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Two different classes of E2 ubiquitin conjugating enzymes are required for the mono-ubiquitination of proteins and elongation by polyubiquitin chains with a specific topology

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Running title: Mechanism of mono- and polyubiquitination

Abbreviations: CHIP, C-terminus of Hsc70 interacting protein; HECT, homologous to E6-AP C-terminus; HH, helical hairpin; Hsp, heat-shock protein; PHD, plant homeodomain; RING, really interesting new gene; TPR, tetratricopeptide repeat; TRAF6, tumour necrosis factor receptor associated factor 6; Ub, ubiquitin; Ubc, ubiquitin conjugating enzyme; Uev, ubiquitin E2 variant.

SYNOPSIS

RING and U-box E3 ligases bridge E2 ubiquitin-conjugating enzymes and substrates to enable the transfer of ubiquitin to a lysine residue on the substrate or to one of the seven lysine residues of ubiquitin for polyubiquitin chain elongation. Different polyubiquitin chains have different functions. K48-linked chains target proteins for proteasomal degradation and K63-linked chains function in signal transduction, endocytosis and DNA repair. For this reason, chain topology must be tightly controlled. Using the U-Box E3 ligase CHIP and the RING E3 ligase TRAF6 with the E2s Ubc13-Uev1a and UbcH5a, we demonstrate that Ubc13-Uev1a supports the formation of free K63-linked polyubiquitin chains not attached to CHIP or TRAF6, whereas UbcH5a catalyses the formation of polyubiquitin chains linked to CHIP and TRAF6 that lack specificity for any lysine residue of ubiquitin. Therefore, the abilities of these E2s to ubiquitinate a substrate and to elongate polyubiquitin chains of a specific topology appear to be mutually exclusive. Thus, two different classes of E2 may be required to attach a polyubiquitin chain of a particular topology to a substrate: the properties of one E2 are designed to mono-ubiquitinate a substrate with no or little inherent specificity for an acceptor lysine residue, while the properties of the second E2 are tailored to the elongation of a polyubiquitin chain using a defined lysine residue of ubiquitin.

Keywords:- ubiquitin; CHIP; TRAF6; E2 conjugating enzyme; Ubc13; UbcH5

INTRODUCTION

The post-translational modification of proteins with mono-ubiquitin or polyubiquitin chains has important functions in almost every aspect of cell biology [1]. The best-studied example is the targeting of proteins for proteasomal degradation, which involves the formation of lysine 48 (K48)-linked polyubiquitin chains comprising at least four ubiquitins [2]. More recently it has been appreciated that polyubiquitin chains can be formed using one of the other six lysine residues of ubiquitin. In yeast all seven lysine residues of ubiquitin seem to be utilized for chain formation [3]. Lysine 63 (K63)-linked polyubiquitin chains play roles in DNA repair, signal transduction and endocytosis and do not appear to target proteins for proteasomal degradation. Thus, polyubiquitin chains with different topologies determine different responses and the topology must be tightly regulated.

The canonical mechanism of polyubiquitin chain formation involves three steps. After an initial activation step catalyzed by the E1, the ubiquitin is transferred in a second step to the E2 ubiquitin-conjugating enzyme. The third step is dependent on the nature of the E3 ubiquitin ligase: RING (really interesting new gene), PHD (plant homeodomain) and U-Box E3 ubiquitin ligases bridge E2 and substrate to enable ubiquitination to occur, whereas HECT (homologous to E6-AP C-terminus) E3s form a thiol ester with the ubiquitin before transferring it to the substrate. Target protein selectivity is provided by the E3, while the E2 determines the specificity for the lysine residue of the acceptor ubiquitin in polyubiquitin chain formation mediated by RING, PHD and U-Box E3s, but probably not by HECT E3s [1].

E2s that direct specificity for K48-linked chains that mark proteins for degradation via the proteasome are for example human Cdc34 or E2-25K, while the formation of K63-linked chains requires a heterodimer of Ubc13 and either of the Ubc-variants Uev1a or Mms2 [4]. Nevertheless, despite more than two decades of research in the field of protein ubiquitination the details of polyubiquitin-chain formation remain elusive [5]. The addition of a polyubiquitin chain of a certain topology involves two steps with very different requirements: Substrate (mono-)ubiquitination and chain elongation (polyubiquitination). Whereas mono-ubiquitination seems to lack an inherent specificity for a particular lysine residue on a substrate, chain elongation can occur specifically on a particular lysine residue of ubiquitin, for example K48 or K63. Two models have been suggested for how different E3-E2 complexes operate. The first model suggests that an E3-E2 complex that can elongate K48-linked polyubiquitin chains efficiently is also able to add the first ubiquitin to the substrate, albeit more slowly, making this the rate-limiting step [6]. A modified version of this model was also suggested for the tumour necrosis factor receptor associated factor 6 (TRAF6)-Ubc13-Uev1a

complex, in which the first ubiquitin was added to TRAF6 in a Ubc13-dependent, but Uev1a-independent reaction, whereas the elongation of K63-linked polyubiquitin chains occurs in a Ubc13-Uev1a-dependent manner [7]. The other model suggests that one E3-E2 pair adds the first ubiquitin and another E3-E2 pair elongates the K48- or K63-linked polyubiquitin chains [8-10].

Recently, we identified CHIP (C-terminal of Hsp70 interacting protein) as a binding partner for the K63-specific ubiquitin-conjugating enzyme Ubc13-Uev1a, demonstrated that it functions as an E3 ubiquitin ligase with Ubc13-Uev1a, and determined the structure of a heterotrimeric complex between Ubc13-Uev1a and the CHIP U-box E3 ligase domain [11]. CHIP is a dimeric ~35 kDa protein containing a C-terminal U-box domain and an N-terminal domain with three tetratricopeptide repeats (TPR) that mediates interaction with Hsp90 and Hsp70 chaperones possessing the C-terminal EEVD-TPR-binding motif [12]. In vitro, we showed that CHIP formed unanchored K63-linked polyubiquitin chains using Ubc13-Uev1a as the E2 [11]. We also demonstrated that CHIP forms polyubiquitin chains in vitro that are neither specific for K63 or K48 when paired with UbcH5a, another E2 conjugating enzyme and that these chains are linked to CHIP [11]. Here, we show that the CHIP-UbcH5a complex lacks specificity for any of the seven lysine residues of ubiquitin, but is able to attach the first ubiquitin to CHIP or the CHIP-interacting protein Hsp90. In contrast, the CHIP-Ubc13-Uev1a complex synthesizes only unanchored K63-linked polyubiquitin chains that are not attached to CHIP or Hsp90. Similar results were obtained using TRAF6 as the E3 ligase. These findings support a model in which the addition of a polyubiquitin chain of a specific topology will frequently require at least two E3-E2 complexes: One for the addition of the first ubiquitin, the second for elongation with a polyubiquitin chain of a specific topology.

EXPERIMENTAL

Materials. An antibody that recognises CHIP was obtained from Abcam, the anti-ubiquitin antibody was from DakoCytomation, the anti-TRAF6 antibody from Santa Cruz and the Colloidal Blue Staining Kit from Invitrogen. The E2 protein UbcH5a was purchased from Biomol. Wild type ubiquitin was purchased from Sigma and methylated ubiquitin from Calbiochem.

DNA constructs. The constructs encoding Ubc13, Uev1a, UBE1, wild type ubiquitin and CHIP have been described previously [11]. HSP90 (NCBI AAQ63401) was amplified from pRSETa His-Hsp90, a kind gift from Lawrence Pearl, Institute for Cancer Research, London, using KOD Hot Start DNA Polymerase (Novagen) then cloned into pCR2.1 (Invitrogen) and sequenced to completion. It was then digested with Not1 and ligated into the same site in pGEX6P-2 to produce pGEX6P-2 HSP90. UbcH4 was amplified from IMAGE 3835609 using KOD Hot Start Polymerase (Novagen), cloned into pCR2.1 (Invitrogen) and sequenced to completion. The insert was then cloned into the Not1 site of pET28b (Novagen) to make pET28 UbcH4. Ubiquitin was RT PCR amplified from peripheral blood total RNA using the Access RT PCR System (Promega). It was cloned into pCR2.1, sequenced and subcloned into the BamH1 site of pGEX6P-1. Each of the mutations was made using the Stratagene QuikChange Method but using KOD Hot Start Polymerase. TRAF6 was amplified from IMAGE 5210798 and cloned as described above into pCR2.1 and then into the BamH1 Not1 sites of pGEX6P-1 to form pGEX6P-1 TRAF6.

Protein expression and purification. A His-tagged ubiquitin-activating enzyme 1 (UBE1) was expressed in insect Sf21 cells and purified by chromatography on nickel-nitrilotriacetate agarose. His-tagged CHIP were expressed in bacteria and also purified by chromatography on nickel-nitrilotriacetate agarose. Ubc13, Uev1a, CHIP, TRAF6, Hsp90, ubiquitin and lysine mutants of ubiquitin were expressed in bacteria with a glutathione-S-transferase (GST) tag at their N-terminus followed by PreScission protease cleavage site. Each protein was purified from bacterial extracts by affinity chromatography on glutathione-Sepharose (Amersham). To remove the GST tags, the glutathione-Sepharose containing the bound GST-fusion was incubated with PreScission Protease to release the cleaved proteins. Each protein was then dialysed against 50 mM Tris-HCl pH 7.5, 270 mM sucrose, 150 mM NaCl, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.2 mM phenylmethylsulphonyl fluoride (PMSF) and 1 mM benzamidine and stored in aliquots at -80°C .

Ubiquitination assay. The formation of polyubiquitin chains was carried out at 30°C for 1 h in a 20 µl reaction mixture containing His₆-E1 (50 nM), E2 ubiquitin-conjugating enzyme (1 µM), ubiquitin (100 µM), CHIP or TRAF6 (1 µM or as indicated in the figure legends), 50 mM Tris-HCl pH 7.5, 5 mM MgCl₂ and 2 mM ATP. The reaction was stopped by addition of LDS sample buffer.

RESULTS

CHIP-Ubc13-Uev1a and CHIP-UbcH5a complexes produce unanchored and anchored polyubiquitin chains, respectively.

We reported previously that CHIP can catalyse the formation of polyubiquitin chains using the different E2 ubiquitin-conjugating enzymes Ubc13-Uev1a and UbcH5a *in vitro* ([11], Fig 1). Interestingly, CHIP-Ubc13-Uev1a formed free polyubiquitin chains not attached covalently to any protein present in the incubation, as shown by the formation of ubiquitin oligomers smaller than CHIP (Fig 1A, upper panel) and by the lack of appearance of slower migrating forms of CHIP during the ubiquitination reaction (Fig 1A, lower panel). In contrast, CHIP-UbcH5a catalysed the auto-ubiquitination of CHIP, as shown by the lack of any ubiquitin oligomers smaller than CHIP (Fig 1B, upper panel) and by the appearance of a ladder of ubiquitinated CHIP species differing in the number of ubiquitin molecules attached (Fig 1B, lower panel). Although highly polyubiquitinated chains were the major species detected by immunoblotting with anti-ubiquitin (Fig 1B, upper panel), the major CHIP species formed was mono-ubiquitinated as shown by immunoblotting with anti-CHIP (Fig 1B, lower panel).

CHIP-Ubc13-Uev1a catalyses the formation of Lys63-linked polyubiquitin chains whereas the polyubiquitin chains created by the CHIP-UbcH5a complex exhibit no specific topology

The complex formed between CHIP and the E2 ubiquitin-conjugating enzyme Ubc13-Uev1a is known to direct the formation of K63-linked polyubiquitin chains. To determine which type of polyubiquitin chain was formed by the CHIP-UbcH5a complex we used a range of ubiquitin mutants with one of the seven lysines mutated to arginine. Surprisingly, all seven mutants were utilized in the auto-polyubiquitination of CHIP, suggesting that the polyubiquitin chains made by CHIP-UbcH5a have no specific topology (Fig 2B). The K6R mutant was used less efficiently than the other mutants for both mono- and polyubiquitination, but was still able to support polyubiquitination at a significant level (Fig 2B, lane 2). The polyubiquitin chains formed by CHIP-UbcH5a and wild-type ubiquitin were identified directly by mass spectrometry, which revealed that they were linked via at least five different lysines, namely K6, K11, K33, K48 and K63 (E. Carrick, M. Windheim and N. Morrice,

unpublished work). In contrast, polyubiquitin chains formed by CHIP-Ubc13-Uev1a are very specific for K63, since only the mutation of this lysine residue to arginine abolished the formation of polyubiquitin chains (Fig 2A, lane 8).

If wild type ubiquitin was replaced by methylated ubiquitin in which all seven lysine residues were modified, incubation with CHIP and Ubc13-Uev1a did not result in the formation of any polyubiquitin chains or multi-ubiquitinated CHIP (Fig 2C, compare lanes 2 and 3). In contrast, incubation of CHIP, UbcH5a and methylated ubiquitin did not produce the high molecular mass forms of polyubiquitinated CHIP seen with wild type ubiquitin (Fig 2C, compare lanes 5 and 6), and instead mono-ubiquitinated CHIP was formed with smaller amounts of a di-ubiquitinated derivative (Fig 2C, bottom panel, lane 6). This last result suggested that CHIP was mainly ubiquitinated at a single lysine residue, which has been reported to be Lys22 in the presence of UbcH5a [13]. We confirmed this result (E. Carrick, M. Windheim and N. Morrice, unpublished work) and also identified Lys221 and Lys255 as sites of polyubiquitination, consistent with the presence of small amounts of di-ubiquitinated CHIP (Fig 2C, bottom panel, lane 6).

Deletion of the N-terminal TPR domain of CHIP suppresses ubiquitination by the CHIP-UbcH5a complex, without affecting ubiquitination by the CHIP-Ubc13-Uev1a complex

We showed recently that CHIP dimerisation involves the helical hairpin domain and the U-Box [11]. We found that the U-Box alone had ubiquitination activity using Ubc13-Uev1a as an E2 (Fig 3, lanes 4 and 14), demonstrating that the U-box is the minimum structural domain needed to support polyubiquitination. Nevertheless, a CHIP mutant consisting of the U-Box and the helical hairpin domain, but lacking the TPR domain, had a significantly higher activity, which was indistinguishable from the full-length protein (Fig 3, lanes 5 and 15 in comparison to lanes 2 and 12, respectively). On the other hand, the CHIP U-Box alone had no detectable ubiquitination activity using UbcH5a as an E2 (Fig 3, lanes 9 and 19) and, even with the CHIP mutant containing the U-Box and the helical hairpin domain, the ubiquitination activity was far lower than with the full-length protein (Fig 3, lanes 10 and 20 in comparison to 7 and 17 respectively). CHIP is ubiquitinated at K22 in the presence of UbcH5a [13] and this lysine residue is missing in the CHIP mutant containing the U-Box and the helical hairpin domain only. The UbcH5a-catalysed ubiquitination is greatly reduced in this CHIP mutant compared to the full-length protein, because it lacks the lysine residue required to anchor the polyubiquitin chains on CHIP. The N-terminal TPR-domain has no effect on ubiquitination mediated by Ubc13-Uev1a, because this E2 does not require K22 to anchor the polyubiquitin chains and forms unanchored polyubiquitin chains.

CHIP-UbcH5a, but not CHIP-Ubc13-Uev1a ubiquitinates Hsp90 in vitro

CHIP is known to bind to Hsp90 via its N-terminal TPR domain [12]. In order to find out whether Hsp90 binding has an effect on the ubiquitination activity of CHIP, we included recombinant Hsp90 in the ubiquitination assays. Interestingly, Hsp90 had no significant effect on the CHIP-Ubc13-Uev1a-dependent ubiquitination (Fig 4, lanes 1-4 and 9-12), but was used as a substrate for UbcH5a-dependent ubiquitination very efficiently (Fig 4, lanes 5-8 and 13-16). Strikingly, almost all the Hsp90 included in the assay became ubiquitinated. As observed before, the CHIP-Ubc13-Uev1a complex only formed unanchored polyubiquitin chains, whereas the CHIP-UbcH5a complex formed polyubiquitin chains anchored either to CHIP or to Hsp90 (Fig 4, right panels).

The RING domain E3 ligase TRAF6 and the U-Box domain E3 ligase CHIP have similar activities when paired with the E2 ubiquitin-conjugating enzymes UbcH5a and Ubc13-Uev1a

The results presented so far showed that the E2 ubiquitin-conjugating enzymes Ubc13-Uev1a and UbcH5a exhibited strikingly different characteristics when paired with the E3 ubiquitin ligase CHIP. To find out whether these findings were specific to CHIP, we used the E3 ubiquitin ligase TRAF6 and investigated its ubiquitinating activity when paired with Ubc13-Uev1a or UbcH5a. As shown with CHIP, Ubc13-Uev1a-dependent ubiquitination using TRAF6 resulted in the formation of unanchored K63-linked polyubiquitin chains, while the TRAF6-UbcH5a complex catalysed TRAF6 auto-ubiquitination (Fig 5). Thus, the characteristics of the E2 ubiquitin conjugating complexes Ubc13-Uev1a and UbcH5a do not depend on the E3 ubiquitin ligase that they are paired with but are intrinsic to the E2 ubiquitin-conjugating complexes. In addition, we tested CHIP and TRAF6 ubiquitinating activity with another E2 conjugating enzyme UbcH4. UbcH4 showed the same characteristics as UbcH5a and was able to support the auto-ubiquitination of CHIP and TRAF6 (M. Windheim, unpublished work).

DISCUSSION

Recently, we [11] and others [14] reported that CHIP is able to form unanchored K63-linked polyubiquitin chains with the E2 conjugating enzyme Ubc13-Uev1a and that the CHIP-UbcH5a complex forms polyubiquitin chains that are anchored to CHIP (Fig 1, [11]) or luciferase [14]. Moreover the polyubiquitin chains that are attached to CHIP (Fig 2) and luciferase [14] have no specific topology. CHIP is a U-Box-containing E3 ligase and fulfils its function by bringing the E2 and the substrate into close proximity to allow ubiquitination to occur. Ubiquitination of CHIP by UbcH5a depends on an acceptor lysine on CHIP, which is predominantly K22 *in vitro* [13]. As a result, the deletion of the N-terminal region of CHIP including the TPR domain, drastically decreased CHIP-UbcH5a mediated ubiquitination, but had no effect on CHIP-Ubc13-Uev1a-mediated ubiquitination (Fig 3). The CHIP-UbcH5a complex also ubiquitinated CHIP-associated Hsp90 *in vitro* (Fig 4), but Hsp90 had no effect on CHIP-Ubc13-Uev1a-mediated polyubiquitination (Fig 4).

It is important to point out that the formation of a substrate-linked polyubiquitin chain of a certain topology involves two steps with very different requirements. For the attachment of the first ubiquitin there seems to be little inherent specificity for any particular lysine residue on the substrate provided that it can be accessed by the relevant E2-E3 complex. However, the interaction between the E3 ubiquitin ligase and its substrate may sometimes determine which particular lysine residue(s) becomes ubiquitinated. For example the ubiquitination of I κ B α occurs at lysines 21 and 22 because the SCF E3 ligase complex binds specifically to I κ B α phosphorylated at Ser32 and Ser36 in such a way that presumably only these two lysines can be accessed [15]. In contrast, the elongation of a polyubiquitin chain with a particular topology is a very specific reaction that must ensure that only one unique lysine residue on ubiquitin and no other on either ubiquitin or any substrate is ubiquitinated. Interestingly, the characteristics of UbcH5a and Ubc13-Uev1a, when paired with CHIP or TRAF6 *in vitro*, match exactly the different requirements that one would predict for the E2s in a two-step model of ubiquitination. UbcH5a has no inherent specificity and can ubiquitinate almost any lysine on a substrate if brought into close proximity. This explains why CHIP is auto-ubiquitinated in the CHIP-UbcH5a complex and why Hsp90 is ubiquitinated in the Hsp90-CHIP-UbcH5a complex. The lack of specificity of UbcH5a also explains why the polyubiquitin chains it forms have no specific topology (Fig 2), because UbcH5a does not differentiate ubiquitin from any other substrate. Therefore, after the mono-ubiquitination of a substrate like CHIP *in vitro*, the CHIP-ubiquitin conjugate that is formed becomes a substrate for UbcH5a and ubiquitin can be transferred from ubiquitin-charged UbcH5a to any or all of the lysine residues of ubiquitin that come into close proximity (Fig 2, [14]). Therefore, it is important to distinguish this reaction from the one catalysed by Ubc13-Uev1a, which

is able to recognise ubiquitin specifically but cannot ubiquitinate any other protein substrate. An important implication of this two-step-model of protein ubiquitination is that after the mono-ubiquitination of the substrate, the polyubiquitination has to be performed by the same E3 using a different E2 or by a completely different E3-E2 complex (Fig 6). Both models are conceivable, since the binding of E1 and E3 to the E2 is mutually exclusive [16], and the E3 and E2 have to dissociate after each ubiquitination cycle.

The lack of specificity of the CHIP-UbcH5a complex determined in the present study is consistent with another report [14], which analysed the polyubiquitin chains formed by UbcH5 paired with three different E3s: CHIP, MuRF1 and Mdm2 [14]. They not only found by mass spectrometry that all seven lysine residues of ubiquitin were utilised for chain formation but also identified “forked chains” with two ubiquitin molecules linked to adjacent lysines of another ubiquitin. This unexpected result can be explained very easily by our model, since E3-UbcH5 complexes lack inherent specificity and any lysine residue on ubiquitin which is attached to a substrate can become ubiquitinated if it gets close enough to the ubiquitin-charged UbcH5. Provided that steric reasons do not prohibit it, there seems no reason why ubiquitination should not take place at any lysine residue, or even on more than one lysine residue of the same ubiquitin molecule, resulting in formation of “forked chains”.

Our findings and those of Kim et al [14] contrast with two reports that the RING E3 ligase BRCA1-BARD1 mainly forms K6-linked polyubiquitin chains with UbcH5c [17, 18]. In our experiments the polyubiquitin chains that were formed with CHIP-UbcH5a were linked via almost every lysine residue of ubiquitin, as shown by mass spectrometry, but the K6 mutant of ubiquitin was not used as efficiently as wild type ubiquitin as shown not only by decreased formation of polyubiquitin but also decreased mono-ubiquitination of CHIP (Fig 2), which should be unaffected by the K6 mutation. Therefore reliance on the use of this mutant in the absence of mass spectrometry data would have been misleading and led to the erroneous conclusion that the ubiquitin linkages formed were mainly via K6. Therefore, either the BRCA1-BARD1 complex is able to restrict the specificity of UbcH5c-mediated polyubiquitin chain formation, perhaps for steric reasons, or the BRCA1-BARD1-UbcH5 mediated polyubiquitin chain formation is not as specific as suggested previously. Ubiquitination reactions mediated by CHIP and TRAF6 when paired with Ubc13-Uev1a or UbcH5a were very similar suggesting that the observed differences between Ubc13-Uev1a and UbcH5a are not dependent on the E3 (Fig 5). We have recently obtained similar results with another E3 ligase Pellino 1, which produced free K63-polyubiquitin chains when paired with Ubc13-Uev1a but polyubiquitinated Pellino or its substrate IRAK1 in the presence of UbcH5a, these chains being linked mainly via Lys11, Lys48 and Lys63 [19].

In another recent publication a different model for TRAF6-Ubc13-Uev1a auto-ubiquitination was proposed [7]. In accord with our results they find that TRAF6 stimulates Ubc13-Uev1a-dependent formation of unanchored K63-linked polyubiquitin chains and UbcH5c-dependent TRAF6 auto-ubiquitination. However, they also found that the TRAF6-Ubc13 complex links ubiquitin to TRAF6 *in vitro* in the absence of Uev1a, a reaction that is prevented in the presence of Uev1a. They therefore proposed a model in which the first ubiquitin is added by the TRAF6-Ubc13 complex while the TRAF6-Ubc13-Uev1a complex subsequently adds the K63-linked polyubiquitin chains. This model requires that there is either an excess of free Ubc13 in cells or that the rather stable Ubc13-Uev1a complex is capable of dissociating *in vivo* under particular conditions, neither of which has been established. In addition, the E3 ligase would have to differentiate between the monomeric Ubc13 and the heterodimeric Ubc13-Uev1a complex to switch from substrate ubiquitination to K63-linked polyubiquitination which would be difficult given the fact that e.g. in the CHIP-Ubc13-Uev1a complex the U-Box of CHIP is only in contact with Ubc13 [11]. Moreover, this model overlooks the possibility that another E2 or another E3-E2 complex could be involved in catalysing the initial mono-ubiquitination reaction and several other publications support the two-step model of ubiquitination illustrated in Fig 6. There are two reports, which indicate that Ubc13-dependent K63-polyubiquitination is preceded by a Ubc13-independent mono-ubiquitination. Genetic evidence from yeast showed that PCNA is mono-ubiquitinated by RAD6 (E2) and RAD18 (E3) and then K63-polyubiquitinated by RAD5 (E3) and Ubc13-Mms2 (E2) [8]. Similarly, the K63-linked polyubiquitination of MHC class I molecules in HeLa cells mediated by the K3 gene product of Kaposi's sarcoma-associated herpes virus (KSHV) involves UbcH5b/c-mediated mono-ubiquitination followed by Ubc13-dependent polyubiquitination [9]. Furthermore, in the case of K48-linked polyubiquitination a two-step model was proposed for the anaphase promoting complex (APC) in yeast with Ubc4 promoting mono-ubiquitination and Ubc1 (the yeast homologue of the human E2-25K protein) promoting chain extension [10].

Structural considerations also support the two-step model of substrate polyubiquitination. Structural analysis of the Ubc13-Mms2 complex revealed the key role of Mms2 in positioning the acceptor ubiquitin to the Ubc13 active site to allow the modification of K63 to occur specifically [20]. Therefore, the Ubc13-Mms2 complex provides a structural explanation for the ability of this E2 to form K63-linked polyubiquitin chains specifically. On the other hand this characteristic makes it unlikely, if not impossible, for this complex to be engaged in the non-specific substrate mono-ubiquitination. There are reports in the literature that E2s specific for a particular lysine residue of ubiquitin can also ubiquitinate substrates *in vitro*, e.g. with TRAF6-Ubc13-Uev1a [21] or CHIP-

Ubc13-Uev1a [14], but this substrate ubiquitination is negligible compared to the formation of unanchored polyubiquitin chains.

Unlike Ubc13-Uev1a, UbcH5a lacks inherent specificity for any particular lysine residue of ubiquitin and makes this E2 a candidate for substrate mono-ubiquitination. We therefore propose that there are different classes of E2s, some that are involved in substrate mono-ubiquitination, e.g. the E2s of the UbcH5 family or UbcH4, and others that are able to form polyubiquitin chains of a specific topology, e.g. human Cdc34 and E2-25K for K48-linked chains and Ubc13-Uev1a (Mms2) for K63-linked chains. Based on this model we predict that E2s exist that are specific for other lysine residues of ubiquitin and have yet to be identified. During the preparation of this manuscript, a publication appeared which suggested a similar model to that proposed here based on results obtained with the RING domain E3 ligase BRCA1-BARD1 and several E2s [22].

A major question arising from this study is how the eukaryotic cell is able to switch from the mono-ubiquitination of the substrate to the elongation of the polyubiquitin chain in such a way as to avoid mixed chain formation or whether under some circumstances mixed chains and “forked chains” are formed *in vivo* and fulfil a particular function that has yet to be identified. Analysis of the complexity of polyubiquitinated proteins *in vivo* by mass spectrometry will be key to a better understanding of these processes.

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Figure legends

Figure 1. The E3 ubiquitin ligase CHIP catalyses the formation of polyubiquitin chains using the E2 ubiquitin conjugating enzymes Ubc13-Uev1a and UbcH5a.

Polyubiquitination was carried out as described under Experimental. An aliquot of the reaction (5 μ l) was subjected to SDS PAGE, transferred to nitrocellulose and immunoblotted using anti-ubiquitin (Ub, upper panels of A and B) or anti-CHIP antibodies (lower panels of A and B). The position where CHIP migrates is also indicated in the upper panels. The experiments were performed with both His₆-CHIP (lanes 2-5 of each panel) and CHIP that have been freed from GST by cleavage with PreScission protease (lanes 6-9 of each panel).

Figure 2. The CHIP-Ubc13-Uev1a complex catalyses the formation of unanchored K63-linked polyubiquitin chains, while CHIP-UbcH5a attaches polyubiquitin chains without any specific topology to CHIP.

Polyubiquitination with CHIP and Ubc13-Uev1a (A) or UbcH5a (B) was carried out as described under Experimental using wild type ubiquitin (WT) or ubiquitin mutants with one lysine (KxR) residue mutated to arginine as indicated. An aliquot of the reaction (5 μ l) was subjected to SDS PAGE, transferred to nitrocellulose and immunoblotted using anti-ubiquitin (Ub, upper panels) or anti-CHIP antibodies (lower panels). (C) Polyubiquitination was carried out with CHIP and Ubc13-Uev1a (lanes 1-3) or UbcH5a (lanes 4-6) with no ubiquitin (-, lanes 1 and 4), wild type ubiquitin (WT, lanes 2 and 5) or methylated ubiquitin (Me, lanes 3 and 6).

Figure 3. Truncated forms of CHIP lacking the TPR domain are as active as full length CHIP when paired with Ubc13-Uev1a, but far less active with UbcH5a.

Polyubiquitination reactions were carried out as described under Experimental using full length (FL) CHIP or deletion mutants as indicated, which contain the U-box alone (U), the helical hairpin and U-box HH+U) or the TPR repeats plus the helical hairpin (TPR+HH). Aliquots of the reactions were subjected to SDS-PAGE and either stained with Coomassie Blue (left hand panel) or transferred to nitrocellulose membranes and immunoblotted with anti-ubiquitin (Ub, right hand panel). The lysine residue N-terminal to the first TPR domain that becomes ubiquitinated by CHIP-UbcH5a (K22) is indicated.

Figure 4. Hsp90 is a substrate for CHIP-UbcH5a, but not of CHIP-Ubc13-Uev1a in vitro

Polyubiquitination reactions were carried out as described under Experimental with the further inclusion of Hsp90 at the concentrations indicated. SDS-PAGE followed by staining with Coomassie Blue (upper left panel) or immunoblotting with antibodies recognising ubiquitin (Ub, lower left panel), Hsp90 (upper right panel) or CHIP (lower right panel). The positions where the unmodified and ubiquitinated proteins migrate are indicated by arrows.

Figure 5. The E3 ubiquitin ligase TRAF6 acts similarly to CHIP when complexed to the E2 conjugating enzymes Ubc13-Uev1a and UbcH5a.

Polyubiquitination reactions were carried out as described under Experimental with either CHIP or GST-tagged TRAF6 as the E3 ligase and Ubc13-Uev1a and UbcH5a as the E2 ubiquitin conjugating enzymes. The experiment was performed as described in Fig 1, except that immunoblotting was additionally performed with anti-TRAF6 antibodies. The positions at which the unmodified and ubiquitinated forms of TRAF6 and CHIP migrate are indicated by arrows.

Figure 6. Model for substrate polyubiquitination by RING and U-Box E3 ligases.

Based on the results presented in this paper and other data in the literature, it is proposed that proteins that are polyubiquitinated through a specific lysine of ubiquitin are formed by a two-step reaction that requires two different E2 ubiquitin-conjugating enzymes. In the first step (1), initial ubiquitin is attached to the protein substrate by an E2 ubiquitin-conjugating enzyme (E2a) with the properties of UbcH5a or UbcH4. To avoid the formation of polyubiquitin chains linked by various lysine residues of ubiquitin, the E2a has to be removed at this stage and replaced in the second step by another E2 conjugating enzyme (E2b) that elongates the polyubiquitin chains with a specific topology, like Ubc13-Uev1a for K63-linked chains. The E3 ubiquitin ligase employed at step 1 (E3a) may be retained at step 2 (2), or replaced at this stage (3) by another E3 ligase (E3b). As a variation of this model a polyubiquitin chain of a specific topology could be preassembled using E2b on the catalytic cysteine residue of E2a and transferred to the substrate in the final step [23, 24].

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Figure 1

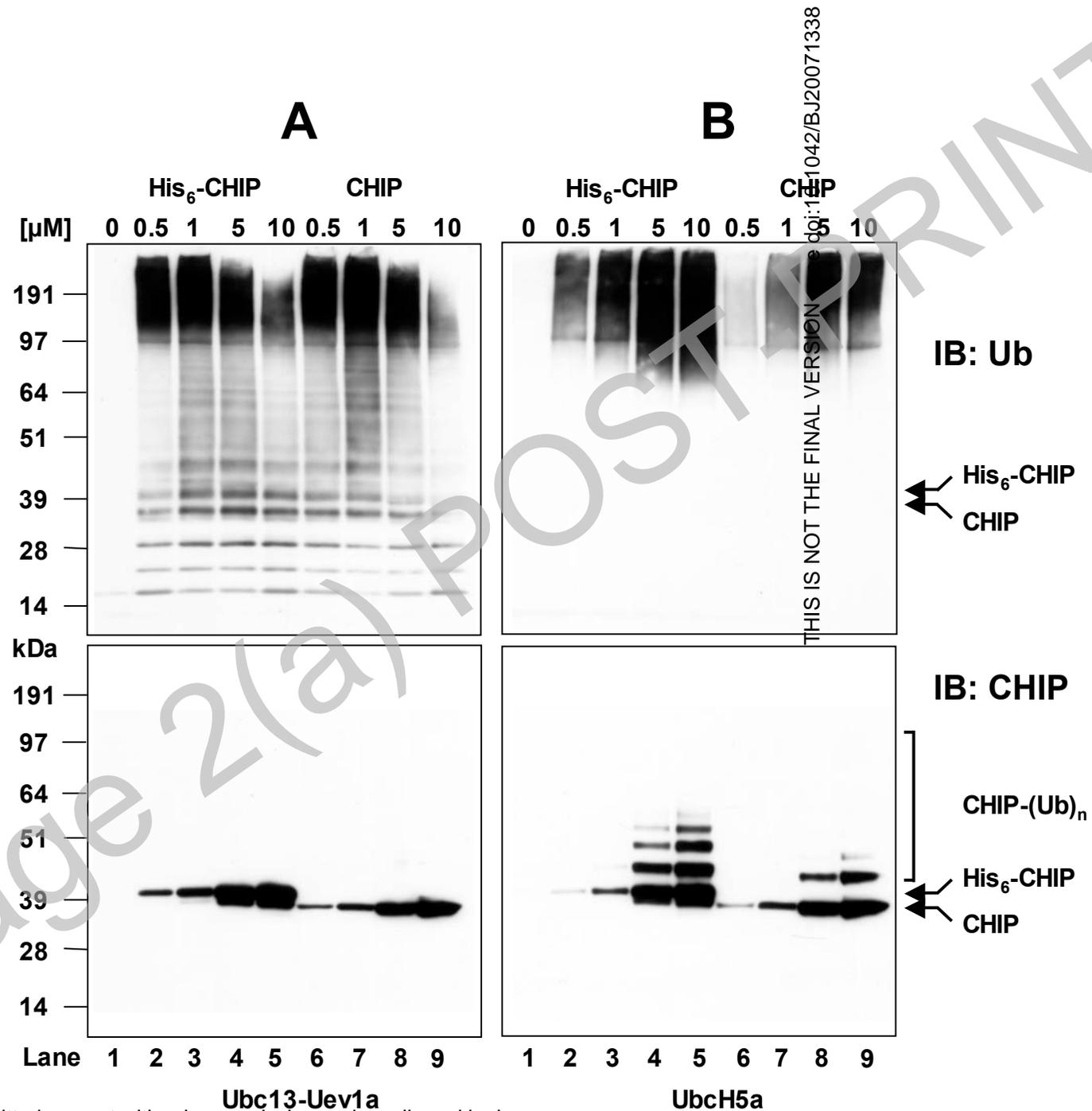


Figure 4

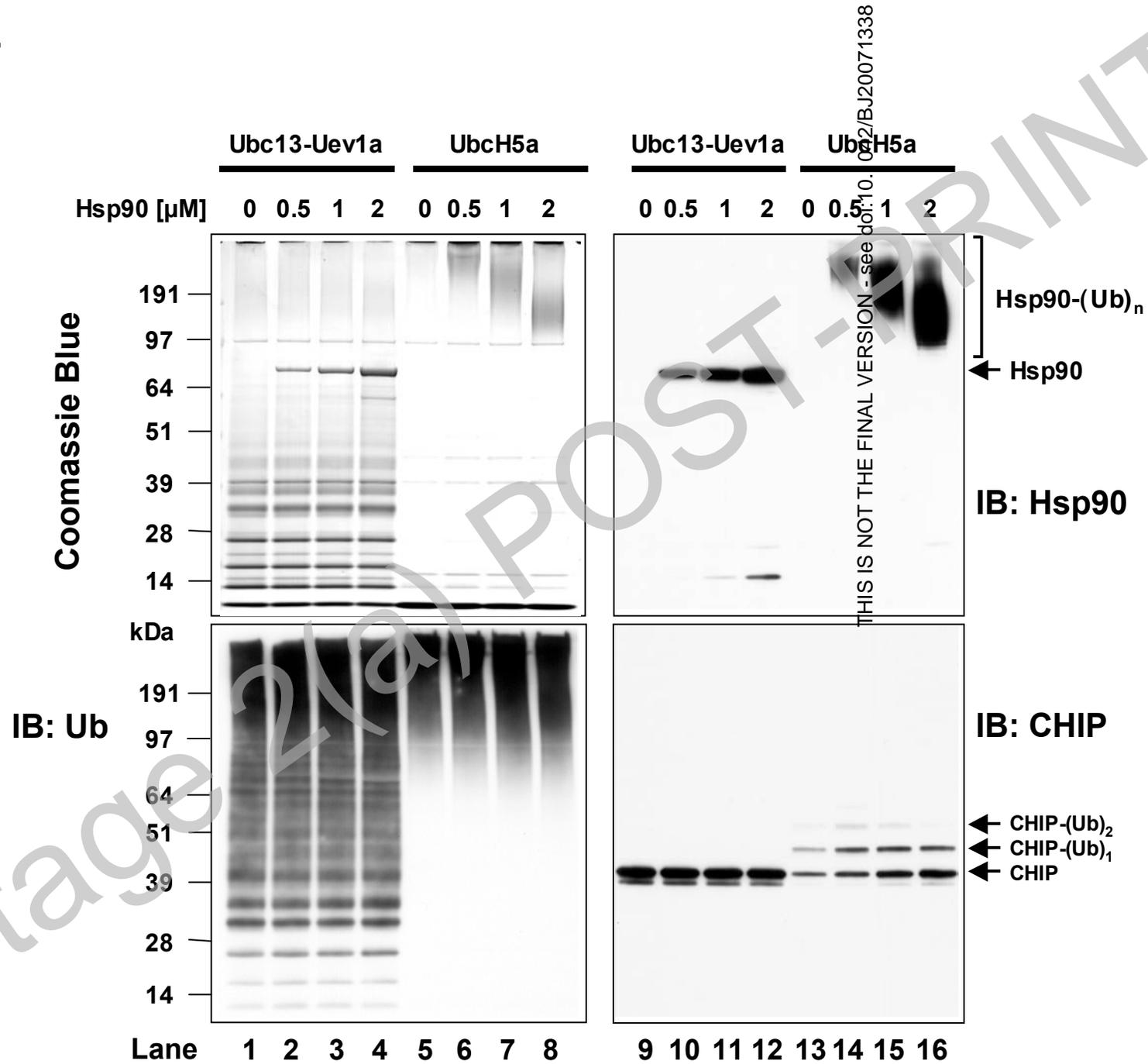


Figure 5

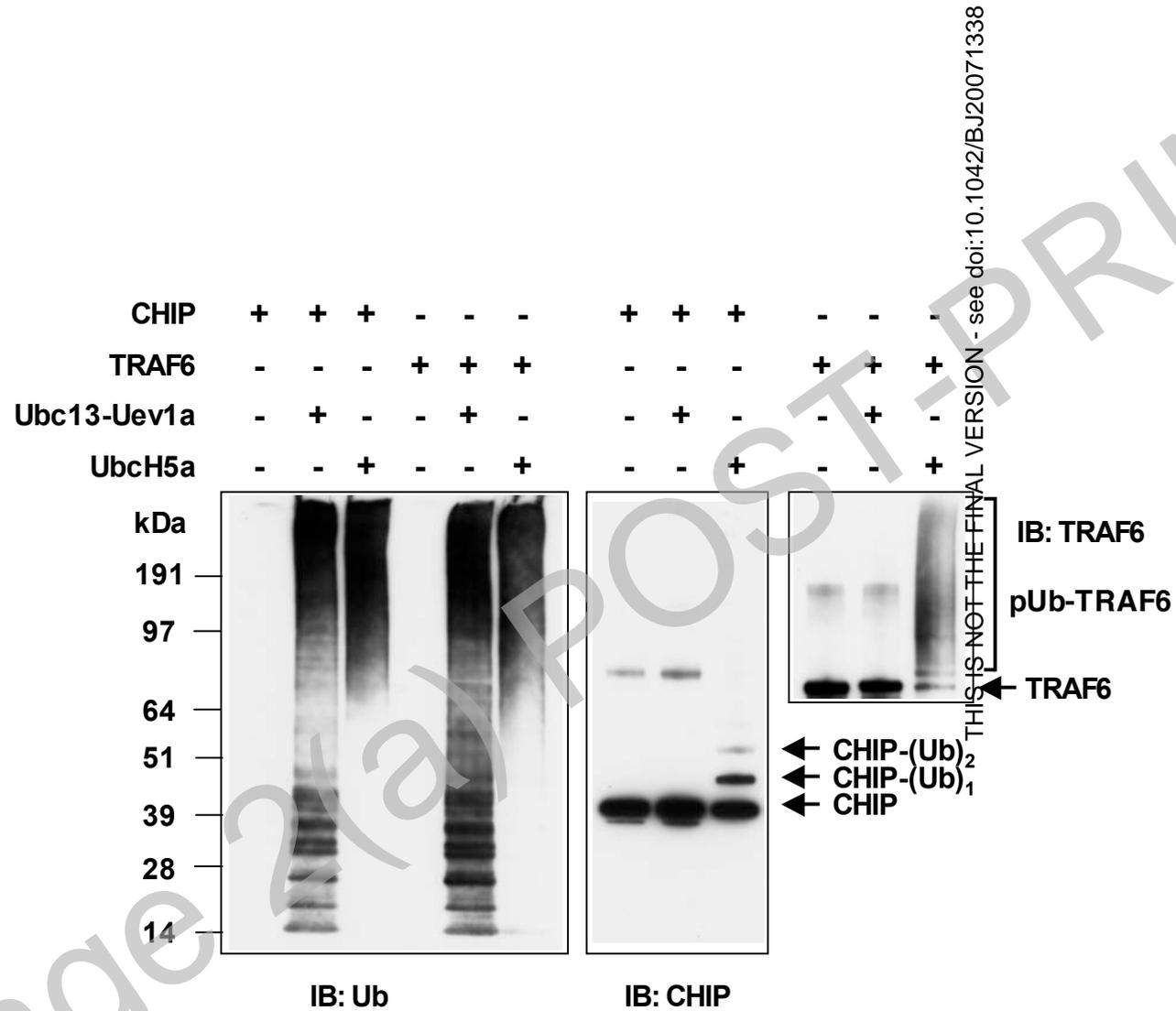


Figure 6

