SIGNAL SPECIFIC UBIQUITINATION AND DEGRADATION OF IκBα

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DEDICATION

This work is dedicated to my mentors Dr. DeMartino and Dr. Chen for supporting me even when I decided to exit with my Master's degree. I would also like to thank my committee, who maintained interest and gave support even after my decision to leave the program. My mother and father must have done many things correctly for me to be here writing a dedication for my Masters thesis. THANK YOU FOR ALL OF YOUR LOVE AND SUPPORT!

SIGNAL SPECIFIC UBIQUITINATION AND DEGRADATION OF IκBα

by

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THESIS

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SIGNAL SPECIFIC UBIQUITINATION AND DEGRADATION OF IκBα

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The transcription factor Nuclear Factor κB (NF-κB) is retained in the cytoplasm by the action of its inhibitor IκB. Upon phosphorylation by the IKK complex, IκB is rapidly ubiquitinated and targeted for 26S proteasome mediated degradation, thus liberating NF-κB for transport to its nuclear destination. The current project was initiated to reconstitute this pathway *in vitro* by using the purified ubiquitination and degradation machinery to degrade IκBα, and activate NF-κB. While signal dependant IκBα ubiquitination was achieved early in the project, this substrate was not degraded by a number of different 26S protein preparations. Instead, an integral or associated isopeptidase activity was observed with each 26S preparation. The development of new 26S protein purification methods has enabled the isolation of highly purified 26S proteins that exhibits low degradative activity towards the ubiquitinated IκBα substrate without excess isopeptidase activity. In an effort to increase substrate degradation, the IκBα ubiquitination reaction was carefully scrutinized. The current literature reports that Ubch5 is the relevant E2 that works in conjunction with the IκBα $SCF^{\beta TrCP}$ E3 complex, however, Cdc34/Ubc3 can also ubiquitinate IκBα, and may also be a relevant E2. While both E2s carry out *in vitro* signal dependant ubiquitination of IκBα, the ubiquitin conjugates made by Ubc3 are specific for Lysine-48 linked isopeptide bonds, whereas Ubch5 is able to utilize a variety of ubiquitin surface Lysine residues in isopeptide bond formation. Because K-48 linked ubiquitin conjugates are believed to target substrates for 26S mediated degradation, it was not surprising to find that my 26S proteasome preparations exhibited higher levels of IκBα degradation when ubiquitin conjugation reactions were carried out with Ubc3 instead of Ubch5. Using small interfering RNA to knock down the protein levels of each E2 *in vivo*, we have found that Ubc3 has no effect on IκBα degradation, whereas the Ubc5/7 double knockdown exhibits partial inhibition of IκBα degradation which is comparable to knocking down the levels of the IκBα E3 specificity factor βTrCP. The completion of this project has established an *in vitro* ubiquitination and degradation system that will be instrumental for future studies aimed at determining how the 26S proteasome unfolds and degrades its protein substrates.

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PRIOR PUBLICATIONS:

Strickland, E.; Hakala, K.; Thomas, P. J.; DeMartino, G. N. (2000) Recognition of misfolding proteins by PA700, the regulatory subcomplex of the 26 S proteasome. *Journal of Biological Chemistry* Vol. 275, No. 8, 5565-5572

Tang, Z.; Li, B.; Bharadwaj, R.; Zhu, H.; Ozkan, E.; Hakala, K.; Deisenhofer, J.; Yu, H. (2001) APC2 Cullin protein and APC11 RING protein comprise the minimal ubiquitin ligase module of the anaphase-promoting complex. *Mol Biol Cell*. (12):3839-51

LIST OF FIGURES

LIST OF DEFINITIONS:

293T HEK – Human Embryonic Kidney cell line expressing the large T antigen

AAA-ATPase – ATPase Associated with a variety of cellular Activities

ATP – Adenosine Tri-Phosphate

βTrCP – β Transducin repeat (a.k.a. WD40 repeats) Containing Protein. The F-box protein that recognizes phosphorylated IκBα.

Cdc34 – Cell division cycle mutant number 34 (a.k.a. Ubc3)

E2-25k – E2 with a molecular weight of 25 kilodaltons

Fraction I – The fraction of S-100 that does not bind to a DEAE anion-exchange column.

Fraction II – The fraction of S-100 that does bind to a DEAE anion-exchange column, and is eluted with 0.5M of either NaCl or KCl

HSP – Heat Shock Proteins. A large class of chaperones up regulated during conditions of stress, including heat shock.

IκBα – Inhibitor of Nuclear Factor kappa B, subtype alpha

IKK – IκB kinase complex

ME3 – Mouse E3 for IκBα (a.k.a. βTrCP)

MG-132 – MyoGenic's (a biotech company) inhibitor of the 20S proteasome compound # 132

Nedd8 – Neuronal precursor cell expressed, developmentally down regulated 8 . This is a small ubiquitin like modifier of the Cullin SCF subunits.

NFκB – Nuclear Factor kappa B

Npl4 – Nuclear protein localization factor 4

p37 – Subunit of the 19S regulatory complex (PA700) believed to be an isopeptidase inhibited by Ub-ald (a.k.a. p40; S12; Rpn8)

p65 – NF-κB family member (a.k.a. RelA)

PBDM – Protein Break Down Mix is an ATP regenerating system, see materials and methods

RJD 1144 – Raymond J. Deshaies yeast strain number 1144

S-100 – The term given to a crude extract that has undergone centrifugation at 100,000 X gravity for one hour at 4°C, which results in a cytoplasmic extract free of all intracellular organelles

SCF – Skp 1, Cul 1, F-box containing modular ubiquitin ligating enzyme (E3)

siRNA – small interfering RNA

TNFα – Tumor Necrosis Factor α is a cytokine that initiates the NF-κB response

Ub – Ubiquitin

Ub-ald – Ubiquitin containing an aldehyde at the C-terminus. Used as a suicide inhibitor against isopeptidase enzymes that cleave ubiquitin chains.

Uba – Ubiquitin activating enzyme (E1)

Ubc – Ubiquitin conjugating enzyme (E2)

Ubch – Ubc from human

Uev1a – Ubiquitin enzyme variant 1a (E2 variant lacking the active site cystine)

Ufd1 – Ubiquitin fusion degradation protein 1

VCP – Valosin Containing Protein. AAA-ATPase that is a chaperone

CHAPTER ONE

INTRODUCTION:

The NF-κB family of transcription factors plays a central role in mammalian inflammatory and innate immune responses. While a host of extracellular events can initiate a number of different signal transduction cascades that activate NF-κB, all pathways lead to the phosphorylation and degradation of the inhibitor of NF-κB, IκB. IκB family members inhibit NF-κB by binding to this dimeric transcription factor and sequestering it in the cytoplasm. Upon phosphorylation by the activated Iκ-kinase (IKK) complex, IκB is recognized by the $SCF^{\beta Trcp}$ E3 ubiquitin ligase complex, ubiquitinated, and subsequently degraded by the 26S Proteasome. This chain of events leaves NF-κB dimmers free to enter the nucleus to activate a variety of genes involved in various aspects of innate immunity (for an excellent review, see Silverman and Maniatis, 2001).

The ubiquitin (ub) proteasome pathway is responsible for the bulk of non-lysosomal protein degradation in the cell (Ciechanover, 1994). Ubiquitin is an 8 kilodalton protein that is ubiquitously expressed in all cells and tissue types (see Hershko and Ciechanover 1982, for history and review). This pathway utilizes the covalent attachment of a poly-ubiquitin chain to the substrate protein as a degradation signal recognized by the 26S proteasome. The poly-ubiquitin chain consists of one isopeptide bond between one of the substrate's lysine residues, and the carboxy terminus of the proximal ubiquitin. Additional ubiquitin proteins are then added to the proximal ubiquitin by another isopeptide bond, this time originating from one of the previous ubiquitin's seven surface lysine residues. Due to the numerous

lysine residues available on the surface of ubiquitin, the isopeptide linkages contained with in the poly-ubiquitin chain represent a wealth of information. While K48-G76 linked chains target proteins for degradation, K63-G76 linked chains are utilized in non-proteolytic cell signaling events (Deng et.al. 2000; for review see Pickart, 2001). Other chain linkages exist, but their physiologic role has yet to be determined.

The ubiquitination machinery is organized in a hierarchical fashion consisting of one or two E1 (ubiquitin activating) enzymes, tens of E2 (ubiquitin conjugating) enzymes, and hundreds of E3 (ubiquitin ligating) enzymes. The ubiquitination cascade begins when the E1 enzyme utilizes ATP and Ub to form a high energy AMP-Ub adduct, which is then transferred to the active site cystine of the E1 enzyme. The "activated" ubiquitin is then transferred to the active site cystine of the E2 enzymes, again, resulting in the formation of a ubiquitin thioester bond. The E2s then cooperate with the E3s, which are responsible for substrate selection, to carry out multi-ubiquitin chain formation (for review see Pickart, 2001).

The 26S proteasome is the multi-catalytic protease responsible for the degradation of ubiquitinated proteins (for review see DeMartino et. al. 1999). This protease consists of a core 20S complex that is capped on the ends by 19S (PA700) regulatory complexes (Eytan et. al. 1989; Walz et. al. 1998). The 20S structure is that of a hollow barrel which sequesters the active sites of the protease on the inside (Lowe et. al. 1995). The 19S regulatory complex consists of a "base" which includes 6 AAA-ATPase family members that form a ring which abuts the ends of the 20S barrel. While the ATPase activity of the 19S is required for its association with the 20S proteasome, it is also believed that these 6 ATPases act to unfold

and translocate substrate proteins into the gated hollow core of the 20S for degradation (Glickman et. al. 1998; Adams et. al. 1998). The additional "lid" complex is attached to the "base" by a single subunit that acts as a hinge to join the two sub-complexes. This complex is believed to be responsible for recognizing and dealing with aspects of the poly-ubiquitin chain component of the substrate proteins (Glickman et. al. 1998; Strickland et. al. 2000).

While it was known that IκB was rapidly degraded after stimulation by a variety of sources, the first clear demonstration that IκB is degraded by the ubiquitin proteasome pathway came in 1995(Chen et. al. 1995). This report demonstrated that phosphorylation of IκB served as a signal for ubiquitination and subsequent degradation by the 26S proteasome. Four years later, the specificity factor for IκB ubiquitination was described as βTrcp (Spencer et. al. 1999). βTrcp works in conjunction with a modular type of E3 known as the SCF complex. These complexes ubiquitinate multiple cellular substrates by associating with different specificity factors. The various SCF complexes bring the substrate together with the proper E2, and catalyze the transfer of ubiquitin to the substrate (for review see Pickart, 2001). The SCF complex for IκB ubiquitination is composed of Skp1, Cull1, Roc1, and βTrcp (for review see Deng et. al. 2003).

It is well established that phosphorylated IκB remains bound to NF-κB (Traenckner et.al. 1994; Finco et.al. 1994; Verma et.al. 1994). In addition, it has been shown that ubiquitinated IκB also remains bound to NF-κB (Chen et. al. 1995). Because of these observations, it is believed that the last step in NF-κB activation is the remodeling of the NF-κB:IκB complex by the 26S proteasome, resulting in the selective degradation of IκB.

(**Figure 1**) To gain insight into the mechanism of this 26S proteasome mediated remodeling, we set out to reconstitute this pathway *in vitro*, using a defined system consisting of purified components.

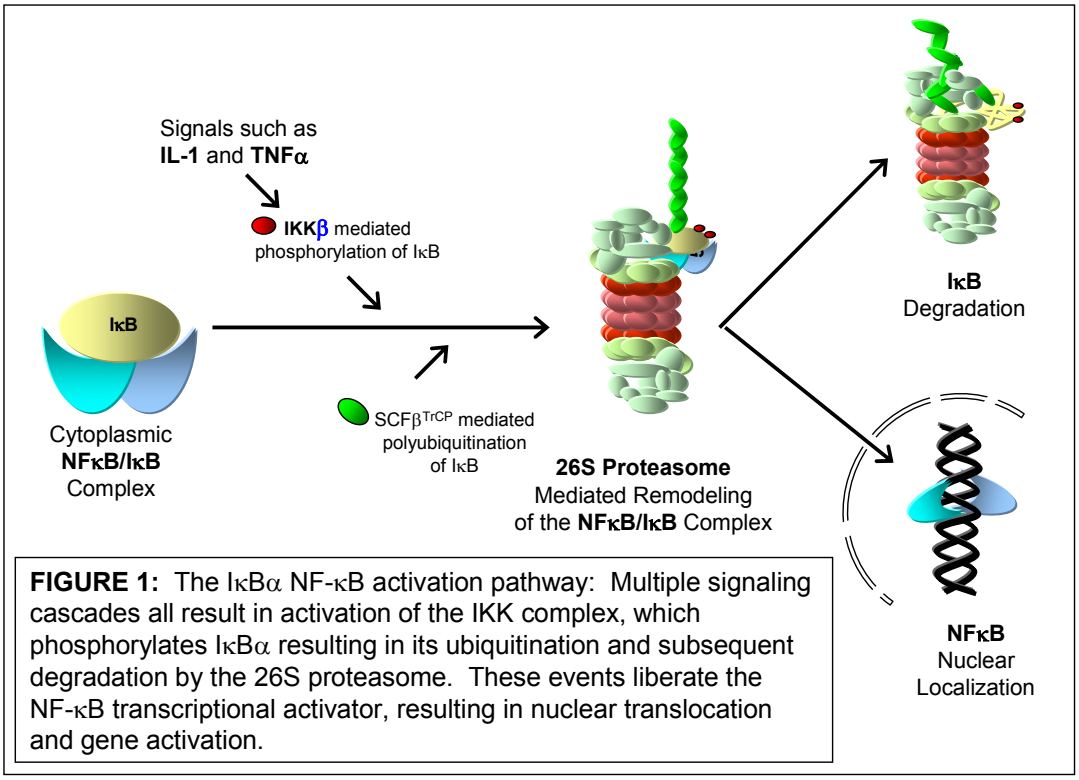

FIGURE 1: The IκBα NF-κB activation pathway: Multiple signaling cascades all result in activation of the IKK complex, which phosphorylates IκBα resulting in its ubiquitination and subsequent degradation by the 26S proteasome. These events liberate the NF-κB transcriptional activator, resulting in nuclear translocation and gene activation.

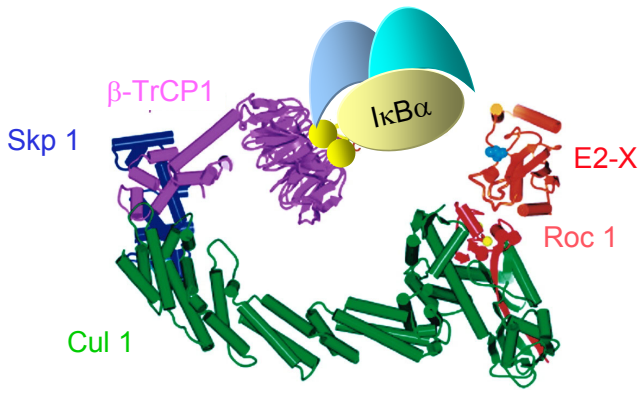

FIGURE 1b: Model diagram of the $SCF^{\beta TrCP}$ E3 complex: This model, adapted from Wu et. al 2003, was made "by superimposing the Skp1-F box portions of the Skp1-β-TrCP1-β-catenin and the SCF^{Skp2} complexes (Zheng et. al., 2002)" (Wu et. al. 2003). The author added the Phosphorylated IκBα:NFκB cartoon diagram. As the literature reports up to three different E2s that work to ubiquitinate IκBα, the E2 has been labeled as "X".

CHAPTER TWO

RESULTS:

The first phase of this project was to faithfully reproduce the signal dependant ubiquitination and degradation of IκB. For this purpose, an alliance was forged between the laboratory of Dr. George DeMartino, who specializes in the large scale purification of the 19S regulator and 20S proteasome, and the laboratory of Dr. Zhijian "James" Chen, who specializes in IKK activation and IκBα ubiquitination. The Chen lab had previously reconstituted IκBα ubiquitination using purified components expressed from a variety of sources (Chen unpublished results), so the first step was to reconstitute the 26S proteasome mediated degradation of IκBα.

Initial degradation reactions were carried out with ^{35}S-IκBα ubiquitinated in IKKβ programmed S100 extracts and immunoprecipitated with anti-p65-agarose (anti-NF-κB) coupled resin. The 26S proteasome was then reconstituted from purified 19S and 20S by adding ATP/Mg^{2+} and incubating at 37°C for 45 minutes as described previously (Ma et. al. 1994). The reconstituted 26S proteasome was added to the immunoprecipitated ubiquitinated complex at various concentrations, but degradation of the radio-labeled IκBα was never observed. Instead, an integral or associated isopeptidase activity was removing all of the ubiquitin conjugates from IκBα (data not shown). This isopeptidase activity was inhibited by ubiquitin aldehyde (Ub-ald), and is thus not the 19S associated metallo-isopeptidase described recently in the literature (Leggett et. al. 2002; Verma et. al. 2002; Yao et. al. 2002). This isopeptidase activity is most likely that of the previously described integral isopeptidase

of the 19S, p37 (Lam et. al. 1997a and 1997b). In addition, adding the 19S preparations directly to the ubiquitinated conjugates resulted in the same isopeptidase activity, as all ubiquitin conjugates were cleaved from the radio-labeled IκBα (data not shown). Because the stoichiometry of 26S reconstitution from 20S and 19S requires a several fold molar excess of 19S, I decided this approach was unfeasible due to this isopeptidase activity. It is also of interest to note that reports in the literature suggest that purified 19S is not the same as 19S dissociated from "bona fide" 26S proteasome (Sawada et. al. 1997).

Due to the problems described above, we decided to purify the native 26S complex from rabbit muscle as described in the original IκB degradation assay (Chen et. al. 1995). Three large scale preparations were undertaken, but each preparation contained the same Ub-ald inhibited isopeptidase activity towards the ubiquitinated IκBα substrate (data not shown). In addition, I was unable to obtain large quantities of highly purified 26S proteasomes from this tissue source. Another approach would have to be taken in obtaining the 26S proteasome.

I next obtained the yeast strain RJD 1144 from Dr. Raymond J. Deshaies, and purified several large scale preparations of affinity tagged yeast 26S proteasome as described (Verma et. al. 2000). While yeast 26S proteasome preparations were considerably easier to make, I was never convinced that my preparations were stoichiometric for the 19S and 20S components, as each preparation appeared to contain an excess of 20S proteasome. In addition, each preparation was contaminated by a several fold molar excess of an unidentified protein running at approximately 40 kDa on SDS-PAGE gels. Although these concerns might have been irrelevant, the yeast 26S proteasome exhibited neither an

isopeptidase activity nor a protein degrading activity towards our ubiquitinated IκBα substrate (data not shown). Because of this, we began developing a new method for the large scale purification of the 26S proteasome.

Bovine red blood cells were a natural choice due to the DeMartino laboratory's extensive experience purifying 19S and 20S from this same source. In addition, red blood cells give the purifier an advantage due to the lack of internalized membrane structures in these specialized cells. We were able to purify large quantities of highly pure 26S proteasome from this source (see materials and methods) (**Figure 2**).

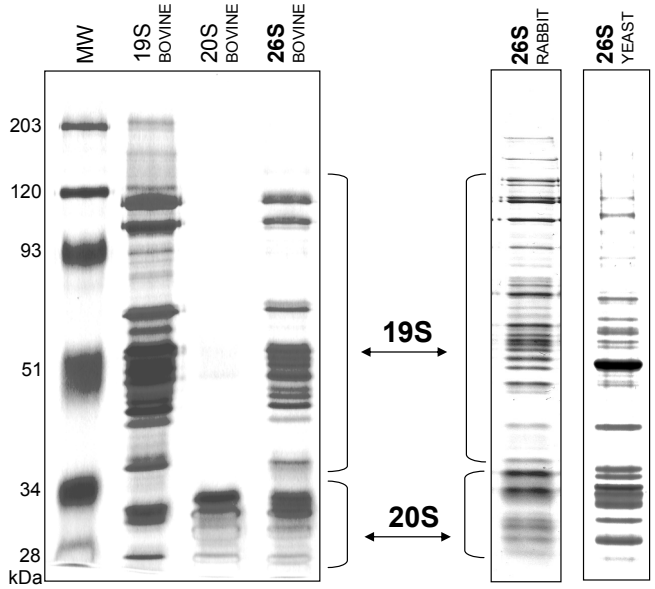

FIGURE 2: 26S proteasome preparations: Approximately 2μg of each sample was run on a 9% SDS-PAGE gel and silver stained. The bands from the Rabbit and Yeast preparation can not be directly compared to the bovine preparations as they were not run on the same gel.

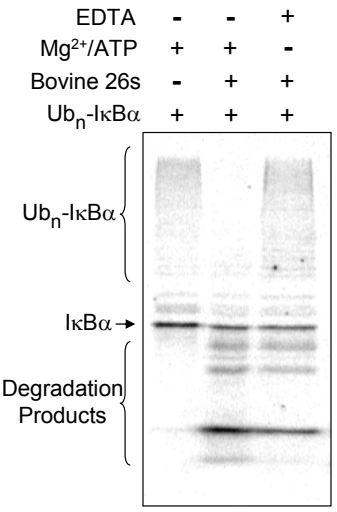

Figure 3: IκBα degradation I: ^{35}S-IκBα was ubiquitinated in S100 extracts as described in materials and methods. Ubiquitin conjugates were then immunoprecipitated with anti-p65 coupled agarose, and 26S proteasome was added with 1X PBDM and incubated at 37°C for 60 minutes. Reactions were analyzed by running on a 9% SDS-PAGE gel followed by autoradiogram.

In addition, these 26S proteasome preparations were fully competent to degrade ubiquitinated IκBα in an ATP/Mg^{2+} dependant fashion, and lacked the Ub-ald inhibited isopeptidase activity of the previous preparations. (**Figure 3**) As seen in the assay, there is a considerable

amount of degradation of the unmodified IκBα. I believe that this is either due to a small but constant amount of free 20S proteasome in the preparations, or due to dissociation of 26S into 19S and 20S, as I can observe the same result by adding purified 20S proteasome (data not shown). With phase I of the project completed, I next set out to reproduce these results using the purified and reconstituted ubiquitination machinery to ubiquitinate IκBα.

The reconstituted ubiquitination system produced much higher molecular weight ubiquitinated IκBα than did the S100 ubiquitin conjugation system. In light of this observation, it was quite unexpected to observe far less degradation of these conjugates than was observed for the S100 created conjugates. In addition, some isopeptidase activity had returned, as observed by the reappearance of unmodified IκBα during the course of the degradation assay. (**Figure 4**) This opened up the possibility that an isopeptidase activity was in our S100 created conjugate degradation assay, but that I am unable to see such an activity due to the presence of unmodified IκBα at time zero.

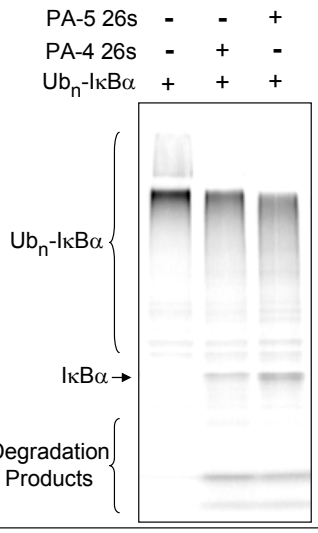

Figure 4: IκBα Degradation II: ^{35}S-IκBα was ubiquitinated using the reconstituted ubiquitination system as described in materials and methods. Ubiquitin conjugates were then immunoprecipitated with anti-p65 coupled agarose, and 26S proteasome was added with 1X PBDM and incubated at 37°C for 60 minutes. Reactions were analyzed as in Figure 2. Two different 26S preparations were used for comparison.

While numerous possibilities existed that could cause these conjugates to be degraded in a less efficient manner, I decided to first confirm that our reconstituted ubiquitination system was signal specific. This was accomplished by omitting IKKβ. As seen in figure 5, Ubch5c

displays some level of non-specific ubiquitination at almost every point in its titration. (**Figure 5**) Subsequent Ubch5 ubiquitination assays have all been performed using the

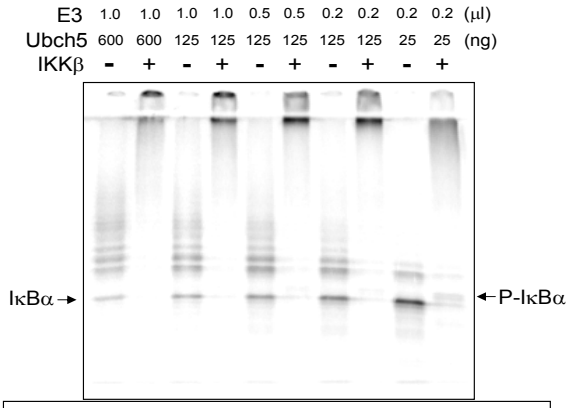

Figure 5: Specificity and titration of conjugation machinery I: Conjugates were prepared as described in materials and methods, but the amounts of Ubch5c and E3 were titrated. The substrate is now purified $_{His6}$p65:IκBα as described in materials and methods. Reactions were analyzed as in Figure 2.

25 ng range of this E2. To further characterize our ubiquitination system, I decided to investigate the isopeptide linkage of the ubiquitin conjugates. This was carried out using various ubiquitin mutants that had been previously expressed and purified by the Chen lab (Deng et. al. 2000). It was surprising that our defined ubiquitination system actually created a variety of ubiquitin isopeptide linkages. (**Figure 6**) In addition, the possibility can not be ruled out that some of the

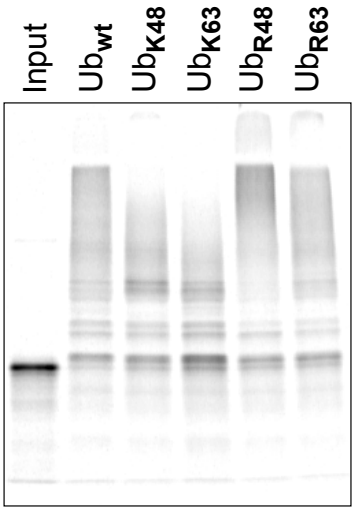

Figure 6: Ubiquitin linkage specificity of Ubch5: Ubiquitination assays were carried out as described in materials and methods, using the indicated ubiquitin mutants. The substrate is purified $_{His6}$p65:IκBα. Reactions were analyzed as in Figure 2.

conjugates have utilized the N-terminus of ubiquitin to create a peptide linkage.

The logic for using Ubch5 as the proper E2 in our IκBα ubiquitination assays stems from the fact that it is found in Fraction I. When HeLa S100 is fractionated into Fraction I and Fraction II, two components must be added back to Fraction II in order to restore the ubiquitination of IκBα. These components were found to be ubiquitin and an E2 from the Ubc4/Ubc5 family

(Chen et. al. 1996; Gonen et. al. 1999). There are numerous reports in the literature that Cdc34/Ubch3 will also support the ubiquitination of IκBα via. the $SCF^{\beta Trcp}$ E3 complex, but because Ubch3 is found in Fraction II, and,

when purified, must be used at much higher concentrations than Ubch5, we favored Ubch5 as the relevant E2 (Vuillard, et. al. 1999; Gonen et. al. 1999; Strack et. al. 2000; Wu et. al. 2000, 2002). In retrospect, there are many explanations for such a negative result, especially in light of the fact that these assays are done in crude extracts. I obtained a Cdc34/Ubch3 clone from the laboratory of Dr. Hongtao Yu, and decided to investigate this E2 in our IκBα ubiquitination and degradation assays.

As seen in figure 7, both Ubch5 and Ubch3 are capable of the signal specific ubiquitination of IκBα in our reconstituted assays. (**Figure 7**) Although it appears that

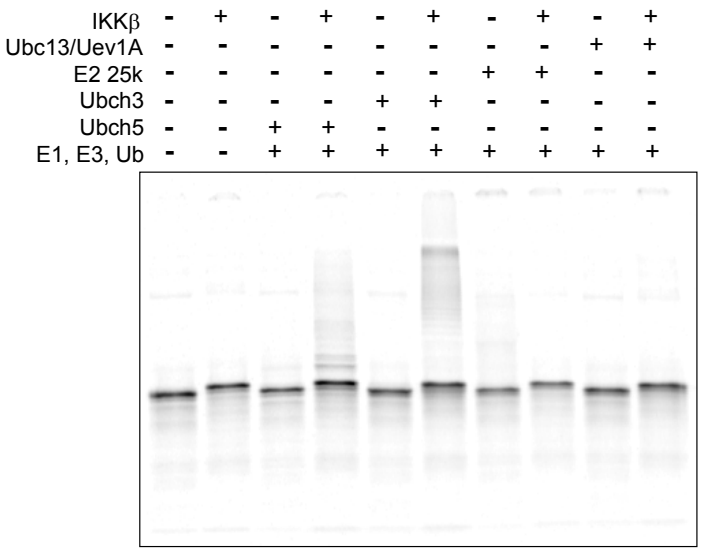

Figure 7: Ubch3 supports signal dependant ubiquitination of IκBα: Ubiquitination reactions were carried out as previously described with the exception of the differing E2 enzymes used. The substrate is purified $_{His6}$p65:IκBα. Reactions were analyzed as in figure 2.

Ubch3 is more efficient at ubiquitination, no conclusion can be made due to the differing concentrations of the various E2 enzymes analyzed in this assay. In addition, two other E2 enzymes were used as controls, and while the results are negative for IκBα ubiquitination, both E2 25k and Ubc13/Uev1a are fully capable of free ubiquitin chain

formation (data not shown). In light of Ubch3's promising activity, I next decided to investigate the isopeptide linkage utilized by the Ubch3 mediated IκBα ubiquitin conjugates, but first needed to determine what concentrations to use the two E2 enzymes at. Figure 8 is a Ubch3 titration similar to that shown in figure 5, with the exception that 0.2 μl of the E3 was used. (**Figure 8**)

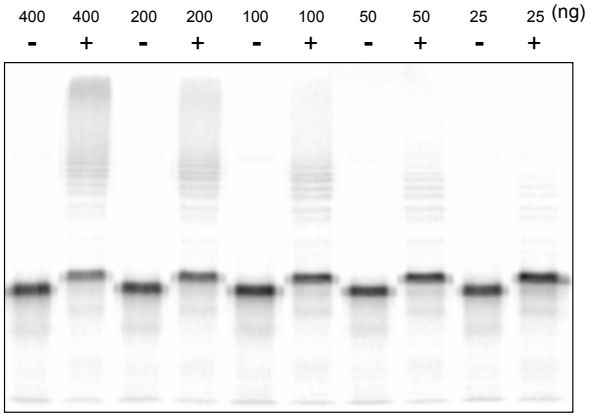

Figure 8: Ubch3 displays no non-specific ubiquitination of IκBα: Reactions Were carried out as in figure 4 with the exception of a constant amount of E3 (0.2 μl). Reactions were analyzed as in figure 2.

Conditions were sought that gave approximately equal amounts of IκBα ubiquitin conjugates as those seen in the last lane of figure 5. While no quantification was carried out, my rough estimate is that a tenfold molar excess of Ubch3 is required to achieve the levels of IκBα ubiquitination seen with Ubch5 (Ubch5 ≅ 20kDa; Ubch3 ≅ 34kDa) in our reconstituted system. One report in the literature compares the rates of IκBα ubiquitin chain formation mediated by $SCF^{\beta Trcp}$ and Ubc5 vs. Ubc3, and concludes that Ubc5 is 19 times more active than Ubc3 (Strack et. al. 2000). As I did not

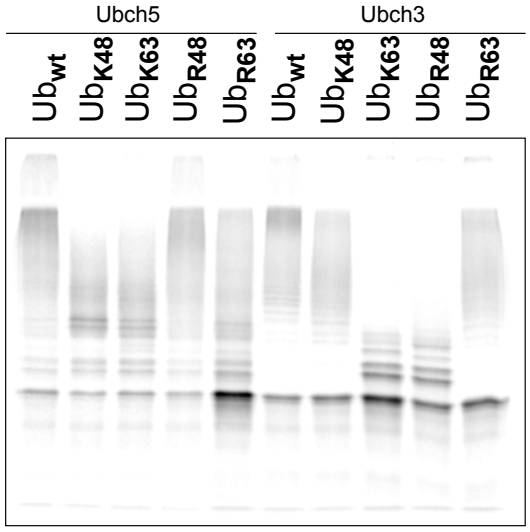

Figure 9: Ubiquitin linkage specificity of Ubch5 vs. Ubch3: Reactions were carried out as in figure 5. Ubch5 is used at 25 ng, and Ubch3 is used at 400 ng. Reactions were analyzed as in figure 2.

investigate the rates of ubiquitin chain formation, one must exercise caution when attempting to compare the two studies. Using 25 ng of Ubch5 and 400 ng of Ubch3, we investigated the isopeptide linkages favored by each E2 in our reconstituted system. As seen in figure 9, the levels of E2 enzymes chosen conjugate approximately equal amounts of wt ubiquitinated IκBα. (**Figure 9**) The striking finding of this assay is that Ubch3 mediated ubiquitination of IκBα is absolutely specific for the K48:G76 isopeptide linkage. I speculate that the observed low molecular weight Ubch3 mediated conjugates formed with Ub_{K63} and Ub_{R48} represent mono ubiquitination products linked to the various surface lysines of IκBα, but I have not yet confirmed this hypothesis with a mutant ubiquitin lacking all lysines. As it is the K48:G76 linkage that is believed to be utilized for 26S mediated protein degradation, I next turned my attention back to IκBα degradation.

Ubch3 mediated IκBα ubiquitin conjugates are preferentially degraded over those mediated by Ubch5. As seen in figure 10, our 26S proteasome degradation assays yielded lower molecular weight degradation products running at and below the dye front in the Ubch3 lanes. (**Figure 10**) This degradation reaction was also carried out in the presence of 20mM KCl, and while I tested numerous salt and buffer

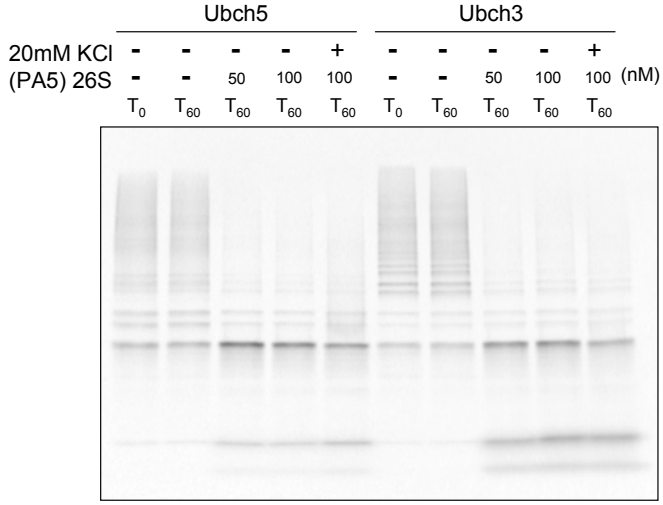

Figure 10: Comparison of Ubch3 and Ubch5 IκBα conjugate degradation I: Ubiquitin conjugation and degradation reactions were carried out as in figure 3, with the exceptions of adjusted E2 enzyme levels, and the addition of KCl in two reactions (see text). Assays were analyzed as in figure 2.

conditions in an attempt to increase the observed degradation of IκBα, no effect was seen (data not shown). The preference of the 26S proteasome for K48:G76 linked ubiquitinated IκBα was observed numerous times, and will be discussed later in this article.

The recent development of RNA interference has provided a means of selectively knocking down the translation of individual proteins *in vivo*. We therefore used this technique to provide *in vivo* data regarding which E2 is physiologically relevant for IκBα degradation. I chose 293T HEK cells to carry out this line of experimentation due to their well documented TNFα initiated NF-κB activation, and their ease of transfection with calcium phosphate. In addition to Ubch5 and Ubch3, I also investigated the possibility that Ubch7 was involved in IκBα ubiquitination due to one report in the literature that identified E2-FI (E2 in Fraction I, a.k.a. Ubch7) as the factor needed to restore the ubiquitination of IκBα in fraction II (Alkalay et. al. 1995). In addition, unpublished data from Dr. Chen indicated that the necessary factor from fraction I might be Ubch7, as it had previously been identified by MALDI-TOF as the required E2. As seen in figure 11, the TNFα stimulated IκBα degradation kinetics are characterized by a slower migrating phosphorylated IκBα species observed at five minutes. At ten minutes, a drop in the levels of both species are observed, and by 20 minutes, the majority of IκBα has been ubiquitinated and degraded. No

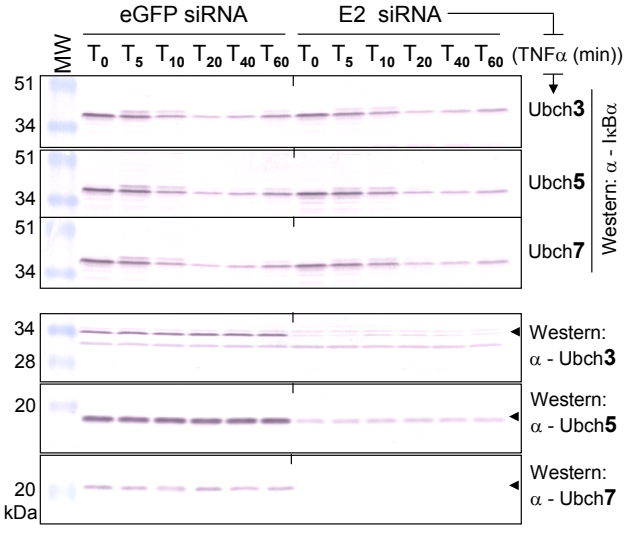

Figure 11: E2 siRNA knockdown studies I – single knockdowns: siRNA knock down and TNFα stimulation was carried out as described in materials and methods. For analysis, 20 μg of each extract was run onto a 12% SDS-PAGE gel, transferred to PVDM, and analyzed by western blot.

single E2 knock down had any effect on the TNFα stimulated IκBα degradation kinetics as assessed by anti-IκBα western blot. (**Figure 11**)

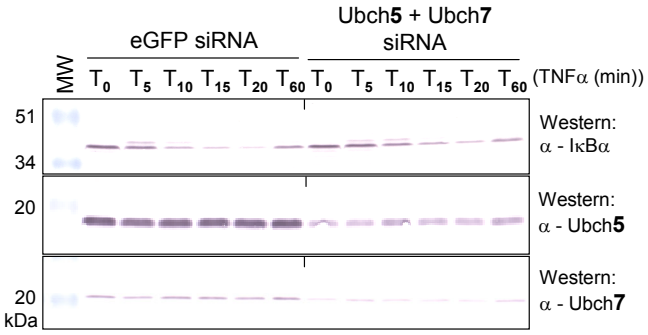

Figure 12: E2 siRNA knockdown studies II – double knockdowns: siRNA knock down and TNFα stimulation was carried out as described in materials and methods. For analysis, 20 μg of each extract was run onto a 12% SDS-PAGE gel, transferred to PVDM, and analyzed by western blot. Note: time points differ that those in figure 10.

This negative result led us to conclude that there exists significant redundancy in the E2 populations used in the ubiquitination of various cellular substrates, including IκBα, as suggested previously. To further investigate any possible redundancy, I proceeded to carry out double and triple E2 knock down experiments. As seen in figure 12, I consistently observed a 5 minute delay in IκBα degradation only with the double knock down for Ubch5 and Ubch7. (**Figure 12**) No difference in IκBα degradation rates were observed for the Ubch3 and Ubch5 double knock down (data not shown). As positive controls, I treated the cells with the proteasome inhibitor MG-132, and conducted siRNA knock down experiments for the E3 specificity factor βTrcp. As seen in figure 13, no siRNA knock down ever approached the inhibition of IκBα degradation achieved through MG-132 treatment, but the βTrcp knock down exactly mirrored the effect seen with the double knock down for Ubch5 and Ubch7. (**Figure 13**) Although we purchased a total of four different

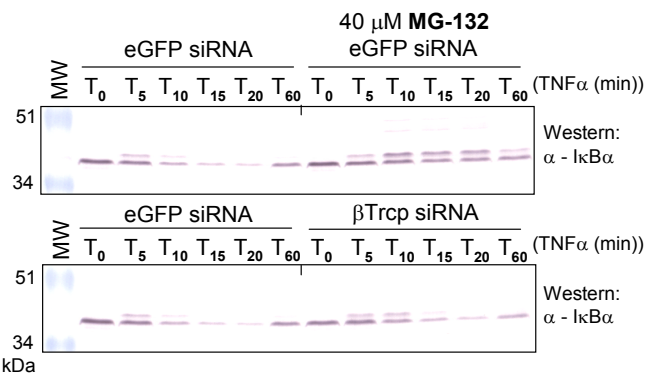

Figure 13: Positive controls for siRNA knock down: siRNA knock down and TNFα stimulation was carried out as described in materials and methods. For analysis, 20 μg of each extract was run onto a 12% SDS-PAGE gel, transferred to PVDM, and analyzed by western blot. MG-132 was added to the media at 40 μM 60 minutes before each TNFα time point. Note: time points differ that those in figure 10.

antibodies against βTrcp, they all failed to detect the protein by western blot in my hands (data not shown). For this reason, I was unable to assess the level of knock down for βTrcp.

Another possible explanation for these results is that the level of Ubch5 knock down is insufficient to affect the kinetics of IκBα degradation. Because Ubch7 is also a member of the Ubc4/5 family of E2 enzymes, knocking down Ubch7 may have funneled the remaining Ubch5 into other substrate ubiquitination pathways in place of Ubch7. This would result in less Ubch5 available for the ubiquitination of IκBα, and hence the observed phenotype. Without further experimentation, it is impossible to form any conclusions from these results.

Because these results were ambiguous, I decided to test whether lysates from the various siRNA knock down cells could support the ubiquitination of radio-labeled IκBα. All extracts were able to ubiquitinate IκBα when programmed with IKKβ, Okadaic acid, and Ub-ald, except for the lysate obtained from the βTrcp knock down cells. This result was consistent, and could not be overcome by increasing the amounts of extract used in the ubiquitination assay (data not shown). To further investigate a possible role of Ubch7 in the IκBα degradation pathway, I returned to our *in vitro* ubiquitination system.

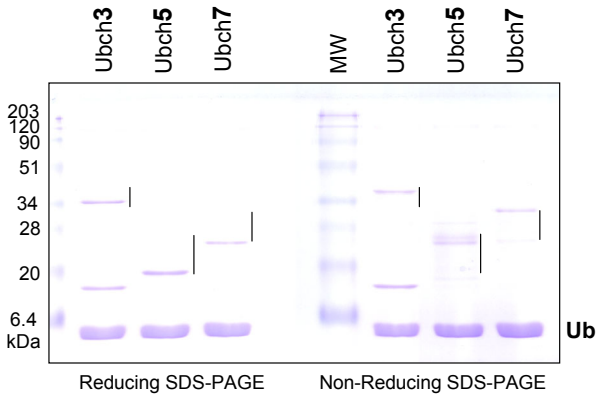

Figure 14: E2 – Ub thioester bond formation: 2 μg of each E2 was incubated With E1 and Ub in PBDM for 30 minutes at 37°C. Half of each 10 μl reaction was then added to either 6 μl reducing or non-reducing 2X SDS-sample buffer, boiled for 2 minutes, and run on a single 12% SDS-PAGE gel for Commassie stain. The lower band in the Ubch3 lanes is a contaminant of the preparation.

Ubch7, which the Chen lab had previously cloned, was expressed as an N-terminal His_{10} fusion protein in *E. coli*, and purified to homogeneity via Ni-agarose resin. Initial experiments were carried out to titrate Ubch7 into our ubiquitination assay as had been previously done with Ubch3 and Ubch5, but all results were negative. Ubch7 was unable to support the formation of any ubiquitin conjugates onto IκBα (data not shown). In an attempt to determine whether or not my Ubch7 preparation had any activity, I carried out E2-ubiquitin thioester bond formation assays. Figure 14 clearly shows that all E2 enzymes utilized in this study are fully capable of interacting with the E1 enzyme to attack the activated ubiquitin, which results in thioester bond formation. **(Figure 14)** In addition, Dr. Xiaodong Wang has utilized our Ubch5 and Ubch7 in his current studies, and has found Ubch7 to be the preferred E2 in his ubiquitination system (personal communication). While these experiments show that Ubch7 is active and capable of making ubiquitin chains, it does little to explain our *in vivo* siRNA knock down data.

In a final attempt to find an effect of Ubch7, I added the enzyme into the Ubch3 and Ubch5 ubiquitination and degradation assays. While Ubch7 displayed no E2 activity on its own, I thought that it might work in conjunction with the other E2s in the system, particularly Ubch5. As seen in figure 15, no effect of Ubch7 was observed in either the ubiquitination or degradation assays. (**Figure 15**)

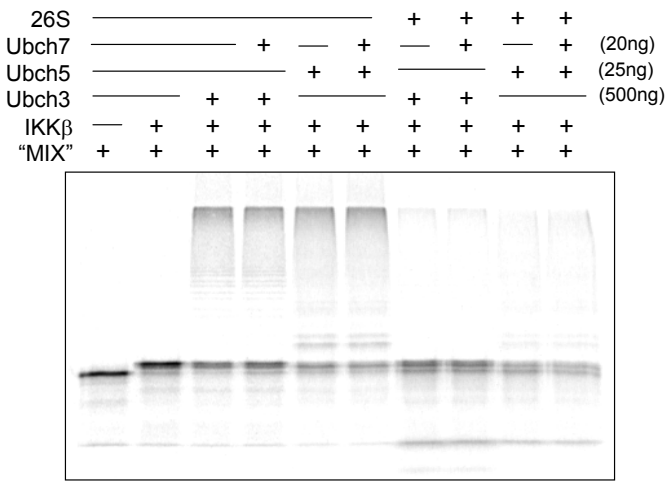

Figure 15: Ubch7 does not enhance ubiquitination of degradation of IκBα: Ubiquitination assays were carried out as in figure 8, with the exception of Ubch7 addition where indicated. "MIX" contains E1, E3, Ub, $_{His6}$p65:IκBα, and PBDM. For the degradation assay, 26S proteasome was added at the start of the assay for a "one pot" ubiquitination and degradation assay. The 26S preference for Ubch3 mediated conjugates is again observed (see text).

CHAPTER THREE

Discussion:

There is currently not enough data to declare either Ubch3 or Ubch5 the physiologic E2 used in IκBα ubiquitination. While much smaller amounts of Ubch5 are needed to support IκBα ubiquitination, the isopeptide linkages created by Ubch3 seem more relevant to 26S proteasome mediated degradation of IκBα. One possibility is that there exists an additional level of E2 regulation. A 1999 report by Gonen et. al. found that both Ubc3 and Ubch5 supported IκBα ubiquitination, but they, like me, were quite cautious about drawing any conclusions. They do however state that an inhibitor of Ubch3 exists in fraction II (Gonen et. al. 1999). We have also observed this inhibitory factor in fraction II, and are currently using our Ubch3 mediated ubiquitination system to purify this putative inhibitor (Yu-Hsin unpublished data). Also of interest is a 2002 report that found phosphorylation of Ubc3B (and Ubc3/Cdc34) by casein kinase 2 (CK2) was required for this E2 to associate with βTrcp (Semplici et. al. 2002). After considering the data, a different interpretation was made. Because the authors used an *in vitro* transcription / translation (TNT) system to express βTrcp, it is highly likely that βTrcp was incorporated into SCF complexes present in the TNT extracts. If this is indeed what happened, then their tagged, phosphorylated, Ubc3 was actually pulling down the entire $SCF^{\beta Trcp}$ complex through Ubc3's interaction with the Roc1 subunit, not the βTrcp subunit. In light of this interpretation, the phosphorylation of Ubch3 might be required to increase its affinity for the $SCF^{\beta Trcp}$ complex. This might

explain why we must use Ubc3 at an approximate 10 fold molar excess over Ubch5 to see efficient ubiquitination of IκBα.

It is clear from the literature that there are additional levels of regulation for the $SCF^{\beta Trcp}$ complex. Numerous reports show that the Cullin 1 subunit of the SCF complex can be modified by the small ubiquitin like protein Nedd8. However, each of these reports attributes a different function to the Nedd8 modification. The first of these reports found that the Nedd8 modification of $SCF^{\beta Trcp}$ enhanced Ubc4's, but not Ubc7's ability to ubiquitinate IκBα (Tanaka et. al. 2001). While the author's did not comment on the phenomenon, it is clear from their data that there is some level of Ubc7 mediated ubiquitination of IκBα, but only when using the Nedd8 modified SCF complex. Another report found that this Nedd8 modification stimulated Cdc34's (Ubc3's) ability to form SCF mediated free ubiquitin chains, but that this stimulation was not found when Ubc4 was assayed (Wu et. al. 2002). In the same year, a similar report found that the Nedd8 modification was needed to recruit Ubc4 to the E3 complex (Kawakami et. al. 2001). Due to these numerous reports, it is clear that we must further investigate the neddylation status of our $SCF^{\beta Trcp}$ complex. While western blot analysis shows only one band for the cullin 1 subunit, the two possibilities are that our complex is either all neddylated, or does not contain the modification (data not shown). Due to these numerous reports, I believe that our assays must be repeated using both forms of the $SCF^{\beta TrCP}$ complex so that some type of conclusion can be reached.

Two reports in 2002 describe an inhibitor of the SCF complex named p120/CAND1 (Liu et. al. 2002, Zheng et. al. 2002). Both reports describe the Nedd8 modification as relieving this inhibition, allowing formation of an active SCF complex. It is unclear if this

inhibitor is the same inhibitor of Ubch3 found in fraction II, as inhibition of the SCF complex by the CAND1 protein should exclude all E2s from binding. The final and most recent report on the Nedd8 modification of the SCF complex states that this modification might stimulate the ubiquitin mediated turnover of the cullin 1 subunit (Morimoto et. al. 2003).

Due to these varying reports about the Nedd8 modification, it is clear that we must increase the complexity of out model system to include the Nedd8 E1 enzyme Uba3, and the Nedd8 E2 enzyme Ubc12 before we can further address which E2 works to ubiquitinate IκBα. In addition, further studies will need to be carried out with crude fractions in an attempt to ubiquitinate IκBα in fraction II alone.

All previous attempts to ubiquitinate IκBα in fraction II used phosphorylation of IκBα to initiate its ubiquitination. This was accomplished by either adding the phosphorylated IκBα directly (Alkalay et. al. 1995), or by adding Ubc4 or Ubch5, which can directly activate the IKK complex by a mechanism that is still not understood (Chen et. al. 1996; Zong-Ping unpublished data). In the current study, the addition of okadaic acid and IKKβ to fraction II was insufficient to stimulate the ubiquitination of IκBα (data not shown). In light of the elaborate signal transduction cascades required to activate the IKK complex, there are multiple possibilities for the control of other components of the IκBα ubiquitination system within this cascade (Deng et. al. 2003). In light of all the possibilities, it would be of great interest to first stimulate HeLa cells with TNFα for five minutes, and then collect the lysate and generate fraction I and fraction II. It is possible that an entirely different result will be obtained with regards to fraction II's ability to ubiquitinate IκBα under these circumstances.

In this report we have reconstituted the ubiquitination and degradation of IκBα *in vitro*. While several aspects of the system remain unclear, we hope that it can be used to help answer several perplexing questions in the ubiquitination and degradation field. Of particular interest is how the 26S proteasome remodels protein complexes, and whether or not other cellular chaperones are involved. Their currently exists two reports in the literature that deal with this particular topic. In 2000, it was reported that a nonproteolytic function of the 26S proteasome was required to dissociate Cdc2 from its complex with ubiquitinated cyclin B (Nishiyama et. al. 2000). Unfortunately, the authors were unable to reconstitute the dissociation with purified 26S, and the immunoprecipitated 26S proteasome used in their studies remains uncharacterized.

A 2001 report from the Deshaies laboratory is the only report to date that demonstrates the ubiquitination and degradation of a substrate using purified components (Verma et. al. 2001). This model system recapitulates the activation of S-phase cyclin dependant kinase (S-Cdk) by the degradation of its inhibitor Sic1. The investigators, using purified components from budding yeast, were able to ubiquitinate Sic1 in a signal dependant manner followed by its rapid degradation with affinity purified 26S proteasomes. While reconstituting 26S proteasome degradation is a feat in itself, this study is even more remarkable in that it demonstrates that the 26S proteasome was able to extract and selectively degrade the ubiquitinated Sic1 from the Sic1:S-Cdk complex, leaving the remaining S-Cdk active and unscathed. In addition, the investigators went to great lengths to demonstrate that the remodeling of the substrate complex was carried out by the 26S proteasome and not by extra-proteasomal un-foldases such as the HSPs or Cdc48/VCP.

In light of the results from the Deshaies laboratory, it is perplexing that our degradation system is not as robust. One possibility is that the nature of the ubiquitinated substrates is not comparable. It has been demonstrated that Sic1 has no discernible structure in solution (Nash et. al. 2001). The 26S proteasome might be capable of the unfolding and translocation of some substrates, but might require extra help for other substrates that contain a more ordered structure.

Numerous reports have implicated the cellular chaperone p97/VCP as being involved in the degradation of ubiquitinated proteins, and a more direct role for this chaperone has been shown in endoplasmic reticulum associated protein degradation (ERAD) (Meyer et. al. 2000; Ye et. al. 2001; Rabinovich et. al. 2002). Of particular interest to this study is a 1998 report that implicates VCP's involvement in the degradation of ubiquitinated IκBα (Dai et. al. 1998). The one major problem with this study is that all data presented was of an indirect nature. With our purified ubiquitination and degradation system, we are in a position to test this hypothesis with the generation of direct biochemical data. To this end, members of the DeMartino laboratory have recently expressed and purified VCP and the Ufd1:Npl4 complex that is believed to target VCP to the ubiquitin and nuclear transport pathways (Meyer et. al. 2002; Heise and Thompson, unpublished data).

As a final note, it has not escaped our attention that we have in out hands a system that can be used to investigate how the 26S proteasome deals with ubiquitinated substrates containing various isopeptide linkages. Vary few studies have been carried out that compare multiple linkages due to the difficulty in obtaining conjugates with a defined linkage. The Chen laboratory has an extensive supply of purified mutant ubiquitins, including K6, R6;

K11, R11; K27, R27; K29, R29; K33, R33; K48, R48; K63, R63; and of course KO and wild type. If the promiscuity of the ubiquitin linkage formed by Ubch5 goes beyond K48 and K63, this could be used to our advantage to create a multitude of different ubiquitin chain linkages. In addition, new mutants could be created containing multiple lysines (for instance K48 and K6; K48 and K27; K48… ect.) that would be used to explore the existence and importance of various isopeptide branches from the main isopeptide linkage that originates from the substrate protein. Such experiments might shed light onto a relatively poorly studied aspect of ubiquitin physiology.

CHAPTER FOUR

Methodology

Protein Purification:

For the IκBα substrate, radio labeled IκBα was transcribed and translated in wheat germ extracts using the Promega TNT expression kit according to the manufacturers specifications. 35S labeled methionine was purchased from NEN (NEG009A). The DNA template was human IκBα cloned into the pcDNA3.1 expression vector. The substrate was further purified by the addition of $_{His6}$p65 (see below), followed by the Ni-agarose (Qiagen) purification of the NF-κB:IκBα complex. The human forms of p65, E1, and IKKβ were expressed as N-terminal His6 fusion proteins in SF9 cells using the Bac-to-Bac Baculoviral expression system from GIBCO. These proteins were purified using Ni-agarose resin (Qiagen), following protocols described by GIBCO. Ubiquitin was purchased from Sigma (U-6253), and resuspended at 10 mg/ml in ddiH_2O. All E2 enzymes used in the study were expressed as either N-terminal GST or His6 fusion proteins in the *E.coli* BL21/pLys strain using either pGEX or pET16b expression vectors. After gluthathione-sepharose purification of GST-Ubch5c and GST-Ubch3, the GST tag was removed via thrombin cleavage, and the resulting GST E2 mixtures were separated on the FPLC using a mono-S column (pH 6.5) for Ubch5, and a mono-Q column (pH 8.0) for Ubch3. All columns and resins were purchased from Amersham Pharmacia, and the manufacturers recommended protocols were followed. The $SCF^{\beta Trcp}$ E3 complex was expressed in SF9 cells using the Bac-to-Bac expression system

from GIBCO. Individual baculoviruses were amplified for human His6-Cul1; His6-Roc1; GST-Skp1, and mouse His6-βTrcp (a.k.a. ME3). These four P2 viruses were then used to simultaneously infect SF9 cells and the resulting $SCF^{\beta Trcp}$ complex was purified using Ni-agarose resin (Qiagen). PA700 and 20S proteasome were purified from bovine Red blood Cells (RBCs) as described (Ma et. al. 1994). Rabbit muscle 26S proteasome was purified as described (Chen et. al. 1995). Tagged yeast 26S proteasome was purified as described (Verma et. al. 2000). Highly pure 26S proteasome was purified from bovine RBCs by a method that will be described in detail in a following publication from the DeMartino laboratory. In brief, fresh bovine blood was obtained and the RBCs were washed extensively in PBS supplemented with glucose. The RBC pellets were then frozen and stored at -80°C. The night before the preparation, the RBC pellets were set at 4°C to thaw, followed by hypotonic lysis in the presence of ATP/Mg^{2+}. Cell pellet debris was removed by centrifugation, and the resulting supernatant was precipitated with 40% saturating ammonium sulfate. This pellet was resuspended and dialyzed overnight in a buffer containing ATP/Mg^{2+}. The dialyzed material was loaded onto a DEAE-Affigel Blue column (Bio-Rad), washed with 40mM NaCl, and eluted with 150mM NaCl. The 150mM NaCl eluted material was concentrated on an XM-300 membrane in a stirred cell concentrator (Amicon), and loaded onto a 12-40% glycerol gradient that was spun at 28,000 RPM for 8 hours. Fractions containing the 26S proteasome were pooled, concentrated, and stored in small aliquots at -80°C. Activity assays were carried out at each step using a fluorescent plate reader (BioTek), and the proteasome specific substrate Suc-LLVY-AMC (BACHEM). This protocol resulted in highly pure 26S proteasomes that were resistant to repeated freeze

thaw cycles as assessed by glycerol gradient re-centrifugation (DeMartino and Liu, unpublished data).

Ubiquitination Assays:

Two different methods were used to ubiquitinate the 35S-labeled IκBα substrates described above. In the first, 1µl TNT IκBα extract was added to 10 to 20µg of HeLa S100 extract in the presence of 10µg ubiquitin, 1µM Okadaic acid, 10 to 20µM Ub-aldehyde, 0.5µg IKKβ, in 1X PBDM. The 10 µl reaction was incubated at 37°C for 1 hour, and either analyzed by SDS-PAGE, or immunoprecipitated with anti-p65 coupled agarose (Santa Cruz) for further use in degradation assays. In the second method, 1µl of TNT IκBα extract (or $_{His6}$p65:IκBα complex) was added to 10 ug ubiquitin, 0.5 µg IKKβ, 0.1 µg E1, 25 to 500 ng E2, 0.2 to 1.0 µl of E3 in 1X PBDM. The 10µl reaction was incubated at 37°C for 60 min. and analyzed by SDS-PAGE and autoradiography. PBDM stands for protein break down mix and is an ATP regenerating system containing 50mM Tris pH 7.6, 5mM $MgCl_2$, 2mM ATP(Sigma A-2383), 10mM creatine phosphate(Sigma P-7936), 3.5 U/ml creatine kinase(Sigma C-3755), and 0.6 U/ml inorganic pyrophosphatase(Sigma I-1643). Okadaic acid is a phosphatase inhibitor, and was obtained from Alexis biochemicals. Ubiquitin aldehyde is an isopeptidase inhibiter, and was prepared in house as described previously (Chen et. al. 1995).

IκBα Degradation Assays:

A variety of methods were used for IκBα degradation. Initially, the ubiquitinated ^{35}S-IκBα:NF-κB complexes were immunoprecipitated with anti-p65 coupled agarose, and the various 26S proteasome preparations (50nM to 150nM) were added directly to the washed beads in 1X PBDM. The 10 μl reactions were incubated at 37°C for 60 min. and analyzed by SDS-PAGE and autoradiography. The second method for degradation involved adding the 26S proteasome preparations directly to the ubiquitination assays. This "one pot" ubiquitination and degradation assay was carried out in a 20μl reaction volume and only employed when using the defined ubiquitination reaction (see above).

siRNA Knockdown Studies:

We chose to carry out the following studies in 293T HEK cells due to their well established TNFα inducible degradation of IκBα, and their ease and low cost of transfection by the calcium phosphate method. Cells were grown on 0.2% gelatin coated 6 well plates in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and penicillin-streptomycin at 100 I.U./ml and 100 μg/ml respectively. All reagents were purchased from Gibco under the Cellgro®

brand name. TNFα was obtained from Sigma (T-6674), and was resuspended in 1x media. Phosphate Buffered Saline (PBS), 1.0M Calcium Chloride, and 2X phosphate buffer (50 mM HEPES, pH 7.05 @ 23°C; 1.26 mM Na_2HPO_4; 140 mM NaCl) were all made in house and autoclaved before use. The pH of the 2X phosphate buffer was optimized before use by the titration of 1M NaOH's effect on β-Galactosidase transfection. Transfection efficiency was routinely >90% (data not shown). All siRNA oligos were obtained from the Center for

Biomedical Invention (CBI) at the University of Texas Southwestern Medical Center. The de-protected rotavaped oligos were resuspended in annealing buffer containing 100 mM Potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM Magnesium acetate. The resuspended oligos were then spun at room temperature for 5 minute @ 10,000 RPM to remove any insoluble material. Sense and anti-sense oligos were annealed at 20 μM each in annealing buffer with a 2 minute incubation at 90°C followed by a 60 minute incubation at 37°C. On day one, 2.0 ml of cells were plated at 200,000 cells/ml. Plated cells came from plates that were 80% or less confluent. On day two, two hours before transfection, the media was removed and 1.7 ml fresh media was added. In a 1.5 ml microfuge tube, siRNA oligos were added to ddiH2O, with the final volume adjusted to 112.5 μl, and the final siRNA oligo concentration at 3.555 μM. To this solution, 37.5 μl of 1.0 M $CaCl_2$ was added. Immediately before adding the oligos to the cells, 150 μl of optimized 2X phosphate buffer was added to the 1.5 ml tube, and the entire mixture was vortexed twenty times, one second each time, with a vortex setting that did not allow the solution to contact the lid of the closed tube. Following vortexing, the solution was immediately added in a drop wise fashion to the particular well to be transfected. This resulted in a final siRNA oligo concentration of 200 nM, which was never exceeded. On day two, the entire procedure was repeated. On day three, 1.0 ml of media was removed one hour before the TNFα stimulation was to occur. Each plate was stimulated and harvested separately to ensure that the various time points were accurate. To each well, at the appropriate time, 2 μl of a 10 ng/μl TNFα stock was added (20 ng/ml final) and the plate was swirled and rocked gently before being placed back into the incubator. At the appropriate time, the 6 well plate was set on an aluminum block in

ice, the media was aspirated and the cells were washed with 1.0 ml ice cold PBS. Following aspiration, 1.0 ml of ice cold PBS was again added to each well and the cells were scraped from the bottoms of the plates followed by centrifugation for 30 seconds at 5,000 RPM. The PBS was aspirated from the cell pellet, and the pellet was immediately snap frozen in liquid nitrogen. The ≅ 50 µl cell pellets were then resuspended in 100 µl of "whole cell" lysis buffer containing 20 mM Tris pH 8.0, 20 mM glycerol-β-phosphate, 20 mM NaF; 0.5 mM Na_3VO_4, 1.0 mM PMSF, 5 µg/ml Leupeptin, 5 µg/ml Pepstatin, 5 µg/ml Chymostatin, 0.2 mM EGTA, 1.0 mM DTT, 300 mM NaCl, 5 mM $MgCl_2$, and 0.5% v/v NP-40. The solution was pipetted up and down 20 times, incubated for 10 minutes on ice, followed by an additional round of pipetting up and down 20 times. Cellular debris was then pelleted by spinning at 14,000 RPM for 10 minutes at 4°C. As with all other protein concentration determinations in this study, the protein concentration was determined by the method of Bradford.

Western Blots:

10 µl of a 2 µg/µl 1X SDS-sample from each lysate was loaded onto the indicated percentage acrylamide gel that contained a 1 cm 5% stacking gel. Acrylamide was purchased from Fisher as a 40% w/v solution of acrylamide : bis-acrylamide (37.5 : 1) (BP 1410-1). Gels were all run at 50V (constant) until all samples had left the wells, whereupon the voltage was raised to 100V until the dye front had entered the resolving phase of the gel. After this, the voltage was raised to 150V for five minutes, followed by 200V until the dye front had reached ≅ 1 cm from the bottom of the gel. All transfers to PVDM membranes

were carried out in solution with BioRad's mini-protein cell electrotransfer apparatus. Blocking, primary, and secondary incubations were all carried out with 10% w/v Carnation® instant non-fat dry milk resuspended in TTBS (Tris buffered saline pH 7.4; 0.05% v/v Tween 20; 0.005% w/v NaN_3) at room temperature. Primary incubations with the following antibodies were either carried out for one hour at RT, or overnight at RT, depending upon the affinity of the antibody [anti-IκBα (C-21) sc-371 rabbit polyclonal @ 1:1000 (Santa Cruz Biotechnology); anti-Cdc34/Ubc3 (H-81) sc-5616 rabbit polyclonal @ 1:500 (Santa Cruz Biotechnology); anti-Ubch5 rabbit polyclonal @ 1:500 was a kind gift from Dr. Allen Weissman; anti-Ubch7 mouse monoclonal U66820 @ 1:1000 (BD-Transduction Laboratories)]. Secondary incubations were for 1 hr using 1:5000 dilutions of Promega's anti-rabbit or anti-mouse IgG alkaline phosphatase (AP) conjugated antibodies (S373B and S372B respectively). Development was carried out with Promega's NBT:BCIP AP substrate as described by the manufacturer (S3771), and the reactions were terminated by washing the blot in dH_2O. All dried blots were scanned into jpeg format on a UMAX Astra 2200 scanner using Adobe Photoshop.

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