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A complex containing βTrCP recruits Ccd34 to catalyse ubiquitination of IκBα

Laurent Vuillard, John Nicholson, Ronald T. Hay*

School of Biomedical Science, BMS Building, University of St. Andrews, St. Andrews, Fife KY16 9ST, UK

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Abstract Activation of transcription factor NF-κB is accomplished by degradation of its inhibitor IκBα. Signal induced phosphorylation of IκBα on serine 32 and 36 targets the protein for ubiquitination on lysine 21 and 22. Here we use a phosphorylated peptide substrate representing residues 20–43 of IκBα to investigate requirements for ubiquitination of IκBα. Phosphorylation dependent polyubiquitination is carried out by a multiprotein complex containing βTrCP, Skp1 and Cdc53 (Cul1). In the presence of ubiquitin activating enzyme and the protein complex containing βTrCP, polyubiquitination of IκBα peptide was dependent on the presence of Cdc34, while Ubc5 only stimulated mono- and di-ubiquitination.

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Key words: IκBα ubiquitination; NF-κB activation; βTrCP; SCF ubiquitin ligase

1. Introduction

In unstimulated cells the transcription factor NF-κB is held in the cytoplasm, in a form that is unable to bind DNA, by IκBα. Exposure of cells to a wide variety of stimuli results in release of the transcription factor from IκBα allowing the active DNA binding form of the NF-κB to translocate to the nucleus where it binds to its recognition sites in the upstream regions of a wide variety of genes. The NF-κB family of proteins share a conserved region known as the Rel homology domain (RHD) which contains the nuclear localisation signal (NLS), as well as the dimerisation and DNA binding functions. Typically, the NF-κB form activated by extracellular signals is composed of p50 and p65. Inhibitor IκB proteins contain ankyrin repeat domains and association with NF-κB not only occludes the nuclear localisation sequence of p50 and p65 leading to cytoplasmic sequestration, but also prevents NF-κB/DNA binding activity. Following signal induction IκBα is rapidly phosphorylated and degraded [1–3]. Released NF-κB translocates from the cytoplasm to the nucleus where it activates responsive genes. As the promoter for IκBα is NF-κB dependent, IκBα mRNA levels rise and IκBα protein is rapidly resynthesised. At this stage cytoplasmic levels of NF-κB are low and free IκBα translocates to the nucleus where it terminates NF-κB dependent transcription. This is accomplished by inhibition of the NF-κB/DNA interaction and export of the NF-κB/IκBα complex back to the cytoplasm [4,5].

Sites in IκBα which are inducibly phosphorylated are located within the N-terminal domain on residues S32 and S36 and mutation of these residues blocks signal induced degradation [6–10]. A common target for inducing signals such as TNFα or IL-1β is the NF-κB inducing kinase (NIK) [11] which is directly upstream from the IκB kinase (IKK) [12]. Transmission of signals to IκBα appears to be facilitated by the scaffold proteins IKAP [13] and NEMO [14] or IKKγ [15] which assemble the various molecules into a physically associated signalling module. Specific inhibition of the proteolytic activity of the proteasome prevents NF-κB activation and results in the accumulation of ubiquitinated forms of IκBα, indicating that IκBα is targeted for degradation by a phosphorylation dependent ubiquitination process [10,16–18]. Mutational analysis has indicated that K21 and K22 are the primary sites for addition of multi-ubiquitination chains with K38 and K47 as secondary sites [19–21]. Although signal induced phosphorylation and ubiquitination of IκBα takes place on the N-terminus of the protein deletion of the C-terminus of IκBα renders the protein resistant to signal induced degradation [7,22–24]. It is thought that the C-terminus of IκBα functions post ubiquitination via interactions with the catalytic core of the proteasome [25].

Ubiquitin addition is accomplished via a thioester cascade with ubiquitin first being activated by a unique E1 enzyme which utilises ATP to adenylate the C-terminal glycine of ubiquitin. Release of AMP accompanies the formation of a thioester bond between the C-terminus of ubiquitin and a cysteine residue in the E1 protein. In a transesterification reaction the ubiquitin is transferred from the ubiquitin activating enzyme to an E2 ubiquitin conjugating enzyme which may, in turn, transfer the ubiquitin to an E3 ubiquitin protein ligase. In many cases it is this enzyme which recognises the protein substrate and catalyses formation of an isopeptide bond between the C-terminus of ubiquitin and the ε-amino group of lysine in the target protein. Proteins destined for degradation via the proteasome are coupled to multiple copies of ubiquitin by formation of further isopeptide bonds between additional ubiquitin molecules and lysine residues in the bound ubiquitin [26]. Here we identify proteins required to carry out polyubiquitination of phosphorylated IκBα. In the presence of E1, cdc34 and a protein complex containing βTrCP, Skp1 and Cul1 a phosphorylated peptide substrate, representing residues 20–43 of IκBα, is efficiently polyubiquitinated.

2. Materials and methods

2.1. Expression constructs

cDNA encoding human βTrCP was obtained from R. Benarous

*Corresponding author. Fax: +44 (1334) 462595.
E-mail: rth@st-and.ac.uk

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(Paris). A modified form of βTrCP linked at its N-terminus to the SV5 Pk epitope tag [27] was constructed by insertion of the βTrCP coding sequence into a pcDNA3 construct containing the coding sequence of the tag [28]. A bacterial expression construct for 6-His tagged cdc34 was obtained from R.J. Deshaies (Caltech). hUbc5 was obtained by PCR amplification of reverse transcribed mRNA and was inserted into pGEX-2T. A baculovirus containing the coding sequence for the human ubiquitin activating enzyme was obtained from M. Rolfe (Mitotix).

2.2. Expression and purification of proteins

Ubiquitin activating enzyme was purified to homogeneity from baculovirus infected insect cells by affinity chromatography on ubiquitin Sepharose essentially as described [29]. 6-His Cdc34 was purified from induced bacteria by chromatography on Ni-agarose. Bound protein was eluted with 250 mM Imidazole HCl pH 6.0, 5 mM betamercaptoethanol and dialysed against 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 mM betamercaptoethanol. GST-Ubc5 was purified from induced bacteria by chromatography on glutathione agarose. Bound protein was eluted with reduced glutathione and cleaved with thrombin. After dialysis against 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 mM betamercaptoethanol GST was removed from the Ubc5 by passage through a column of glutathione agarose.

2.3. Preparation of cell extracts

COS7 cells were grown in 75 cm^2 flasks in DMEM 10% FCS. Transfections with the tagged βTrCP construct and pcDNA3 empty vector were performed using lipofectamine as described [30]. Four to six 75 cm^2 flasks were used for each set of conditions. Where indicated the proteasome inhibitor MG132 (20 μM) was added 45 min before lysis while TNF (10 ng/ml) was added 15 min before lysis. Flasks were washed twice with ice cold PBS then 0.4 ml of lysis buffer were added to each flask. The lysis buffer was 20 mM HEPES, pH 7.5, 0.5% NP40, 1 M NDSB201 [31], 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 20 mM $NaPO_4$ pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate and 1 tablet 'compete mini' protease inhibitor cocktail (Boehringer Mannheim) for 10 ml of buffer. Following lysis extracts were centrifuged at $60\,000\times g$ for 25 min at 4°C. Ten μl of protein A agarose (Pharmacia), to which Mab 336 was crosslinked, was added to the supernatant (approx. 4 ml) and tubes inverted for 2 h at 4°C. Beads were collected by centrifugation at $400\times g$ for 3 min and washed three times in PBS, 1 mM DTT.

2.4. SDS-PAGE and immunoblotting

Immunoprecipitated proteins were fractionated on 10 or 15% polyacrylamide gels containing SDS. Anti-Skp1 antibody (Santa Cruz Inc.) was used at a 1/2500 dilution in PBS containing 5% skimmed milk, 0.5% Tween 20 and bound antibody detected with anti-rabbit-HRP secondary antibody (1/2500, Amersham) and enhanced ECL detection (Boehringer). Anti-Cul1 antibody [32] was a gift from Wilhelm Krek and was used at 1/5000 dilution as described above. Mab 336 [27] was obtained from R.E. Randall. Mab 10B which recognises IκBα was used as described [33].

2.5. Ubiquitination assays

The following peptide H_3N^+LKKERLLDDRHDS(PO_3H_2)GLDS-(PO_3H_2)MKDEEYE-COO^- was synthesised (Bio-Trend Germany), and labelled with ^{125}I (6×10^4 d.p.m./pmol) using the chloramine T method as described [34]. Dephosphorylated peptide was obtained by treatment of the radiolabelled peptide for 1 h at 37°C with alkaline phosphatase immobilised on beads (Sigma). Prior to addition, the beads (20 μl) were washed in 1 ml 0.1 M NaCl, 50 mM Tris pH 8.8, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$. Ubiquitination assays used 10 μl of beads to which the same volume of reaction mix was added. Prior to addition the beads were washed once in reaction buffer. The reaction mix was 0.1 M HEPES pH 7.5, 5 mM ATP, 10 mM $MgCl_2$, 0.12 units/ml inorganic pyrophosphatase (EC 3.6.1.1) 4 μg ubiquitin, 100 ng E1, 200 ng Cdc34 to which 3 ng of radiolabelled peptide was added. Ubiquitination reactions were terminated and the products analysed on 15% polyacrylamide gels containing SDS. Gels were fixed in 10% w/v TCA, 40% v/v ethanol, 1% w/v glycerol, dried and exposed to an image plate. The plate was scanned in a Fuji phosphorimager 1500 and radioactivity quantitated using MacBas software.

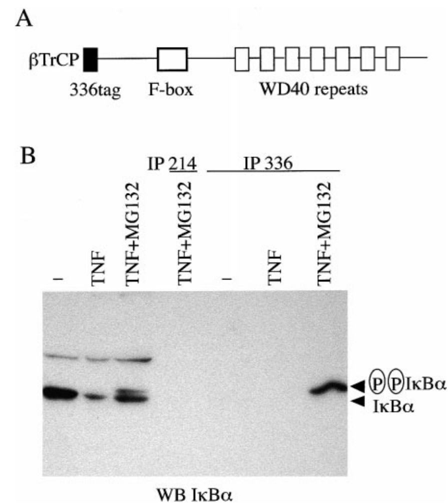

Fig. 1. IκBα, phosphorylated after signal transduction, associates with βTrCP. A: Diagrammatic representation of the tagged version of βTrCP. B: COS7 cells were transfected with a construct expressing the tagged βTrCP and cells either treated with control medium (–), TNF for 15 min or pretreated with MG132 for 45 min prior to treatment with TNF for a further 15 min. Cell extracts were prepared and immunoprecipitated with either Mab 336, which recognises the tag on βTrCP or a control antibody Mab 214. Immunoprecipitates were analysed by Western blotting with Mab 10B which recognises IκBα. The positions of IκBα and the phosphorylated form of IκBα are indicated.

3. Results

3.1. The F-box protein βTrCP associates with phosphorylated IκBα

βTrCP/Slimb is a conserved protein containing an F-box and WD40 repeats. Related proteins are known to be involved in the recognition of phosphorylated proteins which are destined for ubiquitination and proteasome mediated degradation. To determine if βTrCP was involved in the targeting of IκBα for signal induced ubiquitination a plasmid capable of expressing a tagged version of βTrCP was introduced into COS7 cells. Cells were either untreated, treated with TNF alone or pretreated with the proteasome inhibitor MG132 then treated with TNF. Analysis of cell extracts by Western blotting with antibody to IκBα indicates that, as expected, IκBα is degraded in the presence of TNF, but in the presence of TNF and MG132 the phosphorylated form accumulates (Fig. 1B). When these same cell extracts were immunoprecipitated with the 336 anti-tag antibody prior to analysis by Western blotting with an IκBα antibody only the more slowly migrating, phosphorylated form of IκBα, which accumulates in the presence of TNF and MG132, was shown to be associated with βTrCP (Fig. 1B). Thus βTrCP appears to recognise the phosphorylated form of IκBα.

3.2. βTrCP is part of an SCF complex containing Cdc53 and Skp1

To determine if βTrCP was part of an SCF ubiquitin ligase complex the tagged form of βTrCP was introduced in COS7 cells and cell extracts immunoprecipitated with the 336 anti-tag antibody. Extracts of cells transfected with pcDNA3 were used as control. Immunoprecipitated proteins were analysed by Western blotting with antibodies to Skp1 or Cdc53. Both

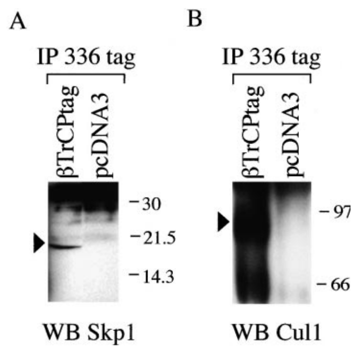

Fig. 2. βTrCP is associated with Skp1 and Cul1. Extracts from COS7 cells transfected with either pcDNA3 or a construct expressing tagged βTrCP were immunoprecipitated with Mab 336 and the immunoprecipitates analysed by Western blotting with antibodies to either Skp1 or Cul1.

Skp1 (Fig. 2A) and Cdc53 (Fig. 2B) were immunoprecipitated with the 336 anti-tag antibody, but were not detected in extracts from the pcDNA3 transfected cells. βTrCP is therefore part of a multiprotein complex containing Skp1 and Cdc53.

3.3. The SCF complex containing βTrCP stimulates ubiquitination of IκBα

To determine if the SCF complex containing βTrCP was directly involved in the ubiquitination of phosphorylated IκBα the tagged version of βTrCP was immunoaffinity purified from transfected COS7 cells which were either untreated, treated with TNF alone or pretreated with the proteasome inhibitor MG132 then treated with TNF. Western blotting with the 336 anti-tag antibody revealed that βTrCP was detected in all conditions, although the level of the protein was elevated in the combined presence of TNF and MG132 (Fig. 3A). As a substrate for ubiquitination a peptide representing residues 20–43 of IκBα was synthesised in which S32 and S36

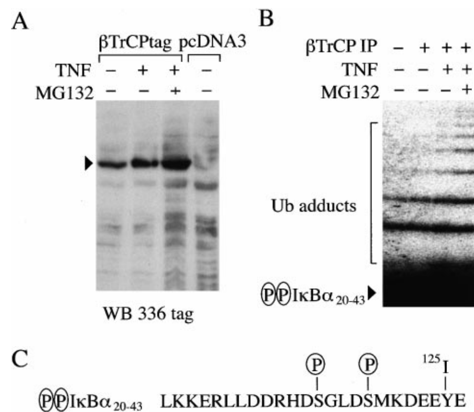

Fig. 3. Immunopurified βTrCP promotes polyubiquitination of a phosphorylated peptide representing residues 20–43 of IκBα. COS7 cells were transfected with a construct expressing tagged βTrCP or pcDNA3 and treated with TNF and MG132 as indicated. A: Expression of βTrCP was monitored by Western blotting with Mab 336. B: A ^{125}I labelled, phosphorylated peptide representing residues 20–43 of IκBα was used as substrate for in vitro ubiquitination assays. In addition to substrate, assays contained ubiquitin, purified E1, purified E2 (Cdc34) and immunoprecipitated tagged βTrCP isolated from cells treated with TNF and MG132 as indicated. The positions of the phosphorylated peptide and ubiquitin adducts were indicated. C: Structure of the phosphorylated IκBα peptide.

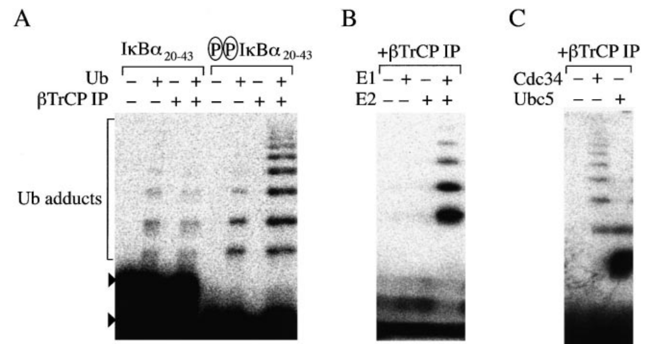

Fig. 4. Polyubiquitination of the IκBα peptide is mediated by Cdc34 and requires phosphorylation on S32 and S36. A: ^{125}I labelled, phosphorylated peptide was dephosphorylated by treatment with immobilised alkaline phosphatase. Untreated or dephosphorylated peptides were used as substrates for ubiquitination either in the presence or absence of ubiquitin and immunoprecipitated tagged βTrCP as indicated. B: ^{125}I labelled phosphorylated peptide was used as substrate for in vitro ubiquitination in the presence of E1, E2 (Cdc34) and immunoprecipitated tagged βTrCP as indicated. C: Comparison of ubiquitinating activity of Cdc34 and Ubc5. ^{125}I labelled phosphorylated peptide was used as substrate for in vitro ubiquitination in the presence of E1, immunoprecipitated tagged βTrCP and either Cdc34 or Ubc5 as indicated.

were phosphorylated (Fig. 3C). The peptide was labelled with ^{125}I and incubated with ubiquitin, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and the immunopurified βTrCP. In the presence of E1 and E2 alone mono- and di-ubiquitinated forms of the peptide are detected. In the presence of immunopurified βTrCP from cells treated with TNF and MG132 extensive polyubiquitination of the peptide substrate is detected (Fig. 3B). βTrCP prepared from untreated or TNF treated cells was less active in promoting polyubiquitination (Fig. 3B).

3.4. Requirements for βTrCP mediated ubiquitination of IκBα

In vivo it is well established that phosphorylation of S32 and S36 is a prerequisite for ubiquitination of IκBα. Therefore to demonstrate the specificity of the in vitro reaction the peptide was dephosphorylated and its substrate activity determined. In the presence of E1, E2 (Fig. 4B) and the immunopurified complex containing tagged βTrCP the phosphorylated peptide is efficiently ubiquitinated, but this activity is severely compromised when the peptide is dephosphorylated (Fig. 4A). Based on electrophoretic mobility the activity remaining is likely to be a consequence of residual phosphorylated material which was not efficiently dephosphorylated. Although it has been reported that Ubc5 is the E2 responsible for IκBα ubiquitination [35–37] many SCF complexes rely on cdc34 to supply the E2 activity. To determine which E2 is responsible for βTrCP mediated ubiquitination of the phosphorylated IκBα peptide, recombinant purified, ubc5 and cdc34 were tested for activity. In the presence of immunopurified βTrCP and E1, cdc34 efficiently catalyses the polyubiquitination of the phosphorylated IκBα peptide, whereas ubc5 stimulated only mono- and di-ubiquitination (Fig. 4C). Control experiments demonstrated that in the presence of E1 Ubc5 and Cdc34 were both active in that they could form thioesters with ubiquitin (data not shown). Thus it appears that in the presence of an SCF complex containing βTrCP cdc34 catalyses the phosphorylation dependent polyubiquitination of IκBα.

4. Discussion

Activation of NF-κB proceeds via signal induced phosphorylation and ubiquitin mediated degradation of the IκBα inhibitor protein. Here we demonstrate that phosphorylated IκBα is recognised by a multiprotein complex containing βTrCP, Skp1 and Cdc53 and is polyubiquitinated by the E2 ubiquitin conjugating enzyme Cdc34. This conclusion is consistent with a number of recent reports [35–39], but while there is general agreement that βTrCP is involved in IκBα ubiquitination there is less agreement on the E2 involved. Here we demonstrate the role of Cdc34 in polyubiquitination of a phosphorylated peptide representing residues 20–43 of IκBα. This ubiquitination is strongly stimulated (15-fold) in the presence of immunopurified βTrCP. An important difference in each of the studies is the substrate used in the assays. While we have used purified peptide as substrate the previously published reports utilised full length IκBα which was phosphorylated in vitro with preparations containing the IκB kinase. The use of a peptide substrate along with purified recombinant E1 and E2 and immunopurified complex containing βTrCP permits the elimination of potentially interfering components and will facilitate biochemical analysis of the ubiquitination reaction. It should be noted that under the reaction conditions employed the ubiquitination reaction is highly specific in that dephosphorylated peptide does not act as a substrate.

In addition to IκBα degradation βTrCP and its Drosophila homologue *Slimb* are involved in the degradation of phosphorylated β-catenin [37,40] and CD4 associated with phosphorylated HIV-1 Vpu [41]. The F-box present in βTrCP connects the bound substrate to Skp1, which is in turn bound to Cdc53. Cdc53 and Skp1 are thus common components of SCF complexes which derive their substrate recognition properties by recruitment of any one of a diverse array of F-box containing proteins. In many cases SCF complexes containing a variety of F-box proteins appear to utilise Cdc34 as the E2 for ubiquitination of the target protein and this is consistent with our observation that Cdc34 is responsible for ubiquitination of phosphorylated IκBα.

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