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Specific Recognition of Linear Ubiquitin Chains by NEMO Is Important for NF-κB Activation

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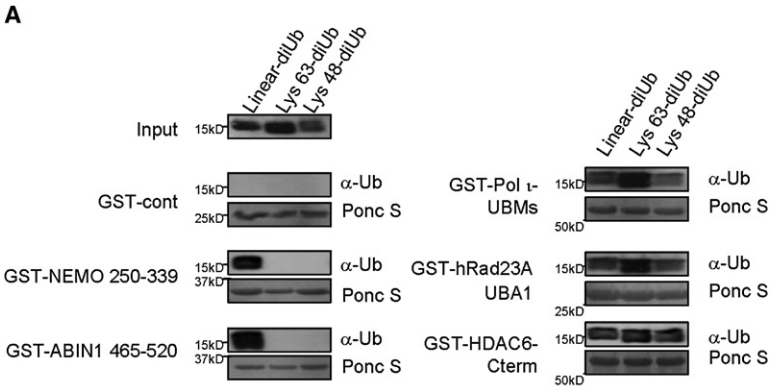
SUMMARY

Activation of nuclear factor-κB (NF-κB), a key mediator of inducible transcription in immunity, requires binding of NF-κB essential modulator (NEMO) to ubiquitinated substrates. Here, we report that the UBAN (ubiquitin binding in ABIN and NEMO) motif of NEMO selectively binds linear (head-to-tail) ubiquitin chains. Crystal structures of the UBAN motif revealed a parallel coiled-coil dimer that formed a heterotetrameric complex with two linear diubiquitin molecules. The UBAN dimer contacted all four ubiquitin moieties, and the integrity of each binding site was required for efficient NF-κB activation. Binding occurred via a surface on the proximal ubiquitin moiety and the canonical Ile44 surface on the distal one, thereby providing specificity for linear chain recognition. Residues of NEMO involved in binding linear ubiquitin chains are required for NF-κB activation by TNF-α and other agonists, providing an explanation for the detrimental effect of *NEMO* mutations in patients suffering from X-linked ectodermal dysplasia and immunodeficiency.

INTRODUCTION

Nuclear factor-κB (NF-κB), a dimeric transcription factor formed by members of the Rel family of proteins, plays essential roles in regulating gene expression during development, skin homeostasis, and immunity. The activity of NF-κB proteins is regulated by a variety of external stimuli, including bacterial lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (Hayden and Ghosh, 2008). In the canonical pathway, NF-κB factors are retained in an inactive state by binding to the inhibitor of NF-κB (IκB), which in response to cell stimulation is ubiquitinated and degraded by the proteasome. Prior to its ubiquitination, IκB is phosphorylated by the IκB kinase (IKK) complex. The IKK complex, consisting of two kinases, IKKα and IKKβ, and the regulatory component NEMO (also known as IKKγ), is activated by an upstream kinase known as TAK1, which is in turn activated after TNF-α or IL-1 receptor stimulation (Häcker and Karin, 2006). Receptor-proximal events appear to initiate a specific type of ubiquitin signal, consisting of Lys63-linked ubiquitin polymers (Hayden and Ghosh, 2008). Attachment of Lys63-linked ubiquitin chains to receptor substrates such as RIP1 (receptor-interacting protein 1, upon TNF-α stimulation), IRAKs (IL-1 receptor-associated kinases, upon IL-1 stimulation), or TRAFs (tumor necrosis factor receptor-associated factors) is critical for activation of NF-κB (Ordureau et al., 2008; Kanayama et al., 2004), a process that is catalyzed by the Lys63-specific E2 complex Ubc13-Uev1a together with a set of E3 ligases, including TRAFs, IAPs (inhibitors of apoptosis), and Pellino (Deng et al., 2000; Ordureau et al., 2008; Varfolomeev et al., 2008). However, the surprising report that Ubc13-deficient mice elicit normal NF-κB activation in response to multiple stimuli (Yamamoto et al., 2006) has led to speculations that additional E2s could also participate in Lys63 chain production in vivo together with TRAF/IAP ligases. Alternatively, other types of ubiquitin chains could mediate activation of the NF-κB pathway. In agreement with the latter hypothesis, an E3 ligase complex, LUBAC (linear ubiquitin chain assembly complex), was recently shown to regulate the canonical NF-κB pathway independently of Lys63 chains (Kirisako et al., 2006; Tokunaga et al., 2009). Yet, it is mechanistically unclear how two differently linked ubiquitin polymers, Lys63 and linear ubiquitin chains, can mediate activation of the IKK complex.

On the basis of the correlative evidence between linkage specificity and cellular functions, it is believed that distinct ubiquitin



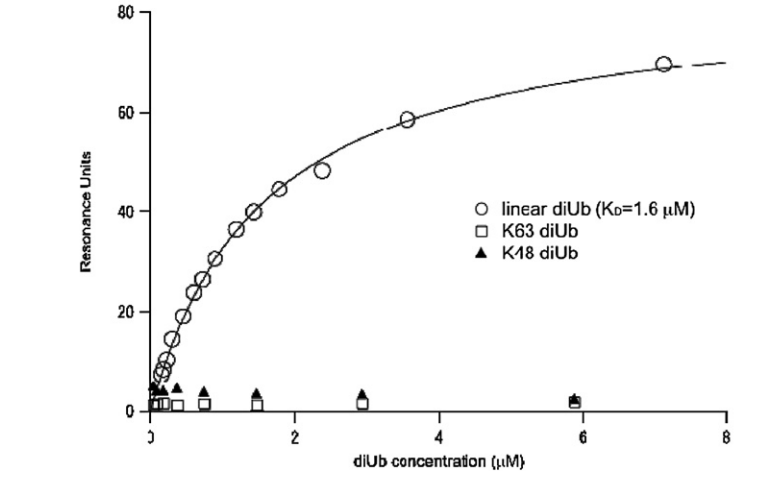


Figure 1. The UBAN Motif of NEMO Binds to Linear Ubiquitin Chains

(A) Bindings of diubiquitin molecules (linear diUb, Lys63-linked diUb, or Lys48-linked diUb) to GST (cont), GST-NEMO CoZi region (NEMO 250–339), GST-ABIN1-UBAN motif (ABIN1 465–520), GST-Polι-UBM, GST-hRad23A-UBA, or GST-HDAC6-NZF containing C terminus (HDAC6-Cterm) were determined by immunoblotting with an anti-ubiquitin antibody. Loading of GST fusion proteins was determined by Ponceau S staining.

(B) Surface plasmon resonance data of the interaction between NEMO and diubiquitin. GST-fused NEMO (250–339) was immobilized on a GST antibody-coated CM5 chip. The linear (circle), K63-linked (square), or K48-linked (triangle) diubiquitin molecules were loaded over the chip. Each measurement was performed at the indicated concentrations, and the fitted curves and calculated K_D values are shown.

binding domains (UBDs) might influence the interpretation of ubiquitin signals within cells (Hurley et al., 2006; Ikeda and Dikic, 2008). In the NF-κB pathway, available biochemical and genetic evidence favors a model in which the adaptor proteins TAB2/3 (TAK1 binding protein 2/3) bind to Lys63-conjugated substrates, resulting in activation of IKKα/β by its upstream kinase TAK1 (Hayden and Ghosh, 2008). Interestingly, several groups have identified a ubiquitin binding domain in NEMO, referred to as NUB (NEMO ubiquitin binding), CoZi (coil-zipper domain), or UBAN (ubiquitin binding in ABIN and NEMO proteins) (hereafter referred to as UBAN), which was thought to specifically bind to Lys63-ubiquitinated substrates (Wu et al., 2006; Ea et al., 2006; Bloor et al., 2008; Wagner et al., 2008). Recently, Lo et al. solved a structure of the NEMO UBAN region and determined that NEMO binds significantly stronger to linear diubiquitin as compared to Lys63-linked chains (Lo et al., 2009). Yet, detailed information how NEMO might distinguish linear from Lys63-linked chains and the importance of this binding event for signaling has been lacking. Here, we provide structural and functional evidence that the UBAN motif of NEMO binds to linear ubiquitin chains, and that this interaction plays a crucial role in TNF-α-induced activation of the IKK complex and NF-κB, but not MAPK pathways in vivo.

RESULTS

UBAN Motifs Bind Selectively to Linear Diubiquitin

Several groups have previously shown that the coiled-coil 2 (CC2) and leucine zipper (LZ) region (abbreviated as CoZi region) is the fragment in NEMO necessary for binding to ubiquitin chains (Ea et al., 2006; Wu et al., 2006; Bloor et al., 2008). The CoZi region contains the centrally located UBAN motif (Figure S1A available online), a conserved domain also present in other ubiquitin binding proteins including the ABINs and optineurin (Zhu et al., 2007; Bloor et al., 2008; Wagner et al., 2008). This fragment was further used to assess NEMO binding to different types of ubiquitin chains in vitro. After confirming that the UBAN motif binds to linear, i.e., head-to-tail linked, tetraubiquitin constructs used in previous studies (Bloor et al., 2008; Wagner et al., 2008), we next compared its ability to interact with different diubiquitin molecules. Interestingly, we found that the UBAN motif could bind linear diubiquitin, but failed to interact with diubiquitin molecules linked via Lys48 or Lys63 obtained from two independent sources (Figures 1A, S1B, and S1C). Similar results were acquired for the UBAN motif of ABIN1 (Figure 1A). These observations are clearly distinct from the NZF domain of HDAC6 (Boyault et al., 2006), the UBM of Polι (Bienko et al., 2005), and the UBA domain of hRad23A, which bind to all diubiquitin chains (linear, Lys63, and Lys48) (Figure 1A). Surface plasmon resonance (SPR) experiments confirmed that the NEMO UBAN motif bound linear diubiquitin molecules with a dissociation constant of 1.6 μM, while binding to Lys63 or Lys48 diubiquitin molecules was below the detection level up to around 5 μM of diubiquitin (Figure 1B).

Structure of the CoZi Region in Complex with Linear Diubiquitin

A challenging task in the ubiquitin field is to understand the specificity of UBD binding to particular ubiquitin chain types. Multiple structures of UBDs in complex with monoubiquitin have been