-ATF4, HA-βTrCP, and HA-βTrCPΔF were obtained following direct subcloning of ATF4, βTrCP, and βTrCPΔF in the pAS1B vector (59). The Myc-His sequence in the pcDNA3.1/Myc-His vector was replaced by the green fluorescent protein (GFP) coding sequence, resulting in pcDNA3-βTrCP-GFP. The pAS2 expression vector for Fbw2 was kindly provided by J. Hsu and P. Jackson.

Coimmunoprecipitation experiments. Samples containing 8×10^6 HeLa cells were transiently cotransfected by electroporation with 10 μg of either pAS1B, pAS1B-ATF4, or pAS1B-ATF4S219N and with 15 μg of the pcDNA3.1A/Myc-His plasmid containing wild-type or mutant βTrCP sequences. Cells were either not pretreated or were pretreated with 20 μM Z-LLL-H (*N*^α-benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal; MG132; Sigma) for 4 h and then cells were harvested 48 h after transfection and lysed in 1% NP-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1 mM EDTA). For immunoprecipitations, cell lysates were incubated with 5 μg of rat monoclonal anti-HA antibody per ml (clone 3F10; Boehringer) or 5 μg of mouse monoclonal anti-Myc antibody per ml (clone 9E10; Santa Cruz) for 90 min and then incubated with protein G-agarose beads (Boehringer) for 1 h. To study the association of ATF4 and βTrCP

endogenous proteins, we performed a coimmunoprecipitation in untransfected 293T cells lysed using the NP-40 lysis buffer. The lysate was precleared with rabbit nonimmune antibodies and protein A-agarose (Sigma) for 90 min. Supernatant was incubated with anti-βTrCP antibody (43) or anti-ATF4 antibody [raised in rabbits by immunization with the fragment 207-351 of human ATF4 expressed in BL21(DE3)LysS bacteria and purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)] or nonimmune rabbit polyclonal antibodies for 90 min and then were incubated with protein A-agarose (Sigma) for 30 min. The beads were washed with lysis buffer. Immune complexes were eluted with Laemmli sample buffer, separated by SDS-12% PAGE, and revealed by chemiluminescence using rat anti-HA (clone 3F10; Boehringer) or mouse anti-Myc (clone 9E10; Santa Cruz) or goat polyclonal anti-βTrCP (C-18; Santa Cruz) antibodies. To demonstrate the specificity of the anti-ATF4 antibodies, we used both immunoprecipitation and Western blot experiments with cells transfected by HA-ATF4 and compared the results with those obtained by Western blotting with untransfected 293T cells. When needed, quantitation of the chemiluminescent signal was performed using NIH Image software. Where indicated, treatment of immunoprecipitates with 32 U of alkaline bovine phosphatase corresponding to a final concentration of 180 μg/ml (P6774; Sigma) was administered to immune complexes for 45 min at 30°C in 50 mM Tris (pH 7.5)–1 mM MgCl₂.

Ubiquitination assay. In vivo ubiquitination was assayed as described by Treier et al. (64). Six-His tag-ubiquitin (10 μg) and HA-ATF4 (8 μg) expression vectors were transiently cotransfected by electroporation in HeLa cells. Twenty-four hours later, cells were lysed with 6 M guanidinium-HCl (pH 8), and His-tagged proteins were purified by nickel resins Ni-nitrilotriacetic acid agarose (Qiagen) as described by Treier et al. (64), eluted with Laemmli sample buffer, and separated by SDS-10% PAGE. Ubiquitin-conjugated HA-ATF4 proteins were revealed by chemiluminescence using anti-HA antibody (clone 3F10; Boehringer).

Immunostaining of cells. For ATF4 and βTrCP localization, HeLa cells were transiently transfected by electroporation with 15 μg of pAS1B-ATF4 and 15 μg of pcDNA3-βTrCP-GFP and seeded onto glass plates at a density of 50,000 cells/plate. At 48 h, cells were washed with phosphate-buffered saline (PBS) and fixed for 20 min in 4% paraformaldehyde, quenched for 10 min with 0.1 M glycine in PBS, and permeabilized for 30 min at room temperature with 0.05% saponin in PBS-0.2% bovine serum albumin. Cells were incubated with anti-HA antibody (1/10 dilution), washed in PBS, and incubated with goat anti-rat Texas-RedR secondary antibodies (1/125 dilution; Jackson Immunoresearch). Confocal microscopy was carried out under fluorescent light.

³⁵S metabolic labeling and pulse-chase experiments. At 24 h after electroporation with various plasmids (pAS1B for ATF4 proteins and pcDNA3 for βTrCP proteins), 10⁶ HeLa cells were incubated for 30 min in Met and Cys-free Dulbecco modified Eagle medium (DMEM), and then 125 μCi of [³⁵S]methionine-cysteine (NENlife) per ml was added to the same medium for 1 h. Similarly, to study the stability of ATF4 endogenous protein, metabolic labeling was performed with 293T cells that were untransfected or were transfected with pcDNA3-βTrCP and were not pretreated or were pretreated with Z-LLL-H (Sigma). Cells were washed in PBS and harvested (time zero) or incubated for 0.5, 1, 2, 3, 3.5, or 4 h in complete DMEM, washed again in PBS, and lysed as described previously. Cell lysates were immunoprecipitated with mouse monoclonal anti-HA (12CA5; Boehringer) or rabbit polyclonal anti-ATF4 antibodies and incubated with protein G-agarose or protein A-agarose (Sigma) beads. Beads were washed with lysis buffer supplemented with NaCl (300 mM final concentration), and immune complexes were eluted with Laemmli sample buffer, separated on SDS-12% PAGE gels, fixed in acetic acid (10%)–methanol (30%), dried, and exposed to Kodak X-Omat film. Quantitation was performed with an ImageQuant phosphorimager (Molecular Dynamics).

Luciferase assays. 293T cells were plated in 6-well flat-bottom plates on the day prior to transfection at a density of 5×10^4 cells/35-mm-diameter well in DMEM. Transfections were performed using the calcium phosphate coprecipitation method with the Mammalian Transfection Kit (Stratagene). We used the luciferase reporter pSS-CRE-LUC containing a sequence of rat somatostatin gene from –71 to 53 (37) (including the CRE site) placed 5' to the gene for luciferase. Cells were cotransfected with 0.2 μg of pSS-CRE-LUC, 3 ng of pRL-TK-renilla (PRL-TK from Promega), and various amounts of plasmids expressing HA-ATF4, HA-βTrCP, or HA-βTrCPΔF as indicated. Six hours prior to harvesting, half of the transfected dishes were incubated in a mixture of 10^{–5} M forskolin (Sigma) and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma). At 24 h posttransfection, cells were lysed and luciferase and renilla activities were measured with luciferase assay reagent (Dual-Luciferase Reporter Assay System; Promega) by using a Lumat LB9507 luminometer (EG&G Instruments).

RESULTS

βTrCP interacts with ATF4. In order to find new substrates of βTrCP, we used the two-hybrid system. The C-terminal substrate-recruiting domain of βTrCP, which contains the seven WD-40 repeats (residues 251 to 569), was chosen as a bait (Fig. 1A) and was fused to the Gal4 DNA binding domain (43). A cDNA library from Jurkat cells, constructed in fusion with the Gal4 activation domain, was screened as previously described (3). Four clones interacting specifically with the WD-40 repeats of βTrCP and which corresponded to human ATF4 cDNA, with different 5' ends within the N-terminal region, were isolated. Interaction between βTrCP and full-length ATF4 is shown in Fig. 1B, lane 1. No other proteins from the ATF/CREB family were isolated from our screen. The structure of βTrCP and ATF4 proteins is schematized in Fig. 1A. Deletion experiments allowed us to map the interacting domains between the two proteins, respectively, on the seven WD-40 repeats at the C terminus of βTrCP and between residues 87 to 279 of ATF4 (data not shown). The deletion of the basic region and the leucine zipper domain of ATF4 (ATF4ΔBZ), both located at the C terminus of the protein, did not affect the interaction with βTrCP (Fig. 1B, lane 2). The interaction of ATF4 with human βTrCP was specific since it was not found with other members of the WD-40 and F-box proteins, such as Fbw2 (the human homolog of mouse MD6; 9, 48) (Fig. 1C, lane 3) or the *Drosophila melanogaster* homolog of βTrCP, Slimb (Fig. 1C, lane 1) (27), although these proteins both interact with Skp1 (Fig. 1C, lanes 2 and 4). The human isoform of βTrCP, βTrCP2, interacts with ATF4 (data not shown).

FIG. 1. Interaction of βTrCP with ATF4 is impaired by mutation on the D218 and S219 ATF4 residues. (A) Scheme of protein structures of βTrCP and ATF4. The diagram of βTrCP shows the F box responsible for proteasome targeting through Skp1 binding and the seven WD-40 repeats involved in binding substrates. For ATF4, the basic region (b) and the leucine zipper domain (Z) are indicated in addition to the DSGXXXS motif. (B) The yeast reporter strain L40 expressing the indicated hybrid protein pairs was analyzed for β-galactosidase expression. βTrCP was fused to the *E. coli* LexA binding domain in the pLex plasmid. ATF4 sequences were fused to the Gal4 activation domain in pGAD13-18. (C) The yeast reporter strains L40 or HF7 expressing the indicated hybrid protein pairs were analyzed for histidine auxotrophy.

Serine 219 of ATF4 is required for interaction with βTrCP.

The phosphorylation motif DSGXXXS required for interaction of βTrCP with its substrates HIV-1 Vpu (43), IκBα, and β-catenin (23, 36, 39, 42, 62, 67, 68) was not found in the ATF4 sequence. However, a closely related motif of the type DSGXXXS occurred at positions 218 to 224 (Fig. 1A). Single amino acid substitutions in this motif, D218N or S219N, were sufficient to impair ATF4 interaction with βTrCP, whereas mutation of ATF4 residues G220 and S224 did not affect such interaction (Fig. 1B, lanes 3 to 6).

ATF4 and βTrCP were tagged with HA and Myc epitopes, respectively, and were transiently expressed in HeLa cells to determine whether the ATF4-βTrCP interaction can take place in vivo. HA-ATF4 was then immunoprecipitated using anti-HA antibody. Wild-type βTrCP was coprecipitated with HA-ATF4 (Fig. 2A, top, lane 2). Deletion of the first WD-40 repeat or the last six WD-40 repeats in βTrCP (βTrCPΔW1 and βTrCPΔW2-7) abolished the association with ATF4, confirming that the WD-40 repeats are the binding site for ATF4 (Fig. 2A, top, lanes 4 and 6). Expression of HA-ATF4 was checked and was shown to be identical irrespective of the βTrCP mutant proteins expressed (Fig. 2A, bottom). In addition, deletion in βTrCP of the F-box motif (βTrCPΔF) or of the N-terminal region upstream of the F-box motif (βTrCPΔN) did not affect association with ATF4 (Fig. 2B, top, lane 4, and Fig. 2A, top, lane 8, respectively). In ATF4, mutation of the serine residue at position 219 again abolished the interaction with βTrCP or βTrCPΔF (Fig. 2B, top; compare lanes 6 and 7 with lanes 2 and 4, respectively), although the level of the ATF4 S219N mutant was higher than that of wild-type ATF4 (Fig. 2B, bottom; compare lane 6 to lane 2). In the presence of the proteasome inhibitor Z-LLL-H, the amount of accumulated ATF4 was fivefold higher (Fig. 2C,

right; compare lane 8 to lane 6), and subsequently, the ATF4- β TrCP interaction was stabilized (Fig. 2C, left; compare lane 4 to lane 2).

Endogenous β TrCP coimmunoprecipitates with endogenous ATF4. In order to check that both endogenous ATF4 and β TrCP proteins were also associated, we performed immunoprecipitations of untransfected cell lysates with anti-ATF4 antibodies, and the immunoprecipitate was probed by Western blotting with anti- β TrCP antibodies. The anti- β TrCP antibodies have been previously characterized by us (43) or by the supplier (Santa Cruz). In order to assess the specificity of the anti-ATF4 antibodies, we checked, as shown in Fig. 2D, that these antibodies can recognize specifically HA-ATF4, in both Western blot and immunoprecipitation experiments (Fig. 2D, lanes 2, 3, and 4). In transfected cells expressing HA-ATF4, two bands were detected by Western blotting using anti-ATF4 antibodies (Fig. 2D, lane 4). The upper band corresponds to the exogenous HA-ATF4 since it comigrates with HA-ATF4 precipitated by anti-HA antibodies (Fig. 2D, lane 3). The lower band, with an approximate molecular mass of 35 kDa, the only one detected in the untransfected cells, corresponds to the endogenous ATF4 (Fig. 2D, lanes 4 and 5). As shown in Fig. 2D, lane 2, anti-ATF4 antibodies efficiently immunoprecipitated HA-ATF4. Thus, these antibodies could be used for coimmunoprecipitation experiments of endogenous ATF4 and endogenous β TrCP proteins.

As shown in Fig. 2E (lane 2), a protein recognized by anti- β TrCP antibodies and migrating as a band with a molecular mass of 60 kDa was present in the anti-ATF4 immunoprecipitate. This band was clearly identified as β TrCP since it was also found in the immunoprecipitate with anti- β TrCP antibodies (Fig. 2E, lane 1) but not in the immunoprecipitate with non-immune antibodies (Fig. 2E, lane 3). Thus, this experiment demonstrates that the endogenous proteins interact in untransfected cells.

ATF4- β TrCP interaction depends on ATF4 phosphorylation. In vivo interaction between ATF4 and β TrCP was confirmed by coimmunoprecipitation of HA-ATF4 with Myc-tagged β TrCP or Myc-tagged β TrCP Δ F by using anti-Myc antibody (Fig. 3, lanes 2 and 4). To assess whether ATF4 that coprecipitated with β TrCP proteins was a phosphorylated form of ATF4, treatment of the immunoprecipitates with alkaline phosphatase was performed prior to denaturation and loading on SDS-PAGE gels. As shown in Fig. 3 (compare lane 3 to lane 2 and lane 5 to lane 4), such phosphatase treatment gave rise to a faster migrating band of HA-ATF4. This result is consistent with the fact that ATF4 which was associated with β TrCP in the anti-Myc immunoprecipitate was phosphorylated. Interestingly, when HA-ATF4 was immunoprecipitated with anti-HA antibody, two bands of HA-ATF4 were detected in the immunoprecipitate (Fig. 3, lane 6). From these results, one can conclude that only the phosphorylated forms of ATF4 can associate with β TrCP.

ATF4 ubiquitination is enhanced in the presence of wild-type β TrCP. To investigate the role of β TrCP as an E3 ubiquitin ligase for ATF4, we decided to establish an in vivo ubiquitination assay of ATF4. HA-ATF4 was transiently coexpressed with a six-His tag-ubiquitin in HeLa cells. Ubiquitin-conjugated proteins were purified using nickel beads, and ubiquitin-conjugated ATF4 proteins were revealed by immunoblotting

FIG. 3. ATF4- β TrCP interaction depends on ATF4 phosphorylation. After transfection of vectors expressing HA-ATF4 (lanes 1 to 6) and β TrCP-Myc (lanes 2 and 3) or β TrCP Δ F-Myc (lanes 4 and 5) or the empty vector pcDNA3 (lanes 1 and 6), anti-Myc or anti-HA immunoprecipitates (IP α -Myc and IP α -HA, respectively) were left untreated (lanes 1, 2, 4, and 6) or were exposed to phosphatase treatment (lanes 3 and 5). Immunoprecipitates were probed with anti-HA antibodies (WB α -HA).

with HA antibodies. In the absence of the proteasome inhibitor Z-LLL-H, no ubiquitin-conjugated ATF4 was detected on the nickel beads (data not shown). In the presence of Z-LLL-H, ubiquitin-conjugated ATF4 was retained on the beads (Fig. 4, lane 2). In the absence of six-His tag-ubiquitin, no HA-ATF4 was detected on the beads after a short exposure (Fig. 4, lane 1, middle) although some background signal could be seen after a long exposure (Fig. 4, lane 1, top). To further confirm the role of β TrCP in ATF4 ubiquitination, we investigated the effect of the expression of β TrCP and of the β TrCP Δ F mutant on ATF4 ubiquitination. In the presence of β TrCP, ATF4 ubiquitination was enhanced (Fig. 4, lane 3, short exposure) and higher-molecular-weight ubiquitinated forms could be detected (Fig. 4, lane 3, long exposure). In contrast, the expression of β TrCP Δ F, previously identified as a negative transdominant able to inhibit degradation of I κ B α , β -catenin, or CD4 (23, 36, 43), decreased ATF4 ubiquitination (Fig. 4, lane 4, short exposure). β TrCP Δ F behaves as a negative transdominant because it binds substrates, but the deletion of the F-box motif impairs its association to Skp1, which is required for its recruitment to the SCF-E3 ubiquitin ligase complex, and the targeting of substrates to the proteasome (23, 36, 43). As a control, we checked that the total amount of ATF4 in the cells was roughly similar (Fig. 4, bottom). Thus, these results further support the notion that SCF $^{\beta$ TrCP is the E3 ubiquitin ligase required for ATF4 ubiquitination. However, most of the ubiquitinated ATF4 that was recovered from nickel beads had a low molecular mass and corresponded in majority to monoubiquitinated ATF4 or to protein-conjugated ATF4 with a very small number of ubiquitin residues. In order to observe higher-molecular-weight forms of ATF4 representing polyubiquitinated protein, the longer exposure was needed. This result raises the question of whether ATF4 is preferentially targeted for monoubiquitination by SCF $^{\beta$ TrCP.

β TrCP colocalizes with ATF4 in the nucleus. The β TrCP-ATF4 interaction was further investigated in HeLa cells by confirming the codistribution of the proteins by confocal microscopy. In transient-transfection assays, we found by indirect immunofluorescence that β TrCP tagged either with GFP at

FIG. 4. ATF4 ubiquitination is enhanced in the presence of wild-type β TrCP. HeLa cells were transfected with HA-ATF4 expression vector (lanes 1 to 4), with plasmids expressing β TrCP (lane 3) or β TrCP Δ F (lane 4) proteins or the corresponding empty vector (lanes 1 and 2), and with a six-His tag-ubiquitin expression vector (lanes 2 to 4). In vivo ubiquitin-conjugated proteins were purified, and ubiquitin-conjugated HA-ATF4 proteins were revealed by immunoblotting with HA antibody after a long exposure (top) or a short exposure (middle) of the film. The lower panel shows a control immunoblotting analysis of whole-cell extracts of HA-ATF4 expression.

the C terminus or HA epitope at the N terminus is mainly in the nucleus (Fig. 5A and B). In these assays, the nucleoli were unlabeled. After cotransfection with vectors expressing β TrCP-GFP and HA-ATF4, which is a nuclear protein (10), β TrCP-GFP and HA-ATF4 were found colocalized in the nucleus (Fig. 5C). All cells examined which had been successfully co-transfected with vectors expressing β TrCP-GFP and HA-ATF4 showed such colocalization of the two proteins in the nucleus.

ATF4 is an unstable protein, and β TrCP modulates its stability. By ³⁵S metabolic labeling and pulse-chase experiments, we studied the stability of the endogenous ATF4 protein in the presence or absence of the proteasome inhibitor

Z-LLL-H in untransfected cells and in transfected cells over-expressing β TrCP. As shown in Fig. 6, the addition of the proteasome inhibitor Z-LLL-H stabilized ATF4 considerably, since most of the protein was still detectable after 3.5 h of chase (Fig. 6A and B). Overexpression of β TrCP resulted in a significant enhancement of the instability of the ATF4 protein, with its half-life decreasing from 30 to 15 min (Fig. 6B). These results demonstrate that the endogenous protein is an unstable protein with a half-life of about 30 min which is stabilized in the presence of proteasome inhibitors. In addition, we found that the protein is further destabilized by overexpression of β TrCP (Fig. 6A and B).

Because of the critical importance of ATF4 residue serine 219 for interaction with β TrCP, we hypothesized that if such interaction plays a role in the control of the stability of this transcription factor, the ATF4 S219N mutant should be stabilized by comparison with wild-type ATF4 when expressed in cells. The ATF4 wild type and mutant S219N, both tagged with the HA epitope at the N terminus, were transfected in HeLa cells. At 24 h after transfection, ³⁵S metabolic labeling and pulse-chase experiments from 0 to 4 h were carried out prior to immunoprecipitation using anti-HA antibody and SDS-PAGE analysis (Fig. 6C). HA-ATF4 and HA-ATF4 S219N half-lives were determined after quantification of the amounts of protein immunoprecipitated by anti-HA antibodies at each time point by using a phosphorimager. The half-life of HA-ATF4 was found to be of about 1 h (Fig. 6D). Of note is that the protein ran as a multiple set of bands, probably because phosphorylation events had taken place. The mutation S219N stabilized the protein, giving rise to a half-life of about 2.5 h (Fig. 6C and D). These results are in favor of a role for the β TrCP-ATF4 interaction in the stability of ATF4. Interestingly, the half-life of the overexpressed exogenous HA-ATF4 protein is significantly longer than that of the ATF4 endogenous protein (1 h instead of 30 min). This observation may indicate that the activity of the SCF ^{β TrCP} machinery to target ATF4 for degradation by the proteasome could be relatively limited in a context of overexpression of exogenous ATF4.

To further confirm the role of β TrCP in the control of ATF4 degradation, we investigated the effect on HA-ATF4 stability of the expression of the β TrCP wild type and of the β TrCP Δ F mutant (Fig. 6E). In the presence of β TrCP Δ F, we observed that wild-type ATF4 was stabilized, with its half-life increasing from 60 to 120 min (Fig. 6F). By contrast, expression of wild-type β TrCP shortened the half-life of the protein from 60 to 45 min (Fig. 6F). We checked that expression of β TrCP or β TrCP Δ F did not affect the half-life of the ATF4 S219N mutant (data not shown). From these results, we conclude that ATF4 behaves like the other substrates of β TrCP (Vpu/CD4, I κ B α , and β -catenin) whose degradations were impaired by the negative transdominant mutant of β TrCP (β TrCP Δ F). Thus, ATF4 can be considered the fourth substrate of the SCF ^{β TrCP} identified so far.

Transcriptional activity of ATF4 is inhibited by β TrCP. To check whether modulation of ATF4 stability by β TrCP had any functional consequences, we examined the effect of β TrCP or β TrCP Δ F expression on the transcriptional activity of ATF4. ATF4 was identified as a transcriptional factor capable of inhibiting or activating transcription mediated through CRE (2, 5, 19, 32, 40, 54, 66). The activity of a luciferase reporter

FIG. 5. β TrCP and ATF4 are codistributed in the nucleus. HeLa cells were transfected with a plasmid expressing β TrCP fused to the GFP protein at its C terminus (β TrCP-GFP) (A), with a plasmid expressing β TrCP fused to the HA epitope at its N terminus (HA- β TrCP) (B), or with both β TrCP-GFP and HA-ATF4 expression vectors (C). (B and C) Cells were stained with a Texas red-conjugated anti-HA antibody. Cells were analyzed by confocal microscopy.

gene under the control of the somatostatin promoter, which contains the classical CRE binding site for the ATF/CREB transcription factors, was analyzed in 293T human embryo kidney cells after transfection with ATF4 and β TrCP or β TrCP Δ F expression vectors and treatment with forskolin as a cAMP pathway stimulator. Consistent with the results obtained by Karpinski et al. (32), overexpression of ATF4 inhibited the CRE-dependent transcriptional stimulation induced by forskolin treatment (Fig. 7A). Expression of β TrCP impaired the repressor activity of ATF4, whereas expression of β TrCP Δ F stimulated its activity (Fig. 7B). These results are consistent with the stimulation of ATF4 degradation provoked by β TrCP overexpression (Fig. 6E) and with the stabilization of ATF4 resulting from inhibition of ATF4 degradation induced by the expression of the β TrCP Δ F-negative transdominant mutant (Fig. 6E).

DISCUSSION

The ubiquitin-proteasome degradation pathway is important at the level of transcription regulation to assure the controlled and timely termination of signaling by irreversible destruction

of the activated transcription factors. Because the E3 ubiquitin ligases are the factors responsible for the specificity of the ubiquitin-dependent proteolysis process, it is essential to identify which E3 targets which transcription factors. Here we have identified SCF ^{β TrCP} as the first mammalian E3 ubiquitin ligase responsible for degradation of ATF4, a transcription factor belonging to the important bZIP family. ATF4 binds to β TrCP, the component receptor of the E3 complex by phosphorylation-dependent interaction, and is colocalized in the nucleus with this protein. We have found that β TrCP controls the stability of ATF4 and subsequently its transcriptional activity. However, the results of our *in vivo* ubiquitination experiments raise the question of whether ATF4 is preferentially targeted for monoubiquitination by SCF ^{β TrCP}.

It was recently reported by Kaiser et al. (29) that the ubiquitination of the transcription factor Met4 mediated by the F-box protein Met30 in yeast would not lead to its degradation as previously shown by Rouillon et al. (56) but nevertheless results in the inhibition of Met4 transcriptional activity. Since, among the yeast F-box proteins, Met30 is the closest homolog to β TrCP, one can wonder whether the same phenomenon of regulation of transcription by ubiquitination without proteoly-

FIG. 6. ATF4 is an unstable protein and β TrCP modulates its stability. (A) Untransfected 293T cells were not treated or were treated with the proteasome inhibitor MG132 (Z-LLL-H) as indicated, cells transfected with expression vector for β TrCP were [35 S]Met-Cys metabolically labeled and chased for 0, 0.5, or 3.5 h, and lysates were immunoprecipitated with anti-ATF4 antibodies and analyzed following SDS-12%PAGE and autoradiography. (B) Scanning of the gel shown in panel A was performed using a phosphorimager. The ratios of the amounts of ATF4 left undegraded at each time point relative to that of the starting material were plotted as a function of time. (C) The ATF4 S219N mutant which has lost its ability to interact with β TrCP is stabilized in HeLa cells. Vectors expressing wild-type HA-ATF4 or HA-ATF4 S219N were transfected, cells were [35 S]Met-Cys metabolically labeled and chased for 0, 1, 2, 3, and 4 h, and lysates were immunoprecipitated using anti-HA antibody and analyzed following SDS-12% PAGE and autoradiography. Immunoprecipitation with a lysate from labeled nontransfected cells (NT) was also performed. (D) Scanning of the gel shown in panel C. (E) Expression of the β TrCP Δ F mutant inhibits ATF4 degradation. ATF4 was transfected in the presence of β TrCP, β TrCP Δ F, or the corresponding empty vector pcDNA3, labeled, and immunoprecipitated as described for panel A. (F) Scanning of the gel shown in panel E.

FIG. 7. Repression transcriptional activity of ATF4 on the somatostatin promoter stimulated by forskolin is controlled by β TrCP. (A) 293T cells were transiently cotransfected with a reporter plasmid expressing luciferase under the control of the somatostatin CRE sequence and different amounts of ATF4 expression vectors. Cells were left untreated or were stimulated in the presence of forskolin and IBMX. Experiments were repeated three times with duplicate samples, and results of a representative experiment are shown. The stimulation factor is reported to the transcription level observed in the cells without transfected ATF4. (B) Increasing amounts of β TrCP or β TrCP Δ F expression vectors were transfected in the presence of the CRE luciferase reporter plasmid and 0.2 μ g of ATF4 vector.

sis described for Met30 could also be observed with β TrCP. Although this cannot be ruled out from our results, we do not favor this hypothesis because in contrast with Met4, which was found to be a relatively stable protein (29), we observed that endogenous ATF4 is an unstable protein with a half-life of 30 min, further destabilized by overexpression of β TrCP, which shortens the half-life of the protein by a factor of two.

ATF4 has been described as both a negative regulator of CRE-dependent transcription (2, 32) and a positive regulator of transcription (5, 40, 66). One possibility for the difference is that forskolin, which activates the PKA pathway, was used in one experiment in which repression was observed (32) but was not used in experiments in which activation was observed. Another possibility is that different amounts of ATF4 were used in the experiments. As suggested previously (40), at high concentrations, ATF4 may repress transcription due to squelching. Alternatively, the difference in activity may be due to dimerization of ATF4 with other bZip proteins (20, 21, 57, 66). In our experiments, we showed that the inhibitory effect of ATF4 on CRE-dependent transcription of the somatostatin promoter is modulated by SCF $^{\beta$ TrCP-mediated ATF4 degradation. It remains to be demonstrated whether the other ATF4 transcriptional activities are also regulated by β TrCP.

Our observation that β TrCP is a nuclear protein differs from the previously reported localization of β TrCP by Winston et al. (67) as being largely cytoplasmic. However, our result is in agreement with the nuclear localization found by Hatakeyama et al. (reference 24 and personal communication). Such discrepancy with the results reported by Winston et al. (67) remains to be further evaluated. One possibility is that the cytoplasmic localization of β TrCP observed by Winston et al. could correspond to that of the β TrCP2 homolog of β TrCP (also called KIAA0696 [18, 26]). Indeed, we also found that β TrCP2, in addition to the nucleus, can be seen in the cytoplasm (unpublished results). The nuclear localization of β TrCP and its involvement in controlling the degradation of ATF4 illustrates the role that E3 ubiquitin ligases of the SCF type could play in the nucleus in mammalian cells. The question then arises whether the degradation of SCF $^{\beta$ TrCP substrates takes place in the nucleus rather than in the cytoplasm, as it is currently thought

for I κ B α and β -catenin, two substrates of β TrCP (31, 53). This hypothesis is consistent with recent results which reveal that tumor necrosis factor alpha can induce the degradation of nuclear I κ B α (28, 55). Interestingly, other components of the ubiquitination machinery have also been seen in the nucleus. These include the E2 ubiquitin-conjugating enzyme UBC9, which has been implicated in the proteolytic control of ATF2 (16), Cdc34, the E2 enzyme typically recruited by SCF complexes (41), Skp1 (17), Cullin1 (41), and the F-box protein Skp2 (41). Because several similar forms of β TrCP have been identified (26, 43), one can speculate that these isoforms could be differentially localized in cells, directing degradation of their substrates in different cellular compartments. For instance, we cannot at this point rule out the hypothesis that ATF4 is transported to the cytoplasm for its ubiquitination and degradation.

Interaction of ATF4 with β TrCP relies on motif DSGXXXS, similar but not identical to that found in the other substrates of β TrCP (HIV-1 Vpu, I κ B α , and β -catenin), which is DSGXXXS (23, 36, 39, 42, 43, 62, 67, 68). It is interesting to note that the first serine residue of this motif seems to play an essential role in this interaction while the second serine residue, which is important in the case of Vpu and β -catenin (unpublished results), is not required for the interaction of ATF4 with β TrCP. The recent discovery of the role of β TrCP in the processing of NF- κ B p105 supports the idea that the DSGXXXS motif is not the only determinant of interaction with β TrCP (50). The β TrCP recognition motif in p105 is very different from the DSGXXXS motif although it contains three phosphorylation sites important for interaction. However, p105 is a special case in the way it undergoes limited processing rather than complete destruction by the proteasome. Further work will be needed to determine which sequence requirements in addition to the DSGXXXS motif are playing a role in interaction with β TrCP.

One crucial step which triggers the interaction of substrates with β TrCP is their phosphorylation by a distinct protein kinase, which thus confers specificity on the ubiquitination process. β -catenin, I κ B α , and Vpu are phosphorylated on DSGXXS by GSK3/axin (53), IKK (31), and casein kinase II (58),

respectively. To understand more of how ATF4 is regulated, it is thus crucial to identify the kinase that phosphorylates ATF4 on its DSGXXXS motif. It could be that ATF4 is phosphorylated by the nuclear protein kinase Zip-kinase/Dlk, recently identified as an ATF4 binding protein, favoring ATF4 interaction with βTrCP (33, 34).

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