Signal-induced ubiquitination of I κ B α by the F-box protein Slimb/ β -TrCP

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Signal-induced phosphorylation of I κ B α targets this inhibitor of NF- κ B for ubiquitination and subsequent degradation, thus allowing NF- κ B to enter the nucleus to turn on its target genes. We report here the identification of an I κ B-ubiquitin (Ub) ligase complex containing the F-box/WD40-repeat protein, β -TrCP, a vertebrate homolog of *Drosophila* Slimb. β -TrCP binds to I κ B α only when the latter is specifically phosphorylated by an I κ B kinase complex. Moreover, immunopurified β -TrCP ubiquitinates phosphorylated I κ B α at specific lysines in the presence of Ub-activating (E1) and -conjugating (UbcH5) enzymes. A β -TrCP mutant lacking the F-box inhibits the signal-induced degradation of I κ B α and subsequent activation of NF- κ B-dependent transcription. Furthermore, *Drosophila* embryos deficient in *slimb* fail to activate *twist* and *snail*, two genes known to be regulated by the NF- κ B homolog, Dorsal. These biochemical and genetic data strongly suggest that Slimb/ β -TrCP is the specificity determinant for the signal-induced ubiquitination of I κ B α .

[**Key Words:** Phosphorylation; NF- κ B; I κ B; ubiquitin; SCF; Slimb; β -TrCP]

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Ubiquitin (Ub) is a small polypeptide that is covalently conjugated to protein substrates, thus committing these proteins for degradation (Ciechanover et al. 1978; Hershko et al. 1980; Wilkinson et al. 1980). Ub conjugation is catalyzed by an enzymatic cascade that begins with the ATP-dependent activation of Ub by a Ub-activating enzyme (E1) to form an E1-Ub thiolester. The activated Ub is then transferred to a Ub-conjugating enzyme (E2 or Ubc). Finally, in the presence of a Ub-protein ligase (E3), the carboxyl terminus of Ub is conjugated via an isopeptide bond to a lysine residue of the protein substrate. Processive conjugation of Ub to a previously conjugated Ub results in the formation of multiubiquitin chains that target the protein substrate for degradation by the 26S proteasome (Chau et al. 1989; for review, see Pickart 1997). In most cases, the specificity of protein degradation is determined by the identity of E3, which is operationally defined as a factor that binds to a specific protein substrate and facilitates its multi-ubiquitination.

Ub-dependent proteolysis plays a pivotal role in the regulation of many biological processes, including cell cycle progression, transcription, and signal transduction (for review, see Hochstrasser 1996; Hershko and Ciechanover 1998). The importance of the Ub pathway in the cell cycle is highlighted by recent studies on the degradation of cyclins and cyclin-dependent kinase (Cdk) inhibitors. Exit of cells from mitosis requires the degrada-

tion of mitotic cyclins, a step that is controlled by the activation of a 20S E3 complex known as anaphase-promoting complex (APC) or cyclosome (Hershko et al. 1994; King et al. 1995). Interestingly, the degradation of Cdk inhibitors such as Sic1, which triggers the entry of cell cycle into the S phase, is regulated by a distinct mechanism. In this case, phosphorylation of Sic1 at the end of G₁ allows this inhibitor to bind to Cdc4, a protein that contains two structural motifs, an F-box at the amino terminus and seven WD40 repeats at the carboxyl terminus. Through the F-box, Cdc4 tethers phosphorylated Sic1 to Skp1, which in turn binds to Cdc53, which then recruits an E2, Cdc34, to ubiquitinate Sic1 (Feldman et al. 1997; Skowyra et al. 1997). This so-called SCF pathway is also responsible for the ubiquitination of several other substrates (for review, see Patton et al. 1998). In each case, the substrate specificity is determined by the presence of a distinct F-box protein. For example, the F-box/leucine-zipper protein Grr1 binds to phosphorylated Cln1 and Cln2, but not Sic1 (Skowyra et al. 1997).

An F-box/WD40-repeat-containing protein called Slimb was identified recently in a genetic screen for recessive mutations that alter adult patterning in *Drosophila* (Jiang and Struhl 1998; Theodosiou et al. 1998). Loss of function of *slimb* causes supernumerary limbs as a result of ectopic activation of the Hedgehog (Hh) and Wnt/Wingless (Wg) pathways. In the Hh pathway, the transcription factor Cubitus interruptus (Ci) is proteolytically processed to a truncated repressor form in the absence of signaling (Aza-Blanc et al. 1997). This processing depends on protein kinase A (PKA) activity, which is

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antagonized by Hh signaling. By analogy to the SCF pathway in yeast (Feldman et al. 1997; Skowrya et al. 1997) and to the processing of NF- κ B1/p105 in mammals (Palombella et al. 1994), it was proposed that in the absence of Hh signaling, PKA phosphorylates Ci, thereby targeting Ci for Slimb-dependent processing via the Ub-proteasome pathway (Jiang and Struhl 1998). Similarly, the Wnt/Wg pathway is also regulated primarily through the stability of β -catenin/Armadillo (Arm), a putative cofactor of the transcriptional activator Lef1/TCF (Nusse 1997). In the absence of Wnt/Wg, β -catenin/Arm is phosphorylated by glycogen synthase kinase-3 (Gsk-3) or Zeste-White 3 (Zw3) and then degraded via the Ub-proteasome pathway (Aberle et al. 1997; Orford et al. 1997). Activation of the Wnt/Wg pathway leads to inhibition of Gsk-3/Zw3, thus allowing for the accumulation of β -catenin/Arm to turn on downstream genes in conjunction with Lef-1/TCF. The ectopic activation of the Wnt/Wg pathway and stabilization of Arm in *slimb* mutant cells suggests that Slimb may be required for the degradation of β -catenin/Arm.

The Gsk-3 phosphorylation sites on β -catenin are strikingly similar to those of I κ B, a family of inhibitory proteins that sequester the transcription factor NF- κ B in the cytoplasm of quiescent cells (for review, see Baldwin 1996; Baeuerle and Baltimore 1996). In response to a variety of stimuli, such as tumor necrosis factor α (TNF α), lipopolysaccharide (LPS) and ultraviolet light (UV), I κ B proteins are phosphorylated rapidly at specific serine residues by a 700-kD protein kinase complex (for review, see Maniatis 1997; Stancovski and Baltimore 1997; Scheidereit 1998). Phosphorylation of I κ B α at serines 32 and 36 targets this inhibitor for ubiquitination at lysines 21 and 22 (Chen et al. 1995; Scherer et al. 1995). Ubiquitinated I κ B α is then degraded specifically by the 26S proteasome, allowing NF- κ B to translocate into the nucleus. Two closely related E2s, Ubc4/5 and Ubch7/E2-F1, are capable of supporting the ubiquitination of I κ B α in vitro (Alkalay et al. 1995; Chen et al. 1996). However, the E3 responsible for I κ B α ubiquitination has remained unknown.

Recently, a human homolog of Slimb, h- β TrCP, was cloned in a yeast two-hybrid screen using human immunodeficiency virus (HIV) Vpu as a bait (Margottin et al. 1998). It was reported that h- β TrCP binds specifically to phosphorylated Vpu, which in turn binds to CD4 on T cells, resulting in the degradation of CD4 in the endoplasmic reticulum (ER). This study, however, did not reveal the function of h- β TrCP in normal cells (not infected with HIV). The structural and functional properties of Slimb/ β TrCP described above led us to hypothesize that it is a component of I κ B-Ub ligase [E3_{I κ B}]. This hypothesis is strongly supported by the evidence presented in this report.

Results

β TrCP binds to phosphorylated I κ B α

We cloned a mouse homolog of Slimb (m β TrCP, GenBank accession no. AF112979) based on two Slimb-re-

lated sequences from the mouse EST database (see Materials and Methods). m β TrCP is 79% (387/485) identical to *Drosophila* Slimb and 98% (560/569) identical to the recently cloned human β TrCP (h β TrCP; Margottin et al. 1998), whose normal physiological function was unknown. As an initial step in determining whether β TrCP binds to I κ B α , we synthesized ³⁵S-labeled I κ B α and m β TrCP by in vitro translation. I κ B α was phosphorylated by a MEKK1-activated I κ B kinase complex (Lee et al. 1997) and then incubated with m β TrCP. The binding of m β TrCP to I κ B α was determined by using a co-immunoprecipitation assay with an I κ B α -specific antibody. As shown in Figure 1A, phosphorylated I κ B α (p-I κ B α) bound to m β TrCP, whereas unphosphorylated I κ B α or the phosphorylation-defective I κ B α mutant (S32A/S36A) was unable to bind to m β TrCP (Fig. 1A, lanes 1–3). The binding of p-I κ B α to m β TrCP was detected under high stringency conditions (1% NP-40, 0.5% deoxycholate, and 0.1% SDS), suggesting a strong interaction. Similar results were obtained when I κ B α was phosphorylated by recombinant IKK β expressed from baculovirus-infected insect cells (data not shown). *Drosophila* Slimb and h β TrCP (kindly provided by Dr. Benarous, INSERM, Paris, France) also bound specifically to p-I κ B α (data not shown), suggesting that Slimb/ β TrCP functions are evolutionarily conserved.

To determine whether m β TrCP binds to p-I κ B α in vivo, 293 cells transfected with Myc-tagged m β TrCP were stimulated with calyculin A, a cell-permeable phosphatase inhibitor that allows for the accumulation of phosphorylated I κ B α (Chen et al. 1995). To block the degradation of phosphorylated I κ B α , cells were pretreated with the proteasome inhibitor MG132 before the addition of calyculin A. Cell extracts were then immunoprecipitated with an antibody against I κ B α , followed by immunoblotting with anti-Myc. The presence of Myc-m β TrCP in the anti-I κ B α precipitates was detected only when cells were stimulated with calyculin A (Fig. 1B, lanes 2,3). Conversely, when cells were immunoprecipitated with anti-Myc, only p-I κ B α but not unphosphorylated I κ B α was coprecipitated (Fig. 1B, lanes 10,11). Thus, m β TrCP binds specifically to p-I κ B α in cells.

Both I κ B α and p-I κ B α are bound tightly to NF- κ B, which is typically a heterodimer of p50 and p65 (for review, see Baldwin 1996). Similarly, the F-box protein β TrCP is likely to be part of a SCF complex that also includes Skp1 (Jiang and Struhl 1998; Margottin et al. 1998). Hence, our observation that p-I κ B α binds to β TrCP raises the possibility that the NF- κ B/p-I κ B α complex might associate with a SCF complex that contains both β TrCP and Skp1. To test this possibility, we examined the presence of p65 and Skp1 in anti-I κ B α and anti-Myc immunoprecipitates by immunoblotting with respective antibodies (Fig. 1B, lanes 2,3,10,11). Moreover, we immunoprecipitated calyculin A-stimulated cell extracts (see above) with a p65-specific antibody and then immunoblotted the precipitates with antibodies against Skp1, Myc, I κ B α , and p65, respectively (Fig. 1B, lanes 6,7). In each case, when cells were stimulated, all four proteins (p-I κ B α , p65, m β TrCP, and Skp1) were detected

in the same precipitates (Fig. 1B, lanes 3,7,11), indicating that they are present in the same complex. In contrast, in the absence of calyculin A treatment, while IκBα remained bound to p65, neither IκBα nor p65 was present in the anti-Myc precipitates (Fig. 1B, lanes 2,6,10, top two panels). Likewise, when cells were not stimulated, mβTrCP remained bound to Skp1, but neither was found in the anti-IκBα or anti-p65 immunoprecipitates (Fig. 1B, lanes 2,6,10, bottom two panels). These results strongly suggest that phosphorylation of IκBα leads to the assembly of a multiprotein complex that contains minimally p-IκBα, p65, βTrCP, and Skp1.

It has been proposed that the F-box mediates the binding to Skp1 of several F-box proteins including Cdc4 (Feldman et al. 1997; Skowyra et al. 1997) and hβTrCP (Margottin et al. 1998). In an effort to generate a dominant-negative mutant of mβTrCP that might allow us to investigate its function in vivo, we deleted the F-box from mβTrCP and tested the ability of this mutant (mβTrCPΔF) to bind to p-IκBα and Skp1, respectively. Deletion of the F-box did not prevent the binding of mβTrCP to p-IκBα either in vitro (Fig. 1A, lane 5) or in vivo (Fig. 1B, lane 5,9,13, top panel). However, this deletion abolished the binding of mβTrCP to Skp1 (Fig. 1B, cf. lanes 10 and 12, bottom panel) and compromised the recruitment of p-IκBα/p65 to a complex containing Skp1 (Fig. 1B, cf. lanes 7 and 9, bottom panel).

Interestingly, both mβTrCP and mβTrCPΔF appeared

to be phosphorylated when cells were stimulated with calyculin A (Fig. 1B, lanes 11,13; also see Fig. 1C, lane 2). Furthermore, calyculin A treatment leads to a weak but detectable binding of mβTrCPΔF to Skp1 (Fig. 1B, cf. lanes 12,13, bottom panel). It is possible that when it is phosphorylated, mβTrCP itself is recruited for ubiquitination by a SCF complex that includes Skp1. In this case, the F-box may be dispensable, as the binding can be mediated by the interaction between certain phosphorylation sites on βTrCP and a SCF complex. Ubiquitination and degradation of other F-box proteins such as Cdc4 and Grr1 within the SCF complexes has been reported recently (Zhou and Howley 1998). We also noted a low level of Skp1 in the anti-IκBα precipitates from mβTrCPΔF-expressing cells (Fig. 1B, cf. lanes 3 and 5, bottom panel). This may reflect a pool of endogenous p-IκBα/βTrCP/Skp1 complexes that were not displaced by mβTrCPΔF.

We noticed that phosphorylated p65 was also detected following calyculin A treatment (Fig. 1B, lane 7). It is not clear whether PKA or another cytokine-inducible kinase is responsible for the phosphorylation of p65 in this case (Zhong et al. 1997; Wang and Baldwin 1998). Notwithstanding this uncertainty, the observation that p65 is phosphorylated raises the possibility that phosphorylated p65 may bind to βTrCP directly, whereas p-IκBα binds to βTrCP indirectly through p65. To test this possibility, we transfected 293 cells with the Myc-tagged

m β TrCP expression construct, together with Flag-tagged I κ B α (F-I κ B α) or a phosphorylation-defective I κ B α mutant lacking the amino-terminal 36 residues (F-I κ B α Δ N; Brockman et al. 1995). Following treatment with MG132 and calyculin A, cell extracts were immunoprecipitated with a Flag-specific antibody and then immunoblotted with anti-Myc or anti-Skp1 antibodies (Fig. 1C). Like endogenous I κ B α , transfected F-I κ B α associated with Myc-m β TrCP and Skp1 when cells were stimulated with calyculin A (Fig. 1C, lane 2). We also observed a low but detectable level of binding between transfected F-I κ B α and m β TrCP in the absence of calyculin A treatment (Fig. 1C, cf. lanes 1 and 2). This may be due to overexpression of F-I κ B α , a small but significant fraction of which may be phosphorylated at the signaling sites even in the absence of calyculin A. In contrast to F-I κ B α , transfected F-I κ B α Δ N did not co-immunoprecipitate with either Myc-m β TrCP or Skp1 even when cells were treated with calyculin A (Fig. 1C, lanes 3,4). However, F-I κ B α Δ N remained bound to p65. Therefore, p65 is recruited to a β TrCP/Skp1-containing complex by virtue of its association with p-I κ B α , implying that p-I κ B α binds to β TrCP directly. Further supporting this idea, unphosphorylated p65 was present in the anti-Myc immunoprecipitates, whereas only p-I κ B α coprecipitated with Myc-m β TrCP (Fig. 1B, lanes 11,13).

We also found that the electrophoretic mobility of F-I κ B α Δ N was reduced slightly when cells were treated with calyculin A (Fig. 1C, cf. lanes 3 and 4), which is indicative of phosphorylation outside the inducible amino-terminal phosphorylation sites. However, phosphorylation at noninducible sites did not lead to the binding of I κ B α to m β TrCP or Skp1, suggesting that the binding of m β TrCP to I κ B α is strictly dependent on signaling and that m β TrCP does not simply bind to phosphorylated proteins indiscriminately.

Dominant-negative β TrCP blocks I κ B α degradation and NF- κ B activation

The ability of m β TrCP Δ F to bind to p-I κ B α , together with its inability to bind to Skp1, suggests that this mutant might be a dominant-negative inhibitor of I κ B α degradation, provided that β TrCP is involved in this pathway. To test the *in vivo* function of m β TrCP, 293 cells were transfected with m β TrCP Δ F, together with a Flag-tagged I κ B α expression construct. The transfected cells were stimulated with TNF α for 30 min, and the degradation of transfected and endogenous I κ B α analyzed by immunoblotting with antibodies specific for Flag or I κ B α , respectively (Fig. 2). With increasing concentration of m β TrCP Δ F, there was a concentration-dependent stabilization of the phosphorylated forms of both transfected and endogenous I κ B α . High concentrations of wild-type m β TrCP also led to a slight stabilization of p-I κ B α (data not shown). This finding may be explained if some of the overexpressed m β TrCP is not incorporated into a functional SCF complex but still binds to p-I κ B α . The sequestered p-I κ B α may be protected from ubiquitination.

Figure 2. β TrCP is required for TNF α -induced degradation of I κ B α . 293 cells were transfected with pCDNA3-Flag-I κ B α (20 ng), together with increasing concentration of pcDNA3-m β TrCP Δ F (lanes 1,2, vector only; lanes 3,4, 0.01 μ g; lanes 5,6, 0.1 μ g; lanes 7,8, 1 μ g). After treatment of cells with or without TNF α (20 ng/ml) for 30 min, cell extracts were analyzed by immunoblotting with antibodies against Flag, I κ B α , or Myc, respectively.

To investigate the role of β TrCP in NF- κ B activation, we transfected 293 cells with a m β TrCP Δ F expression construct, together with a luciferase reporter gene, which is under the control of three tandem repeats of NF- κ B binding sites (Fig. 3A). As a control, we also examined the expression of a GAL4-dependent reporter gene. TNF α -induced expression of the NF- κ B reporter, but not GAL4VP16-activated expression of the GAL4 reporter, was severely inhibited with increasing concentration of m β TrCP Δ F. This result suggests that β TrCP is required for TNF α -induced activation of NF- κ B.

Degradation of I κ B α is required for NF- κ B activation by many different stimuli (for review, see Baldwin 1996). If β TrCP is an obligatory component that mediates I κ B α degradation, it is expected that interference of β TrCP function should compromise the activation of NF- κ B by multiple stimuli. To address this possibility, we examined the effects of m β TrCP Δ F on the induction of NF- κ B reporter gene by interleukin-1 β (IL-1 β), NIK, MEKK1, and IKK β , respectively (Fig. 3B). In each case, NF- κ B activation was inhibited markedly by m β TrCP Δ F, strongly suggesting that β TrCP participates in the activation of NF- κ B by multiple signaling pathways.

Slimb is required for Dorsal-dependent activation of twist and snail in *Drosophila* embryos

Although the cell culture experiments shown above support the involvement of β TrCP/Slimb in NF- κ B activation, it is imperative to determine whether the same conclusion can be reached in animal models that are amenable to genetic manipulations. In this regard, *Drosophila* embryos provide an excellent model system, not only because *slimb*-deficient embryos can be generated (Jiang and Struhl 1998) but also because there is a highly conserved signaling pathway in *Drosophila* that is analogous to that of NF- κ B/I κ B (for review, see Morisato and Anderson 1995). In *Drosophila* early embryos, dorsoventral patterning is established by a nuclear concentration gradient of the Dorsal morphogen, a homolog of NF- κ B. In the ventral region of the *Drosophila* embryo, local

Figure 3. Dominant-negative β TrCP inhibits the activation of NF- κ B-dependent transcription. (A) Effect of m β TrCP Δ F on induction of the NF- κ B-dependent reporter gene by TNF α . 293 cells were transfected with pcDNA₃-m β TrCP Δ F (0.01–3 μ g as indicated), together with a NF- κ B-dependent (●) or GAL4-dependent (□) luciferase reporter construct (p[κ B]₃-tk-Luc, or Gal4-E1b-Luc, 10 ng each). The NF- κ B reporter gene was stimulated with TNF α (20 ng/ml) for 6 hr prior to harvest, whereas the GAL4 reporter gene was activated by cotransfection with the strong activator GAL4-VP16 (200 ng). Reporter activity is expressed as fold induction of normalized luciferase activity in stimulated cells relative to that of unstimulated cells. TNF α stimulated the NF- κ B reporter by 14-fold, whereas the GAL4-VP16 activation was >3000-fold. (B) Effect of m β TrCP Δ F on the activation of NF- κ B by IL-1 β , NIK, MEKK1, and IKK β . 293 cells were transfected with pcDNA₃-m β TrCP Δ F and p[κ B]₃-tk-Luc as described in A, together with 100 ng of NIK, MEKK1, and IKK β expression constructs, respectively. Stimulation of cells with IL-1 β (10 ng/ml) was carried out for 6 hr prior to harvest. Luciferase activity was determined and normalized as described in Materials and Methods.

activation of Toll, a homolog of mammalian IL-1 receptor, results in degradation of the I κ B-like protein Cactus. Consequently, Dorsal translocates into the nucleus where it activates downstream genes that include *twist* (*twi*) and *snail* (*sna*) (for review, see Morisato and Ander-

son 1995). To explore the role of Slimb in Dorsal activation in vivo, we generated *slimb*-deficient embryos and examined the expression of *twi* and *sna* by whole-mount in situ RNA hybridization. As shown in Figure 4, wild-type embryos expressed *twi* and *sna* in the ventral region (Fig. 4A,C). In contrast, *slimb*-deficient embryos expressed markedly reduced levels of *twi* and *sna* in most of the ventral region (Fig. 4B,D). The residual expression of *twi* and *sna* at the posterior pole may be due to modification of the Dorsal/Cactus pathway by terminal signaling, such that reduced dosage of Dorsal is sufficient to activate the polar expression of *twi* and *sna* (Ray et al. 1991). This staining pattern in *slimb*-deficient embryos is reminiscent of what has been described in *cactus* gain-of-function mutant embryos (Roth et al. 1991), strongly suggesting that Slimb/ β TrCP is required for Cactus/I κ B degradation in vivo.

Slimb/ β TrCP is a component of I κ B-Ub ligase (E3_{I κ B})

To demonstrate directly that β TrCP is a component of E3_{I κ B}, we expressed Myc-tagged m β TrCP in 293 cells by transient transfection and then purified m β TrCP containing complex by immunoprecipitation using a Myc-specific antibody. The immunoprecipitates were used directly as the source of E3 in a reconstituted I κ B α ubiquitination assay (Chen et al. 1995). The reconstituted system contains in vitro-translated ³⁵S-labeled I κ B α phosphorylated by the MEKK1-activated I κ B kinase complex, recombinant p50/p65 (to form a complex with I κ B α), purified E1, recombinant Ubch5 (which was shown previously to support the ubiquitination of I κ B α in vitro), Ub, and ATP. The presence of Ubch5 in the reaction led to the formation of low molecular mass ubiquitinated I κ B α even in the absence of any E3 (i.e.,

Figure 4. Slimb is required for the Dorsal-dependent activation of *twi* and *sna* in *Drosophila* embryos. Wild-type (A,C) and *slimb* mutant (B,D) embryos were hybridized with *twi* (A,B) or *sna* (C,D) antisense RNA probes to visualize their expression. Embryos were oriented with anterior to the left and dorsal up. Wild-type embryos at the blastoderm stage express the Dorsal target genes *twi* and *sna* in the ventral region (A,C). In contrast, *slimb* mutant embryos at the same stage show diminished expression of both *twi* and *sna* in most of the ventral region, with residual expression detectable at the posterior pole (arrows in B and D).

mono-ubiquitinated I κ B α was evident in Fig. 5A, lanes 1 and 6, but not in lane 4). However, it has been shown that proteins bearing one or a few Ub molecules are poor substrates for the 26S proteasome (Chau et al. 1989). Only high molecular mass conjugates, whose synthesis usually requires E3s, can be degraded efficiently. As shown in Figure 5, addition of immunoprecipitates containing m β TrCP to the reconstituted system led to efficient multi-ubiquitination of phosphorylated I κ B α . In contrast, the phosphorylation-defective I κ B α mutant (S32A/S36A) was not ubiquitinated. A control IgG failed to immunoprecipitate any Ub ligase activity from the same extracts. Similarly, immunoprecipitates containing m β TrCP Δ F were also unable to support the ubiquitination of p-I κ B α , most likely because of the inability of this mutant to bind to Skp1 (Fig. 5A, bottom panel).

It has been shown previously that mutation of lysines 21 and 22 of I κ B α impair the ubiquitination and degradation of I κ B α significantly, suggesting that these two lysines are the primary ubiquitination sites on I κ B α (Scherer et al. 1995). We examined whether m β TrCP immunoprecipitates could ubiquitinate I κ B α at specific sites in the reconstituted assay. As shown in Figure 5, the I κ B α mutant in which lysines 21 and 22 were mutated to arginine (K21R/K22R) was only weakly ubiquitinated (~40% of wild type; Fig. 5B), consistent with its weaker dominant-negative effect on NF- κ B activation than the S32A/S36A mutant (Scherer et al. 1995). In contrast, mutation of the nearby lysines 38 and 47 (K38R/K47R) did not impair the ubiquitination of I κ B α in the reconstituted system. Therefore, m β TrCP not only binds specifically to p-I κ B α but also promotes the ubiquitination of phosphorylated I κ B α at the physiologically relevant lysine residues in the presence of E1 and E2, thus fulfilling the major criteria for a bona fide E3_{I κ B}.

Discussion

In this report we have shown that β TrCP/Slimb exhibits several critical features expected of the substrate recognition subunit of I κ B-Ub ligase (E3_{I κ B}). First, β TrCP binds specifically to phosphorylated, but not unphosphorylated, I κ B α , both in vitro and in vivo. Second, a dominant-negative β TrCP mutant blocks the signal-induced degradation of I κ B α and the activation of NF- κ B. Third, *Drosophila slimb*-deficient embryos fail to activate *twi* and *sna*, two genes regulated by Dorsal, the *Drosophila* homolog of NF- κ B. Finally, a β TrCP-containing complex ubiquitinates phosphorylated I κ B α in the presence of E1 and Ubch5.

β TrCP/Slimb is an F-box protein and is likely to function within a SCF complex that serves as an E3 for I κ B. Deletion of the F-box abolishes the binding of β TrCP to Skp1 and also abolishes its ability to support the ubiquitination of p-I κ B α in vitro (Fig. 5A). Three F-box proteins involved in the ubiquitination of cell cycle proteins in yeast, including Cdc4, Grr1, and Met30, function as part of SCF complexes (Patton et al. 1998). In all three cases, the role of F-box is to mediate binding to Skp1, which in turn binds to Cdc53, which recruits an E2

Figure 5. β TrCP promotes ubiquitination of phosphorylated I κ B α in vitro. (A) Reconstitution of I κ B α ubiquitination. ^{35} S-labeled I κ B α or mutants were incubated in an ATP-supplemented reconstitution system containing E1, Ubch5, Ub, p50/p65, I κ B kinase and MEKK1 Δ , as described in Materials and Methods. Ubiquitination was initiated by the addition of immunoprecipitated beads containing Myc-m β TrCP, which was expressed in 293 cells. Ubiquitinated products were analyzed by SDS-PAGE following immunoprecipitation with an anti-p65 antibody, which coprecipitated I κ B α , as well as ubiquitinated I κ B α (Chen et al. 1995). (Lane 1) No E3 was added; (lane 2) immunoprecipitated Myc-m β TrCP Δ F was used in place of Myc-m β TrCP; (lane 3) normal rabbit IgG instead of Myc antibody was used to precipitate Myc-m β TrCP; (lane 4) no E2 was added; (lanes 5–8) Myc-m β TrCP (FL) beads were used to ubiquitinate wild-type and mutant I κ B α as indicated; (lanes 9–12) 293 cell extracts (S100) were used to ubiquitinate wild-type and mutant I κ B α as indicated. An aliquot of the immunoprecipitated beads used in the ubiquitination reaction was analyzed by western blotting using antibodies against Myc (middle panel) or Skp1 (bottom panel). (B) Quantitation of I κ B α ubiquitination. Ubiquitinated I κ B α shown in A was quantitated with the aid of PhosphorImager. Because only multiubiquitinated I κ B α are degraded efficiently by the 26S proteasome, Ub_n-I κ B α containing more than two Ub units ($n > 2$) was quantitated and expressed as the percentage of total radioactivity in each lane.

(Cdc34) to the SCF complex. Based on evidence presented in this report and by analogy to the SCF complexes involved in cell cycle, we propose a model suggesting that a β TrCP-containing SCF complex is responsible for the signal-induced ubiquitination of $\text{I}\kappa\text{B}\alpha$ (Fig. 6). In this model, β TrCP associates with Skp1, which in turn binds to a Cdc53-like protein whose identity remains to be determined. When cells are stimulated with NF- κ B agonists, $\text{I}\kappa\text{B}\alpha$ is phosphorylated at serines 32 and 36 by an $\text{I}\kappa\text{B}$ kinase complex. Phosphorylated $\text{I}\kappa\text{B}\alpha$ is recruited to the $\text{SCF}^{\beta\text{TrCP}}$ complex through its binding to β TrCP. An E2, such as Ubch5, binds to the SCF complex and ubiquitinates the nearby $\text{I}\kappa\text{B}\alpha$ at lysines 21 and 22. Because of the high-affinity binding of p- $\text{I}\kappa\text{B}\alpha$ to β TrCP, ubiquitinated $\text{I}\kappa\text{B}\alpha$ is not released from SCF and is processively multi-ubiquitinated to form a multi-Ub chain. Multi-Ub chains on $\text{I}\kappa\text{B}\alpha$ recruit the 26S proteasome to degrade $\text{I}\kappa\text{B}\alpha$, allowing NF- κ B to translocate into the nucleus, where it activates target genes.

Two components in this $\text{SCF}^{\beta\text{TrCP}}$ pathway remain to be identified. First, what is the third component of the $\text{SCF}^{\beta\text{TrCP}}$ complex? It is now known that there is a large family of Cdc53-related proteins belonging to the Cullin family (Jackson 1996; Kipreos et al. 1996). Further work is needed to determine whether a mammalian homolog of Cdc53 or a distinct member of the family is involved in the assembly of the $\text{SCF}^{\beta\text{TrCP}}$ complex. Second, what is the identity of $\text{E2}_{\text{I}\kappa\text{B}}$? Although we and others have shown that Ubch5 or Ubch7 can ubiquitinate $\text{I}\kappa\text{B}\alpha$ in vitro (Alkalay et al. 1995; Chen et al. 1996), this does not rule out the possibility that other E2s might also be involved. It remains to be determined which E2 (or E2s) functions in the signal-induced ubiquitination of $\text{I}\kappa\text{B}\alpha$ in vivo.

Another important question concerns the dynamics of $\text{SCF}^{\beta\text{TrCP}}$ complex assembly and substrate binding. Co-immunoprecipitation experiments showed that β TrCP is associated with Skp1 regardless of signaling (Fig. 1), suggesting that $\text{SCF}^{\beta\text{TrCP}}$ is a preexisting complex in cells and that this complex binds further to p- $\text{I}\kappa\text{B}\alpha$ /NF- κ B to form a larger complex upon signaling. This dynamic assembly process is consistent with the previous report that $\text{I}\kappa\text{B}\alpha$ eluted as part of a high molecular weight

complex when TNF α -stimulated cell extracts were fractionated by gel filtration (Yaron et al. 1997). The dynamic nature of SCF complex assembly is underscored further by the recent finding that several F-box proteins in yeast are short-lived as a result of their own ubiquitination within SCF complexes (Zhou and Howley 1998). The rapid turnover of F-box proteins may provide an opportunity for different F-box proteins to compete for a limited pool of core SCF components such as Skp1. It is not known at present whether and how this combinatorial assembly of SCF complexes is regulated. The rapid degradation of F-box proteins also raises the possibility that these proteins might be limiting factors that control the rate of degradation of their target proteins. This may explain why overexpressed $\text{I}\kappa\text{B}\alpha$ is not degraded efficiently in response to signals despite efficient phosphorylation (Traenckner et al. 1995).

Ubiquitination of $\text{I}\kappa\text{B}\alpha$ by the β TrCP-containing complex in vitro occurs primarily at lysines 21 and 22, and is strictly dependent on its phosphorylation at serines 32 and 36, thus recapitulating the in vivo setting (Fig. 5). Interestingly, the specificity of $\text{I}\kappa\text{B}\alpha$ ubiquitination is compromised when it is not bound to NF- κ B (Z.J. Chen, unpubl.). For example, free $\text{I}\kappa\text{B}\alpha$ mutants (i.e., S32A/S36A) can be ubiquitinated in the reconstituted system, albeit much more weakly. It has been shown that free $\text{I}\kappa\text{B}\alpha$ is a short-lived protein that can be stabilized by binding to NF- κ B (Scott et al. 1993). The binding of NF- κ B to $\text{I}\kappa\text{B}\alpha$ is mediated primarily through the ankyrin repeats of $\text{I}\kappa\text{B}\alpha$, which encompasses the bulk of the molecule (Haskill et al. 1991). It is therefore possible that NF- κ B masks the majority of the $\text{I}\kappa\text{B}\alpha$ molecule except for the amino-terminal regulatory sequence, thus rendering the degradation of bound $\text{I}\kappa\text{B}\alpha$ dependent on signaling. The binding of NF- κ B to $\text{I}\kappa\text{B}\alpha$ may also minimize the accessibility of lysine residues to ubiquitination enzymes, thereby facilitating the ubiquitination of $\text{I}\kappa\text{B}\alpha$ at specific lysine residues. This may explain why $\text{I}\kappa\text{B}\alpha$ is one of the few proteins in which ubiquitination sites can be defined (Scherer et al. 1995).

β TrCP/Slimb has now been implicated in the ubiquitination of several proteins, including $\text{I}\kappa\text{B}$ /Cactus, β -catenin/Arm, CD4 (through HIV Vpu), and Ci. How-

Figure 6. A proposed SCF pathway for the signal-induced ubiquitination of $\text{I}\kappa\text{B}\alpha$. β TrCP/Slimb associates with Skp1 and another protein (possibly Cdc53-like) to form a $\text{SCF}^{\beta\text{TrCP}}$ complex. Upon phosphorylation of $\text{I}\kappa\text{B}\alpha$ by an $\text{I}\kappa\text{B}$ kinase complex, β TrCP recruits $\text{I}\kappa\text{B}\alpha$ to the SCF complex, allowing the associated E2, such as Ubch5, to ubiquitinate $\text{I}\kappa\text{B}\alpha$. Following ubiquitination, $\text{I}\kappa\text{B}\alpha$ is rapidly degraded by the 26S proteasome, allowing NF- κ B to translocate into the nucleus where it activates gene transcription. NF- κ B is represented by a heterodimer of p50 and p65, whereas a multiubiquitin chain is represented by the branch structure on $\text{I}\kappa\text{B}\alpha$.

ever, I κ B α is the only protein so far shown to be directly ubiquitinated by the β TrCP-containing complex in vitro. Strikingly, the phosphorylation sites among Vpu, I κ B, and β -catenin are very similar, with a minimal consensus sequence of DSG Ψ -S (Ψ represents a hydrophobic residue). Although the serine residues of these proteins appear to be phosphorylated by distinct kinases, the fates of these proteins after phosphorylation are likely to be the same in terms of their binding to β TrCP/Slimb and subsequent ubiquitination and degradation. The only exception is Vpu, which does not appear to be degraded. Instead, it was proposed that Vpu targets its cognate partner CD4 to the ER degradation pathway (Margottin et al. 1998). It will be important to determine whether Vpu or CD4 is ubiquitinated to understand why and how Vpu escapes degradation by the SCF pathway.

The potential involvement of β TrCP/Slimb in the NF- κ B, Hh, and Wg raises the exciting possibility that these divergent pathways may in fact be interconnected and that β TrCP/Slimb may be deployed from one pathway to another to allow for integration of these pathways in response to signals. Further studies on β TrCP/Slimb may provide another avenue for modulating the activity of these pathways, all of which have been implicated in several human diseases, including cancer.

Materials and methods

Plasmids, antibodies, and chemicals

cDNAs encoding I κ B α and mutants (S32/36A, Δ N, K21/22R, K38/47R) were gifts of Dr. Dean Ballard (Vanderbilt University, Nashville, TN) and have been described previously (Brockman et al. 1995; Chen et al. 1995; Scherer et al. 1995). A luciferase reporter construct containing three tandem repeats of NF- κ B binding sites [p κ B]₃-TK-Luc) was a gift of Dr. Shigeki Miyamoto (University of Wisconsin, Madison). Rabbit anti-I κ B α (C15 and C21), anti-Myc (A14), anti-Skp1 (H163), and goat anti-p65 (C20G) polyclonal antibodies, as well as mouse anti-Myc monoclonal antibody (9E10), were purchased from Santa Cruz Biotechnology. Mouse anti-Flag antibodies (M2 and M5) were from Eastman Kodak. MG132 (z-Leu-Leu-Leu-H) was purchased from Calbiochem. Calyculin A and okadaic acid were purchased from Alexis.

Proteins

Ub was purchased from Sigma. Ub aldehyde (Ubal) was produced by periodate oxidation of Ubdiol, which was synthesized using the carboxypeptidase Y method (Lam et al. 1997). Ubch5 was derived from GST-Ubch5 (Chen et al. 1996) by thrombin cleavage and then purified to apparent homogeneity by MonoS fast performance chromatography (FPLC, Pharmacia). E1 was purified from calf thymus by covalent affinity chromatography on Ub-Sepharose. Recombinant (His)₆-MEKK1 Δ was purified from baculovirus-infected Sf9 cells as described previously (Lee et al. 1997; baculovirus provided by Drs. Frank Lee and Tom Maniatis, Harvard University, Cambridge, MA). I κ B kinase complex was partially purified as described previously, except that calf thymus instead of HeLa cells was used as the source of the kinase. The kinase activity from the Superdex fractions can be activated by ubiquitination (Chen et al. 1996), by MEKK1

(Lee et al. 1997), or by NIK (Z.J. Chen, unpubl.), and was used for in vitro phosphorylation of I κ B α at serines 32 and 36. Further purification and characterization of the I κ B kinase complex will be reported elsewhere. GST-p50 and p65-His₆ were expressed from *Escherichia coli* and purified as described previously (Thanos and Maniatis 1992). To allow for the formation of p50/p65 heterodimer, these proteins were mixed in the presence of buffer A (20 mM Tris at pH 7.5, 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM PMSF, 0.1 M NaCl) plus 6 M urea and then dialyzed against the same buffer overnight. The proteins were renatured by step-wise reduction of urea concentration to 4 M, 2 M, and finally no urea in buffer A. In some cases, the proteins were further purified by glutathione chromatography. The functionality of the renatured p50/p65 heterodimer was confirmed by its ability to coimmunoprecipitate in vitro-translated ³⁵S-labeled I κ B α .

cDNA cloning of m β TrCP and m β TrCP Δ F

A BLAST search of the EST database for mammalian homologs of Slimb (Jiang and Struhl 1998) and *Xenopus* β TrCP (Spevak et al. 1993) identified two clones (AA197590 and AA033076) that together encompass ~370 amino acids corresponding to the carboxyl terminus of the mouse homolog of Slimb/ β TrCP (m β TrCP). PCR primers derived from these two clones (5'-GCGGTCGACCGTCAGGACGGACTCTCTGTGG-3' and 5'-AGTGCGGCCGCTTATCTGGAGATGTAGGTGTA-3') were used to amplify a 1.1-kb fragment (m β TrCP Δ N) from a mouse E8.5 (embryonic day 8.5) cDNA library in λ ZAPXR (Stratagene). The 5'-coding region of m β TrCP was amplified from the same library by PCR using T7 primer and a reverse m β TrCP primer (5'-AGTGCGGCCGCGAGCTTTTCCACAGCATGCC-3'). A 650-bp fragment was subcloned into *Bam*HI and *Not*I sites of pBluescript (Stratagene) and sequenced. This fragment contains the full-length 5'-coding sequence plus 60 bp of 5'-untranslated sequence. To generate a full-length m β TrCP that is fused in-frame with five tandem repeats of amino-terminal Myc epitopes, the amino terminus of m β TrCP was amplified by PCR using the following primers: 5'-CGCCCATGGACCCGGCAGAGGCGGTG-3' and 5'-CGCTCTGCCAGGCCTCGCCACAGA-3'. A 600-bp fragment was subcloned into the *Nco*I and *Stu*I sites of pSK-Myc-Slimb (Jiang and Struhl 1998) to replace the amino terminus of Slimb. The carboxyl terminus of Slimb was subsequently replaced with a *Stu*I-*Not*I fragment (1.1 kb) of m β TrCP Δ N. Following partial digestion with *Kpn*I and *Not*I, the full-length Myc-m β TrCP fragment (~1.9 kb) was subcloned into pcDNA₃. The entire coding region was confirmed by DNA sequencing.

To delete the F-box from m β TrCP, the amino terminus of m β TrCP up to the F-box was amplified by PCR (5'-CGCCCATGGACCCGGCAGAGGCGGTG-3' and 5'-GGCGACGTCGCAGTTATGAAATCCCTCTG-3'). The amplified fragment (450 bp) was then cloned into the *Nco*I-*Aat*II sites of pSK-Myc-m β TrCP to replace the amino terminus of m β TrCP, which includes almost the entire F-box (34 of approximately 40 residues; Bai et al. 1996). Following confirmation by DNA sequencing, the resulting m β TrCP Δ F, including the amino-terminal Myc coding sequence, was subcloned into the *Kpn*I-*Not*I sites of pcDNA₃.

Cell culture, transfection, and reporter assays

Human embryonic kidney 293 cells were grown on 0.1% gelatin-coated plates in DMEM supplemented with 10% fetal calf serum. Transfection of 293 cells was carried out by the calcium phosphate precipitation method (Sambrook et al. 1989), which

yielded nearly 100% transfection efficiency (data not shown). For reporter gene assays, 293 cells were seeded into six-well plates (4×10^5 cells/well) and then transfected with 10 ng of p[κ B]₃-TK-Luc reporter construct, 10 ng of pCMV-*lacZ*, and various amounts of other expression constructs. The total DNA concentration was kept constant at 3 μ g by supplementing with pcDNA₃ vector. After 40 hr, cells were treated with or without TNF α (20 ng/ml) for 6 hr prior to harvest for luciferase assay (Promega). Transfection efficiencies were normalized by β -galactosidase activities, and the reporter gene activity was expressed as the mean of normalized luciferase activity from duplicated experiments.

In vitro translation, immunoprecipitation, and immunoblotting

³⁵S-Labeled I κ B α and its mutants were synthesized in wheat germ extracts programmed with the corresponding RNA using TNT in vitro translation kits (Promega). ³⁵S-labeled m β TrCP and m β TrCP Δ F were produced similarly, except that rabbit reticulocyte lysates were used in place of wheat germ extracts. To analyze the binding of I κ B α to m β TrCP in vitro, ³⁵S-labeled I κ B α or S32A/S36A mutant was phosphorylated by MEKK1-activated I κ B kinase as described previously (Lee et al. 1997) and incubated with equal amounts of ³⁵S-labeled m β TrCP or m β TrCP Δ F at 4°C in 0.15 ml of RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate) plus 0.1% SDS. After 90 min of incubation, 0.1 μ g of anti-I κ B α antibody (C21, Santa Cruz) preadsorbed to protein A-Trisacryl (Pierce) was added, and the mixture was incubated further at 4°C for 1 hr with end-over-end rotation. The beads were then washed three times with 0.75 ml of RIPA/0.1% SDS and resuspended in Laemmli/SDS sample buffer. Proteins bound to the beads were resolved by 9% SDS-PAGE and analyzed with the aid of the PhosphorImager (Molecular Dynamics).

For analysis of I κ B α binding to m β TrCP in vivo, 293 cells were plated on 10-cm dishes (10^6 cells/plate) and transfected with up to 10 μ g of total DNA containing various expression constructs. Forty-eight hours after transfection, cells were pretreated with 20 μ M of MG132 for 30 min, followed by stimulation with 0.1 μ M calyculin A for 10 min. After washing with cold PBS, cells were resuspended in lysis buffer (20 mM Tris at pH 7.5, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM NaVO₃, 0.5 mM PMSF, 5 μ g/ml leupeptin, 0.2 mM EGTA) and lysed on ice for 15 min. Cell debris was removed by centrifugation, and the supernatant was used for immunoprecipitation with appropriate antibodies as described above, except that lysis buffer was used in place of RIPA buffer. Immunoprecipitated proteins were analyzed by immunoblotting and visualized by either colorimetric (Promega) or enhanced chemiluminescence (ECL, Amersham) methods.

In vitro ubiquitination assays

Myc-tagged m β TrCP was expressed in transiently transfected 293 cells and then purified by immunoprecipitation using anti-Myc polyclonal antibody (A14, Santa Cruz) bound to protein A-Sepharose. After extensive washing with lysis buffer, the beads were washed further with buffer A (10 mM Tris-HCl at pH 7.5, 0.5 mM DTT, 1.5 mM MgCl₂, 10 mM KCl, 5 μ g/ml leupeptin) and aliquoted into assay tubes to be used as E3 for ubiquitination reactions. The reaction conditions were modified from what was described previously (Chen et al. 1995; Scherer et al. 1995) to accommodate the reconstitution with E1, E2, and E3. Briefly, ³⁵S-labeled I κ B α or mutants were incubated with recom-

binant p50/p65 (50 ng) to form a complex in a reaction mixture (20 μ l) containing an ATP-regenerating buffer (Chen et al. 1995), Ub (60 μ M), Uba1 (1.5 μ M), okadaic acid (3 μ M), MG132 (10 μ M), MEKK1 (5 nM), I κ B kinase complex (75 ng/ μ l), E1 (0.1 μ M), and UbcH5 (0.6 μ M). Reactions were initiated by adding the reaction mixture into the tubes containing m β TrCP beads. After 1 hr of incubation at 37°C, 150 μ l of cold lysis buffer containing 0.4 μ g of anti-p65 bound to protein A/G-Trisacryl was added to the reaction, and the mixture was incubated at 4°C for 1 hr with end-over-end rotation. The beads were washed once with 0.7 ml of lysis buffer, and the bound proteins analyzed by 9% SDS-PAGE followed by fluorography. Ubiquitinated I κ B α was quantitated by PhosphorImager.

Generation of slimb mutant Drosophila embryos and whole-mount in situ hybridization

An EMS-induced allele of *slimb*, *slimb2*, was used to generate embryos deficient in both the maternal and zygotic *slimb* function. These mutant embryos were generated by the dominant female sterile technique (Chou and Perrimon 1992). Females of the genotype *y*, *hs-flp121/y*; *FRT82 slimb2/FRT82 ovoD* were subjected to multiple heat shock at pupal stages to induce germline clones, which were then mated with *FRT 82 slimb2/TM2* males. Embryos were collected from such crosses for whole-mount in situ hybridization with *twi* or *sna* antisense RNA probes as described previously (Jiang et al. 1991).

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Note

The GenBank accession number for the mouse β TrCP cDNA sequence reported in this paper is AF112979.

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