MODULATION OF TRANSCRIPTION FACTOR ACTIVITY BY MONO-UBIQUITIN

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DEDICATION

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MODULATION OF TRANSCRIPTION FACTOR ACTIVITY BY MONO-UBIQUITIN

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The Ubiquitin-Proteasome Pathway plays both proteolytic and non-proteolytic roles in the regulation of transcription. We recently reported that the ATPases of the 26S proteasome can destabilize activator-DNA complexes in a non-proteolytic manner that requires direct interactions between the Rpt4 and 6 subunits with the activation domain of the activator. Remarkably, mono-ubiquitylation of the activator blocks this repressive activity. In this study, we probe the mechanism of this protective effect. Using novel label transfer and chemical cross-linking techniques, we show that ubiquitin contacts the ATPase complex directly, apparently via Rpn1 and/or Rpt1, and that this interaction

results in the dissociation of the activation domain-ATPase complex via an allosteric process. We also provide *in vivo* evidence demonstrating the importance of mono-ubiquitylation in inhibition of activator-DNA destabilization. A model is proposed in which activator mono-ubiquitylation serves to limit the lifetime of the activator-ATPase complex interaction and thus the ability of the ATPases to unfold the activator and dissociate the protein-DNA complex.

TABLE OF CONTENTS

TABLE OF CONTENTS viii

LIST OF FIGURES xii

LIST OF TABLES xv

LIST OF DEFINITIONS xvi

CHAPTER ONE 1

THE UBIQUITIN-PROTEASOME SYSTEM 1

TRANSCRIPTION AND THE *GAL* SYSTEM 10

THE UPS IN TRANSCRIPTION 18

CHEMICAL CROSS-LINKING AS A TOOL TO STUDY PROTEIN-PROTEIN INTERACTIONS 31

CHAPTER TWO 36

SUMMARY 36

INTRODUCTION 38

MATERIALS AND METHODS 41

RESULTS 52

CHEMICAL CROSS-LINKING OF THE GAL4-AD TO GAL80 52

DOPA-GAL4 AD CROSS-LINKING TO THE 26S PROTEASOME 55

IDENTIFICATION OF THE CROSS-LINKED PRODUCTS 57

THE FLASH BASED SYSTEM 65

INTERACTIONS BETWEEN KNOWN BINDING PARTNERS 66

FLASH FOR USE WITH MULTI-PROTEIN COMPLEXES 73

DISCUSSION 75

CHAPTER THREE 78
SUMMARY 78
INTRODUCTION 80
MATERIALS AND METHODS 82
RESULTS 89
THE gal4D MUTATION RESULTS IN A DEFECT IN PROMOTER OCCPANCY *in vivo* 89
Gal4D IN NOT UBIQUITYLATED EFFICIENTLY 91
IDENTIFICATION OF A SUB-DOMAIN OF THE AD REQUIRED FOR MONO-UBIQUITYLATION 95
UBIQUIYLATION OF Gal4 *in vivo* 96
Gal4D FUNCTION CAN BE RESCUED BY GENETIC FUSION OF UBIQUITIN 101
DESCREASED 'STRIPPING' ACTIVITY OF Sug 1-1-CONTAINING PROTEASOMES 103
DISCUSSION 107
CHAPTER FOUR 114
SUMMARY 114
INTRODUCTION 116
MATERIALS AND METHODS 118
RESULTS 124
UBIQUITIN ANTAGONIZES PROTEASOME-MEDIATED DESTABILIZATION *in trans* 124

UBIQUITIN BINDS DIRECTLY TO THE 19S RP 129
IDENTIFICATION OF UBIQUITIN-PROXIMAL PROTEINS
IN THE 19S RP 133
UBIQUIYLATED Gal4-VP16 BINDS THE PROTEASOMAL ATPASES
WEAKLY 137
THE ADs OF Gal4 AND VP16 COMPETE FOR PROTEASOME
INTERACTION 142
IMPORTANCE OF THE HYDROPHOBIC PATCH OF
UBIQUITIN *in vivo* 147
DISCUSSION 151
CHAPTER FIVE 158
CROSS-LINKING 158
MONO-UBIQUIYLATED ACTIVATORS 161
BIBLIOGRAPHY 167

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LIST OF FIGURES

FIGURE 1-1 The UPS 3

FIGURE 1-2 Ubiquitin: monomeric and chains 10

FIGURE 1-3 General transcription regulation 13

FIGURE 1-4 Gal4 protein domains 15

FIGURE 1-5 Regulation of the *GAL* system 18

FIGURE 1-6 Role of the UPS in transcription 26

FIGURE 1-7 Bifunctional cross-linkers 33

FIGURE 1-8 DOPA mediated chemical cross-linking 35

FIGURE 2-1 The cross-linking and label transfer scheme 41

FIGURE 2-2 Periodate mediated cross-linking of peptides to Gal80 54

FIGURE 2-3 Periodate mediated cross-linking of peptides to 26S proteasome 56

FIGURE 2-4 Identification of cross-linked product of 26S proteasome and N-term DOPA-Gal4 AD peptide 58

FIGURE 2-5 Identification of cross-linked product of 26S proteasome and Gal80 BP 60

FIGURE 2-6 Identification of cross-linked product of 26S proteasome and C-term DOPA-Gal4 AD peptide 62

FIGURE 2-7 The Gal4 AD and Gal80 BP compete for the proteasome 64

FIGURE 2-8 The biotin-DOPA-FlAsH reagent and cross-linking scheme 66

FIGURE 2-9 DBF transfer from GST-FRP-G80BP to His6-Gal80 68

FIGURE 2-10 Yield of DBF-mediated label transfer from GST-FRP-G80BP to Gal80 70

FIGURE 2-11 His6-Gal80 protein pull-down assay with glutathione bead-bound GST-FRP-G80BP and GST-G80BP-FRP 72

FIGURE 2-12 DBF transfer from GST-Gal4-VP16-FRP to 26S proteasome 74

FIGURE 3-1 Affect of sug1-1 mutation on Gal4D promoter occupancy *in vivo* 91

FIGURE 3-2 The mini-Cla Gal4 protein 92

FIGURE 3-3 Gal4D mono-ubiquitylation 93

FIGURE 3-4 Evidence that Gal4 is ubiquitylated *in vivo* 97

FIGURE 3-5 Gal4 ubiquitylation *in vivo* 100

FIGURE 3-6 Fusion of mono-ubiquitin to Gal4D 102

FIGURE 3-7 The destabilization assay 104

FIGURE 3-8 sug1-1 containing 26S proteasome destabilization activity 105

FIGURE 3-9 A model for activator mono-ubiquitylation promoting function 109

FIGURE 4-1 Destabilization of activator-DNA complexes by the 26S proteasome in the presence of ubqiutin and related proteins 126

FIGURE 4-2 Effect of free ubiquitin on the destabilization of activator-DNA complexes by the 26S proteasome 127

FIGURE 4-3 Effect of exposure of the hydrophobic patch of ubiquitin in inhibiting destabilization 129

FIGURE 4-4 The DBF cross-linking and label transfer scheme 131

FIGURE 4-5 Mono-Ub cross-links to subunits of the 19S proteasome 133

FIGURE 4-6 Mono-Ub labels subunits of the proteasome 135

FIGURE 4-7 Mono-Ub interaction with recombinant ATPases of the proteasome 137

FIGURE 4-8 Mono-Ub and other activities of the 26S proteasome 138

FIGURE 4-9 Effect of activator mono-ubiquitylation on the interaction of the GST-Gal4-VP16 activator with the proteasome 141

FIGURE 4-10 The ADs of VP16 and Gal4 compete for proteasome binding..... 144

FIGURE 4-11 Effect of mono-Ub on the interaction between the Gal4 AD and the proteasome..... 147

FIGURE 4-12 Mutation of the hydrophobic patch prevents rescue of Gal4D 149

FIGURE 4-13 The I44A mutation abolishes the ability of ubiquitin to promote Gal4D activity 151

FIGURE 4-14 A'hand-off' model for how activator mono-ubiquitylation facilitates activator function 155

LIST OF TABLES

TABLE 3-1 Primers used in ChIP analysis..... 86

TABLE 3-2 Primers used in mRNA analysis 87

TABLE 4-1 Primers used for cloning 119

LIST OF ABBREVIATIONS

AD – Activation domain

APIS- A subcomplex involved in transcription containing the proteasomal ATPases

BAL – British anti-Lewisite, 2,3-dimercaptopropanol

ChIP – Chromatin immunoprecipiation

CP – 20S Core Particle

CTR – C-terminal region

DBD – DNA binding domain

DBF – DOPA-biotin-FlAsH cross-linking reagent

DOPA – 3,4-Dihydroxyphenylalanine

DTT-Dithiothreitol

FRP – FlAsH Receptor Peptide (CCPGCC)

GST-Glutathione S-transferase

Gal80 BP – Gal80 Binding Peptide

HAT – Histone acetyl transferase

IMAC – immobilized metal-affinity chromatography

LTR –Long terminal repeat

MS – mass spectroscopy

NA – NeutrAvidin

NTR – N-terminal region

PTM-Post-translational modification

qPCR – quantitative PCR

RP – 19S Regulatory Particle

SAGA-Spt-Ada-Gcn5-Acetyltransferase, a chromatin modifying complex

TBP-TATA binding protein

Ub – ubiquitin

UAS – Upstream Activating Sequence

UBA –Ubiquitin-associated domain

UBL – Ubiquitin-like domain

UPS – Ubiquitin-Proteasome System

CHAPTER ONE

INTRODUCTION

The Ubiquitin-Proteasome System

The Ubiquitin-Proteasome System (UPS) is responsible for most of the non-lysosomal protein degradation in the cell (1). The components of the UPS include a multi-protein protease, a small protein that is used to mark target proteins for degradation, and the machinery used to attach and remove the degradation tag. A similar, but much simpler, protease is present in archeabacteria (2) and some eubacteria (3); though these organisms lack the tag and tagging machinery, instead relying on a short peptide sequence to mark proteins for degradation (4). A complete UPS is known in the simple eukaryotic, *Sacchoromyces cerevisiae*, and upwards throughout the remainder of the evolutionary tree.

The 26S proteasome is a ~ 2 MDa multi-protein machine responsible for proteolysis of proteins (Figure 1-1) (1). The proteolytic active sites are located on the interior of the barrel shaped 20S or core particle (CP). Structural studies have shown that the CP is made up 14 different proteins that are arranged in two different heptamer rings stacked with top to bottom symmetry. The alpha

subunits (α1-7) make up the two outer rings and the beta subunits (β1-7) make up the two inner rings to give the $\alpha\beta\beta\alpha$ architecture (5). The proteolytic sites are contained within the beta subunits with trypsin-like activity associated with Beta 2, chymotrypsin-like with Beta 5, and caspase-like with Beta 1 (6-8). This results in two copies of each catalytic site, giving a total of 6 sites. The alpha rings flank the beta rings and form the top and bottom of the barrel structure. Entrance to the interior of the barrel is through a small pore located in the center of the alpha rings (9). This pore is restricted by the N-termini of the alpha subunits, which allows only small polypeptides or unstructured proteins to enter the CP interior, but not larger, folded proteins (5). The pore in the alpha ring is opened or "gated" by interaction with different capping subunits that can associate with the CP. The interaction of the capping subunit with the alpha ring to open the gate and the activities of the capping complex are both needed for entry of folded proteins into the CP (10).

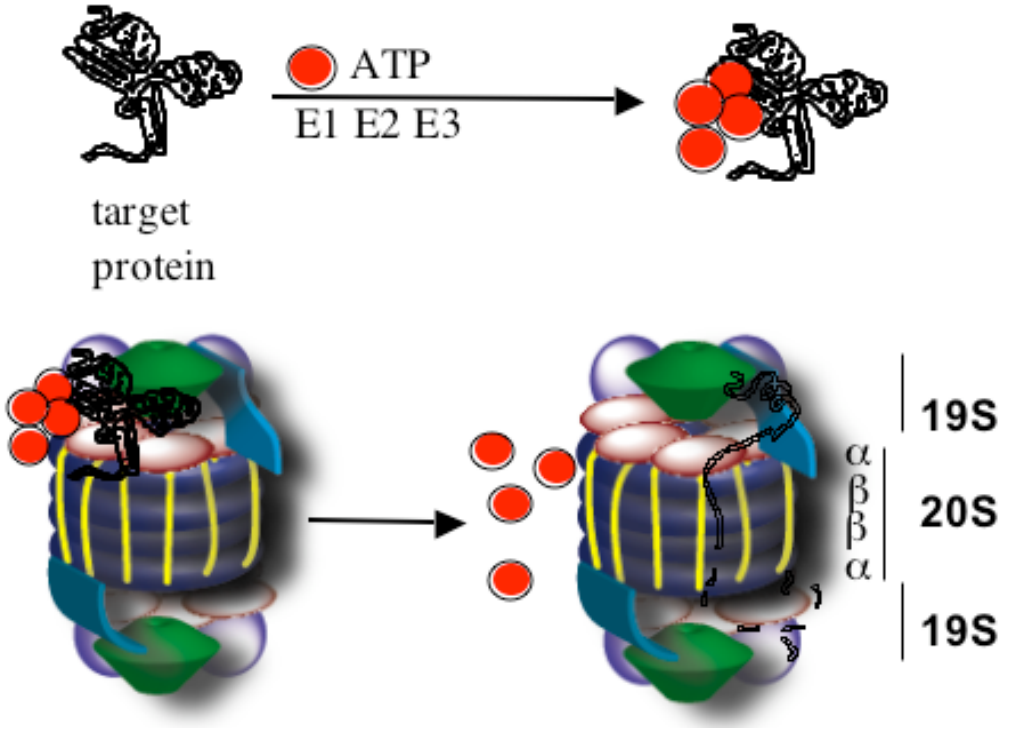

Figure 1-1 The UPS. The top panel shows a protein being targeted for proteasomal degradation by attachment of an ubiquitin (red circle) chain by the E1, E2, and E3 enzymes. This target protein is the recognized by the 26S proteasome (bottom panel), which destroys the target protein and recycles the ubiquitin monomers.

The main capping complex required for the bulk of protein degradation is the 19S proteasome or regulatory particle (RP). The RP can associate with either or both of the alpha rings of the CP to form singly or double capped proteasomes (10). Under non-physiological high-salt conditions the RP can be further divided into the lid and the base subassemblies (11). Unlike the CP, there is no high-resolution structure of the RP, only low-resolution EM structural studies. These EM studies, along with various 2-hybrid, mutational, IP and mass spectroscopy

and cross-linking studies, have given some insight of the organization of the RP. The base of the RP is made up of a single member ring containing 6 ATPases (Rpt1-6) as well as two non-ATPase subunits (Rpn 1 and Rpn 2). Rpn10 is sometimes thought of as a member of the base or alternatively as a "hinge" linking the base and lid together as it seems to associate with members of both complexes. The remainder of the non-ATPase subunits (Rpn 3-9, 11, 12) make up the lid of the RP.

Functionally, the base of the RP is important for gating the entrance to the CP and also in the unwinding and translocation of substrates for degradation. Gating is accomplished by the actual contact between the RP base and the alpha ring. The gating mechanism was first studied in detail using a chimeric system with a eukaryotic CP with the PAN capping complex (12), a smaller archaebacterial complex that resembles that base of the RP. These studies demonstrated that the C-termini from the PAN subunits were required for gate opening. The C-termini of some of the subunits actually insert into the subunits of the alpha ring, resulting in a structural change that causes the N-termini of the alpha ring subunits to move and open the pore. Later, it was demonstrated that these small C-terminal peptides from PAN (or from the RP ATPases themselves) could induce gate opening even when removed from the remainder of the protein (13).

The intact RP requires ATP binding, but not ATP hydrolysis, for interaction and gating of the CP (14). The translocation of substrates into the CP also depends on ATP binding but again is independent of ATP hydrolysis. However, the unfolding of substrates to linear polypeptides requires both ATP binding and hydrolysis. This unfolding, or reverse-chaperone activity, is required for the entry and subsequent degradation of most proteins targeted to the 26S proteasome. The amount of ATP seems to depend on how 'well-folded' the target is. For example, in the bacterial ClpX/ClpP system, a multi-protein machine that is homologous to the more complex 26S proteasome, the amount of ATP hydrolyzed by the ClpX ATPase in unfolding a protein correlated to how resistant the protein was to denaturation by GuHCl (15). A stable structure of the titin protein required much more ATP to unfold than a point mutant of the same protein that was shown to be much more easily denatured. The binding and, in some cases, hydrolysis of ATP is vital to the function of the RP base.

Another major role of the RP is in recognition of the ubiquitylated substrates and maintaining ubiquitin homeostasis. Ubiquitylation of substrates is the main mechanism for targeting proteins for degradation. Purified Rpn10 (and the human homologue S5a) is the only subunit known to bind tightly to ubiquitin chains outside of the context of the 26S proteasome (16). However, in the context of the 26S proteasome the ubiquitin binding site on Rpn10 seems to be masked. Deletion of Rpn10 only slightly decreases the ability of the 26S proteasome to

degrade ubiquitylated substrates, suggesting there must be other mechanisms for substrate recognition (17). Another possible poly-ubiquitin binding protein is the ATPase Rpt5, which has been shown to cross-link to poly-ubiquitin chains in the context of the proteasome (18). Another mechanism by which poly-ubiquitylated proteins may be recognized by the 26S proteasome is by 'shuttle proteins'. These proteins are able to bind to poly-ubiquitin chains and the 26S proteasome simultaneously. Examples include Rad23 and Dsk2, both of which contain an ubiquitin-like domain (UBL) and an ubiquitin-associated domain (UBA) (19-21). Deletion either of these proteins does result in an accumulation of model proteasome substrates and high molecular weight ubiquitylated proteins, but yeast with a *rad23* deletion are still able to degrade proteins that are degraded rapidly (22). Rpn10 and Rpn1 have both been implicated as possible binding sites of UBL-UBA containing proteins (23). Finally, some E3 ligases (the final enzyme in the cascade to ubiquitylate proteins) such as Hul5 and Ufd4, are known to interact with the 26S proteasome (24,25). These proteins could function similarly to the UBL-UBA proteins to tether proteins to the 26S proteasome for degradation. The only difference would be UBL-UBA proteins recognize poly-ubiquitylated proteins and the E3 ligase would presumably recognize the actual protein. Alternatively, the E3 ligase could function to ubiquitylate proteins and directly transfer them to the 26S proteasome. Deubiquitylases (DUB) function to remove poly-ubiquitin chains from substrates as well as disassemble chains.

DUB activity serves to help maintain the free ubiquitin pool for use in cellular activities. The 26S proteasome subunit Rpn11 contains DUB activity as well as a protein that is known to co-purify with the proteasome, Ubp6 (24,26).

The post-translational attachment of ubiquitin is used to target proteins for 26S proteasome-mediated destruction in *S. cerevisiae* (27), although some unfolded or damaged proteins can be degraded without being ubiquitylated (28). Ubiquitin is a small (8.5 kDa) protein that folds into a small globular protein (Figure 1-2). It is usually attached through an isopeptide bond from its C-terminus residue to a lysine on the target protein by 3 different enzymes (27). The ubiquitin activating enzyme (E1) forms a thioester bond with ubiquitin, expending ATP in the process. The ubiquitin is then transferred to the ubiquitin conjugation enzyme (E2) by transthioesterification reaction and ultimately onto the target protein with the help of an ubiquitin ligase (E3). E3's come in two main types, a ligase containing a HECT-domain, or those containing the RING fold. A HECT ligase will accept the ubiquitin from the E2 through its own cysteine residue and then pass it onto the target protein (29). In contrast, the RING finger ligase does not actually bind the ubiquitin but instead is thought to function more as a scaffolding protein to bring the E2-ubiquitin molecule and the target substrate together (30).

The attachment of a single ubiquitin (mono-ubiquitylation) has important roles in biology, which will be covered in more detail later. Ubiquitin, through its

seven lysine residues, has the ability to be attached to other ubiquitins, forming poly-ubiquitin chains. The lysine used in the linkage results in very different functional outcomes for the target protein (31). Linkage through lysine 63 is associated with DNA repair, translational activation, and other functions, while lysine 48 linkage is the target signal for 26S proteasome-mediated degradation. Additionally, the number of ubiquitin monomers linked together will also determine functional outcomes. The minimum recognition signal appears to be at least 4 monomers linked together (K48 Ub_4) (32). Model substrates tagged with a single monomer are not degraded by the 26S proteasome, while a substrate tagged with a five ubiquitin chain is degraded readily, Chains containing less than 4 monomers bind poorly to the 26S proteasome, but increasing the chain length beyond four monomers had only a small effect on the ability to bind to the 26S proteasome.

A possible explanation for the difference in functional outcomes may be structural. The quantenary structure of K63 and K48 linked Ub_4 chains is very different. A K63-linked chain is open and extended, each ubiquitin monomer is exposed to solvent (or other interacting proteins) and relatively free (33). The K48-linked chains are more compact with interactions between the monomers that can bury the interfaces (34). There is some disagreement between X-ray crystal structures, NMR structural data, and functional results of mutational data but the most recent data indicate K48-linked ubiquitin chains appear to be dynamic,

alternating between a ‘closed’ or tightly packed confirmation and an ‘open’ or less tightly packed confirmation . In the closed confirmation the hydrophobic patch, consisting of L8, I44, and V70, was buried between the second and third monomers but in the open confirmation the hydrophobic patch is exposed (35). Mutational data suggests that the hydrophobic patch is required for recognition of K48- Ub_4. Mutation of the residues in the hydrophobic patch to alanine renders the ubiquitin chain unable to bind to 26S proteasomes or the Rpn10 subunit (36) (37). It is unclear if this a consequence of disruption of the quantanary structure that requires an intact hydrophobic patch or disruption of the recognition site used to recognize K48-Ub_4.

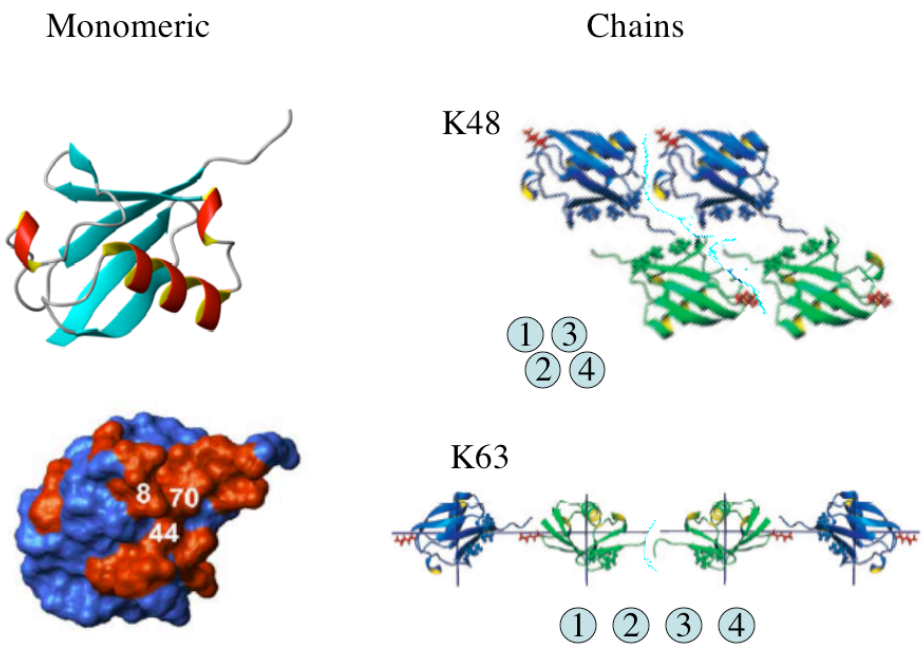

Figure 1-2 Ubiquitin: monomeric and chains. The structure of monomeric ubiquitin is shown in the left panel. The three residues that make up the 'hydrophobic patch' are highlighted at the bottom left. The right panel shows two confirmations of two different linkages of ubiquitin chains. K48 linked chains are more tightly packed compared to K63 linked chains

Transcription and the *GAL* System

The first step in the production of proteins needed by an organism to carry out their cellular activities is to take the information stored in the DNA sequence and transcribe it into the mRNA that serves as a template for protein production. The process of transcribing genes involves a diverse and growing list of proteins involved in recognizing specific DNA sequences, basal transcription machinery,

co-activators and co-repressors, and chromatin modification. This complex 'dance' of proteins and protein machines providing positive and negative input must be tightly regulated at many points to insure efficient and correct transcription of genes.

One of the first places this regulation is seen is with the transcription factor or transactivator. Transcription factors are proteins that help define the open reading frame and are involved in recruitment of the necessary transcriptional components. These proteins are modular and require a DNA binding domain (DBD) and an activation domain (AD) to function as transactivators (38). The DBD binds to a specific sequence of DNA, often located 5' to the transcription start site. In *S. cerevisiae*, these specific sequences of DNA, or upstream activating sequence (UAS), are usually located within a few 100bp of the gene to be transcribed (39). However, in some higher eukaryotic systems the UAS can be over 1kbp from the start site (40). The AD is involved in recruiting the transcriptional machinery necessary for transcribing DNA. This machinery includes the RNA polymerase II (RNAPII), which transcribes the gene, and a host of accessory factors, such as the general transcription factors (TFIIA, B, D, E, F, and H) and the Mediator complex (Figure 1-3) (41). These factors are recruited to the core promoter by the transactivator to form the pre-initiation complex (PIC) on the TATA box. Additionally, there are several other

types of co-activators that are involved in transcription often depending on which transactivator is being used.

Assembling the PIC and transcribing the gene is not as easy as just recruiting the required proteins and sending the RNAPII on its way. That is because DNA is associated with protein in a structure called chromatin. DNA is wrapped around a histone octomer, made up of two copies of four different histones (H2A, H2B, H3, and H4), to form a nucleosome, with H1 bound to the DNA outside of the octomer structure (42). Depending on how tightly packed the chromatin is it can be inaccessible (heterochromatin) or more open and accessible (euchromatin) (43). To move down the gene, the nucleosomes must be removed or modified so the elongating RNAPII holoenzyme can pass. This is accomplished by nucleosome remodeling complexes that work to alter and then replace the nucleosome organization (44). The histones can also be modified by a host of post-translational modifications (PTM), usually on the N-terminal tails of H3 and H4 (45). This chromatin modification can be used to change the chromatin structure or 'mark' the chromatin, often by recruiting certain proteins to the PTM.

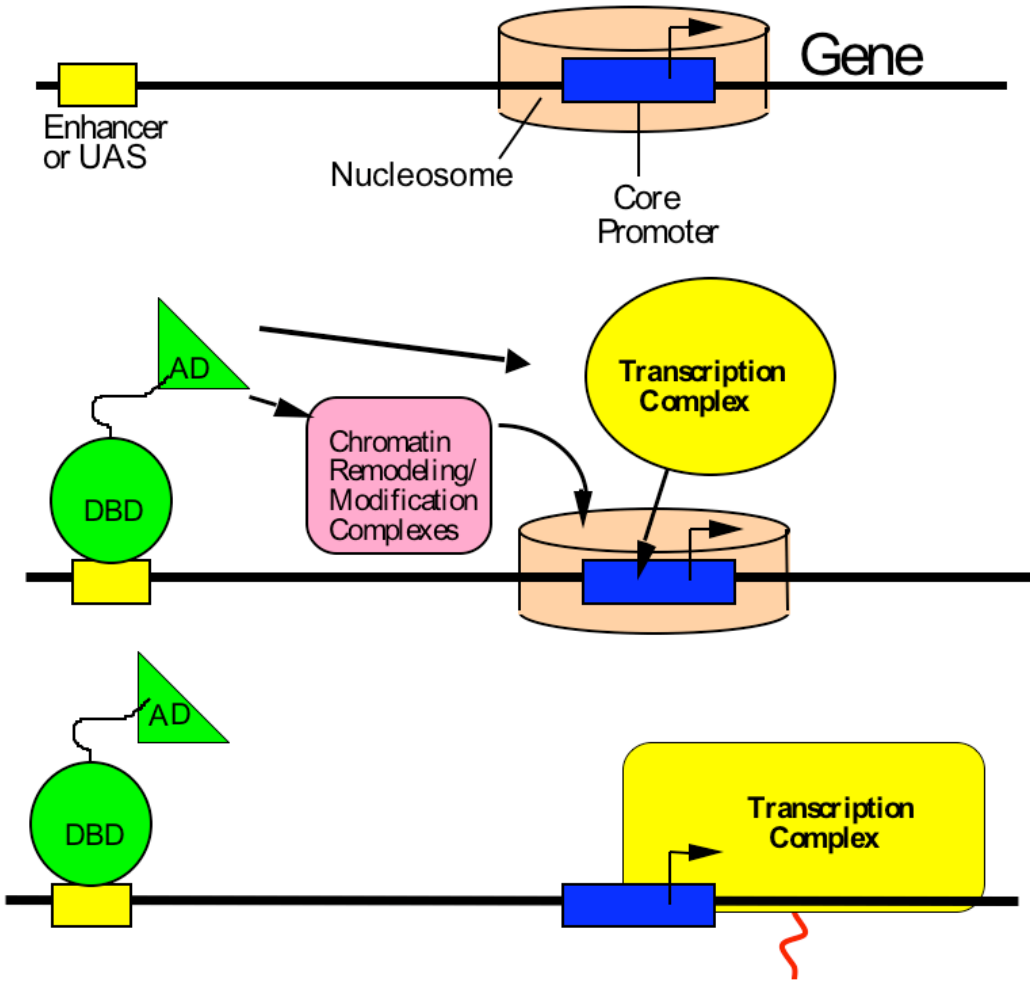

Figure 1-3 General transcription regulation. The upper panel shows an inactive gene associated with a nucleosome. Binding of a transcription factor (green) recruits chromatin modification complexes and transcriptional components (middle panel). After modification and assembly of the transcription complex, the gene is transcribed (lower panel).

One of the most studied eukaryotic expression systems is the *GAL* system in *S. cerevisiae*. Yeast are able to utilize multiple carbon sources to meet their needs for growth and survival. In order to use galactose as a carbon source, the

GAL regulon must be produced (46,47). These are a collection of genes that have both structural (*Gal1, Gal2, Gal5, Gal7, Gal10, and Mel1*) as well as regulatory (*Gal3, Gal4, Gal80*) roles, most of which are under control of the transactivator Gal4 (48). Gal4 is an 881 amino acid protein that binds specifically to a 17 bp upstream activating sequence (UAS_G, CGG-N_{11}-CCG) in the promoters of the *GAL* genes to induce their transcription (49). Different domains in Gal4 comprise the different activities needed to function as a transcriptional activator (Figure 1-4). A Zn-Cys cluster DBD present in the N-terminal first 57 amino acids of Gal4 is responsible for the specific binding of Gal4 the UAS_G (50). The AD of Gal4 is made up of 34 amino acids at almost the extreme C-terminus of the protein (51). The AD functions by recruiting proteins and complexes to the DNA to assemble a transcription complex. Many proteins have been shown to bind directly to the AD such as TBP, TFIID, Gal11 (a component of Mediator), and Tra1 (a component of the SAGA histone acetyltransferase complex, HAT) as well as others (52-55). The best *in vivo* evidence for physiological relevant interactions is for SAGA and Mediator (56,57). Similar to many other transactivators, these domains are modular in nature, such that the DBD of Gal4 can be fused with another AD to produce a chimeric protein that will bind and transcribe from the UAS_G sequences and vice versa (58,59). These domains can also be broken apart and fused to other proteins and will still function as a DNA specific transcriptional activator if brought back together by a protein-protein interaction between the fused proteins.

This characteristic forms the basis of the yeast 2-hybrid method, useful for screening for protein-protein interactions.

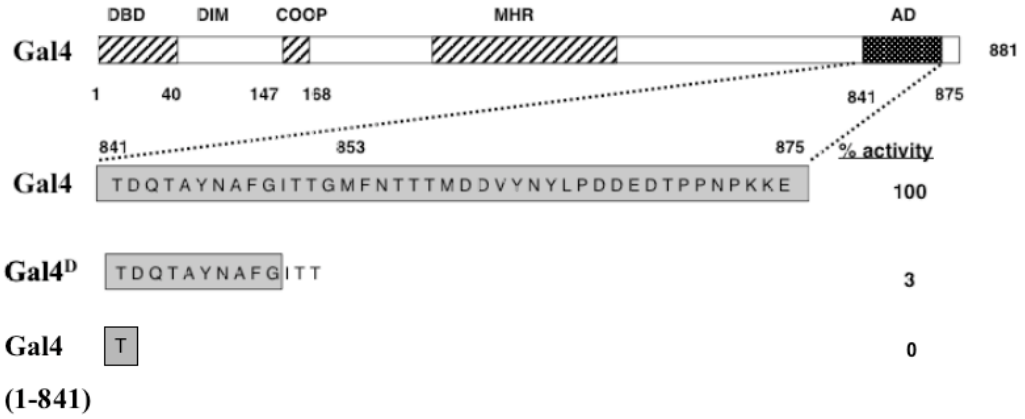

Figure 1-4 Gal4 protein domains. The 881 amino acid Gal4 protein is shown at the top. The N-terminal DNA binding domain (DBD) and C-terminal activation domain (AD) are highlighted. The lower panels show the residues of the AD for Gal4 and two different truncation mutations. Transcriptional activity is shown as a percentage of Gal4 activity.

The regulation of the *GAL* system is mainly done by modifying the activity and availability of Gal4, as is common for other inducible transcription systems (Figure 1-5). This can be done at both the DNA and protein level. The Gal4-responsive genes have between 1 and 4 UAS_G sites in their promoters (60). The CGG triplets and the 11 nucleotide spacing between them is absolutely required for Gal4 binding to the UAS_G, but the sequence of those 11 nucleotides can be modified. This results in differential binding affinity for the sites, with the highest affinity for those that most resemble the consensus UAS_G and a lower

affinity for sites that deviate from the consensus. Gal4 binds cooperatively to the low affinity UAS_G *in vitro* and this can lead to synergistic activation *in vivo* (61,62). Thus, the number and affinity of the UAS_G sites present in the promoter will influence the occupancy and ultimately the activity of Gal4 on those promoters. Additionally, the accessibility of the DNA can be altered depending on chromatin structure and the position of the nucleosomes in the promoter. The UAS_G sequences are free of nucleosomes in the *GAL1-10*, *GAL7*, and *GAL80* promoters (63). This allows Gal4 to bind freely to the promoters on these genes without prior removal of the nucleosomes. However, the TATA boxes and initiation sites are occupied and only become free upon galactose induction which suggests that Gal4 must recruit some sort of chromatin remodeling enzymes to assemble the PIC of these genes.

Besides the regulation that occurs at the DNA and chromatin level, regulation also occurs at the protein level. In the presence of glucose, the *GAL* system is repressed. This is done by the constitutively expressed protein, Mig1. Mig1 functions by binding DNA sequences in the promoter to prevent the expression of those genes (64). There are Mig1 binding sites in *GAL4, GAL1, MEL1* and *GAL3* promoters. Thus, Mig1 can indirectly repress all of the *GAL* genes by directly repressing the transcription of the activator Gal4 as well as directly repress a few of the genes. In carbon sources such as raffinose or glycerol/lactic acid the *GAL* system is poised for activity, but is kept inactive by

the repressor protein Gal80 (65). Mig1 no longer represses *GAL* promoters and Gal4 is able to bind and occupy its UAS_G, but its AD is not able to function because of interaction with Gal80. Gal80 has been shown to inhibit interaction of the AD with TBP and the SAGA complex, thus inhibiting transcription by preventing the AD from recruiting the factors necessary for transcription complex formation. However, when galactose is available to the yeast the repression by Gal80 is relieved and the system begins robust transcription of the *GAL* genes. The exact mechanism is still unclear, but Gal3 is involved in relieving the Gal80 mediated repression (66). *In vivo* studies indicate that Gal80 is most likely still bound to the AD, so the hypothesis is that Gal3 functions to alter or move the Gal80/Gal4 AD interaction so Gal4 can bind the Mediator and SAGA complexes, in turn recruiting the remainder of the transcriptional components (67).

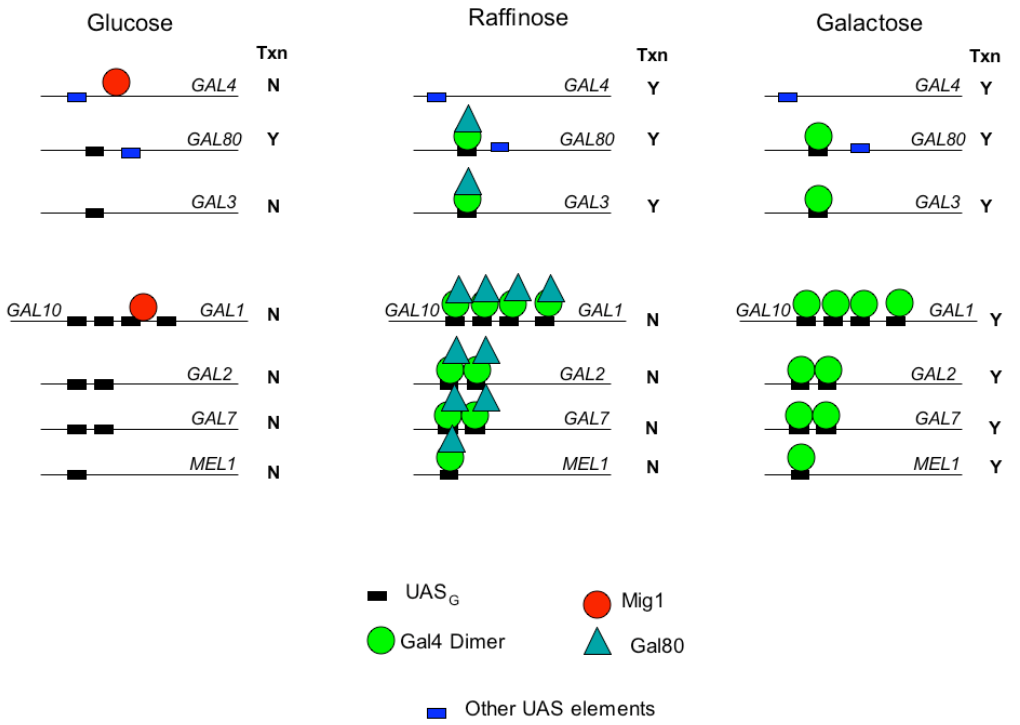

Figure 1-5 Regulation of the *GAL* system. The transcription of the *GAL* regulon is controlled by the activator Gal4 and the two repressors Mig1 and Gal80. The occupancy of these factors on each of the *GAL* genes and the transcription of the genes is shown for the three different states of the system.

The UPS in Transcription

The UPS has an essential role in the cell in the turnover of short-lived proteins and in the degradation of damaged proteins. However, the UPS also plays a very important role in the transcriptional process that extends beyond the control of protein level and degradation of 'checkpoint' proteins (Figure 1-6). Over 15 years ago, before these proteins were identified as part of the 26S proteasome, it was discovered that proteasomal ATPase proteins TBP-1 (Rpt5) and MSS1 (Rpt1) were important for HIV-1 Tat-mediated transcription (68,69).

Additionally, two different repressive suppressors of a truncation of Gal4 (*sug1-1* and *sug2-1*) (47,70) were also later identified as members of the 26S proteasome (71) (72). These two systems provided the first clues that the UPS was involved in transcription in ways not previously expected. More recently, ChIP to chip protocols (chromatin immunoprecipitations (ChIP) followed by microarrays) using antibodies raised against the proteasome subunits demonstrated that the 26S proteasome and different subcomplexes were associated throughout the yeast genome (73,74). While many of the functions of the UPS in transcription are proteolytic there are also now several non-proteolytic roles of the UPS in transcription.

One way to regulate transcription is to regulate the abundance of the transactivator. If the cell maintains a low level of activator, this results in a low level of transcription from that activator. This occurs with the transcription factors Jun, p53, Myc, and others (75-77). Several systems can destroy proteins, but the UPS is responsible for the majority of the degradation of these short-lived proteins. The signal for UPS mediated destruction of Myc seems to be related to its function as a transactivator. It was discovered that the AD of Myc and the sequence that signals for its UPS-mediated destruction (degron) overlap in the N-terminus of Myc (77). The overlap between AD and degrons was found in many other unstable transcription factors as well (78). Fusion of an AD to stable proteins made these proteins unstable, and fusion of degrons to a DBD containing

protein turned them into a transactivator. This overlap suggested a close link between transcriptional activation and degradation by the UPS. This idea was extended by demonstrating that Myc-mediated transcription was dependent upon Skp2, a component of the E3 ligase complex that signaled for the degradation of Myc (79).

The UPS is also important for degrading other molecules involved in transcription other than the transactivators. The UPS can stimulate transcription by degrading inhibitory molecules. One example of this is an inhibitory molecule that represses NFκB-mediated transcription (80). NFκB is held in the cytoplasm in an inactive state by the IκB repressor molecule. When the extracellular receptor receives a signal that activates NFκB, a signaling cascade begins. This cascade results in the phosphorylation and ubiquitylation of IκB, which is recognized and degraded by the 26S proteasome. NFκB is then free to translocate to the nucleus and start transcription.

Another example of degradation of molecules besides transcription factors is in degradation of subunits of the RNAP II. It is not uncommon for the RNAP II to pause or stall at sites in the coding region that have been damaged (81). If left unresolved, the paused RNAP II complex will block the passage of other molecules and inhibit transcription. To overcome this, the RNAP II is poly-ubiquitylated and the 26S proteasome is recruited to the pause site (82). The 26S proteasome degrades the poly-ubiquitylated subunit and this clears the blockage

caused by the paused RNAP II. Moreover, the 26S proteasome is recruited to the terminator pause site at the 3' end of coding regions in a transcription dependent manner and functions to resolve the paused RNAP II.

For several different inducible systems, inhibition of the UPS by chemical inhibitors such as MG132 resulted in a decrease or abolishment of the transcriptional output of those systems. The transcription and degradation of the transactivator were coupled together. This seems to be the case for the hormone inducible estrogen receptor (ER) alpha in mammalian systems (83,84) and the general regulator of transcription (Gcn4) in yeast (85). ERα was seen to cycle on and off its promoter and the transcriptional machinery also cycled in response to ERα. Interestingly, the proteasomal ATPases also cycled on and off the promoter. Chemical inhibition of the 26S proteasome abolished this cyclic activity and inhibited ERα-mediated transcription. Surprisingly, ERα protein level was stabilized by inhibition of RNAP II. The authors proposed that ERα was ubiquitylated in the process of transcription activation. The ubiquitylation lead to recruitment of the proteasomal ATPases, removal from the DNA, and degradation by the 26S proteasome. The act of transcription by ERα leads to a chain of events that leads to its degradation.

Another example of an ubiquitylation dependent activation followed by degradation by the UPS is for the co-activator protein, Src-3. This protein functions as a co-activator for several different transcription factors in mammalian

cells. It is also an oncogene, found overexpressed in ~10% of breast cancer tumors and over half of all tumors (86). Src-3 is known to undergo post-translational modification by both ubiquityaltion and phosphorylation (87,88). In response to the Akt signaling pathway, Src-3 is phophorylated by the kinase Gsk3. This phosphorylation leads to multiple mono-ubiquitylations and the C-terminus of Src-3 from the $SCF^{Fbw7\alpha}$ ligase (89). The result of this phosphorylation and mono-ubiquitylation of Src-3 is increased binding to transcriptional activators and increased transcriptional activation. As an example, estradiol induces rapid phosphorylation of Src-3 and targeting to promoters containing ER, which recruits other co-activators to potentiate transcription. During transcription, the mono-ubiquitins on Src-3 are extended into poly-ubiquitin chains that results in degradation by the UPS. Addition of transcriptional inhibitors such as Actinomycin-D prevents the ubiquitylation and degradation of Src-3. This effect of transcriptional inhibitors is similar to the studies on the cyclic nature of ERα on promoters, where transcriptional inhibitors disrupted the cycling on and off promoters similar to 26S proteasome inhibitors.

This coupling between transcription activation and degradation is also seen for the activator Gcn4. Destruction of Gcn4 by the UPS begins with the phosphorylation of Gcn4 by the kinase, Srb10 (90). Srb10 is a component of the Mediator complex, which associates with the transcriptional machinery on the promoter (91). Srb10 is important for phosphorylation of several different

proteins important for transcription, including the C-terminal domain (CTD) of RNAP II. The phosphorylated Gcn4 is recognized by an E3 ligase, SCF^{DCD4}, which results in poly-ubiquitylation and degradation by the 26S proteasome (92).

The two examples above demonstrate the direct coupling of transcription and destruction. In both cases, inhibition of proteolysis lead to a decrease in transcriptional activation, although the link to proteolysis almost seemed to be a by-product of the act of activation. Perhaps the UPS was important for 'resetting' the system for continued transcription. However, there are examples of activators where the ability to activate transcription is dependent upon a UPS dependent modification, which is followed later by the destruction of the transcriptional activator. The licensing of transcription is best understood with the activator LexA-VP16 (93). This chimeric activator is made of the bacterial LexA DBD fused with the viral VP16 AD. When this protein was expressed in yeast it would promote robust transcription from a reporter gene containing LexA binding sites. Deletion of the E3 ligase, Met30, resulted in loss of regulation of the protein level of LexA-VP16 and the steady-state level of the protein became very high. However, transcriptional activation by LexA-VP16 in the Met30 deletion background was abolished. This surprising result indicated that the UPS did not regulate transcription solely by controlling the activator level and suggested something else must be involved in the regulation. The authors found that genetic fusion of mono-ubiquitin to the LexA DBD would restore transcriptional activity

to the LexA-VP16 even in the Met30 deletion background. They hypothesized that the mono-ubiquitylation of LexA-VP16 functioned as a licensing event that allowed the transcriptional activator to function. At some time in the future this mono-ubiquitylation would be extended into the poly-ubiquitin chain that would be recognized by the 26S proteasome and target the activator for degradation, thus acting as a brake or checkpoint on transcriptional activation. The initial licensing event, followed by poly-ubiquitin and degradation was termed the timer or clock model of transcriptional activation. The mechanism by which ubiquitin could promote activity is unknown, but there are examples of mono-ubiquitylation promoting recruitment of co-activators and increasing the interaction of transactivators with DNA.

The overlap of AD and degrons and the licensing followed by degradation of activators highlight the coupling of transactiavtion and degradation by the UPS. In some cases, this coupling is not so clear. For instance, the class II transactivator (CIITA) is a transcription factor important for expression of the major histocompatibility complex in higher eukaryotes (94). Ubiquitylation of CIITA resulted in increased transcription of the endogenous MHCII genes and increased interaction with the co-activators important for expression of these genes and the chromatin (95). This effect seems to be specific to mono-ubiquitylation because in some studies the authors used a form of ubiquitin that contained mutations of all the lysine residues so now chain coupling could occur.

The ability of ubiquitylation to enhance transcription is also seen for Tat-mediated transcription. Tat is the transcription factor important for HIV-1 transcription. Tat is ubiquitylated by Mdm2, but this does not target Tat for degradation by the 26S proteasome (96). Instead it enhances the ability of Tat to transactivate the HIV-1 long terminal repeat (LTR). This is another example of an E3 ligase functioning as a positive regulator of transcription, but unlike the Myc/Sk2 example Tat is not destabilized as a consequence of the modification.

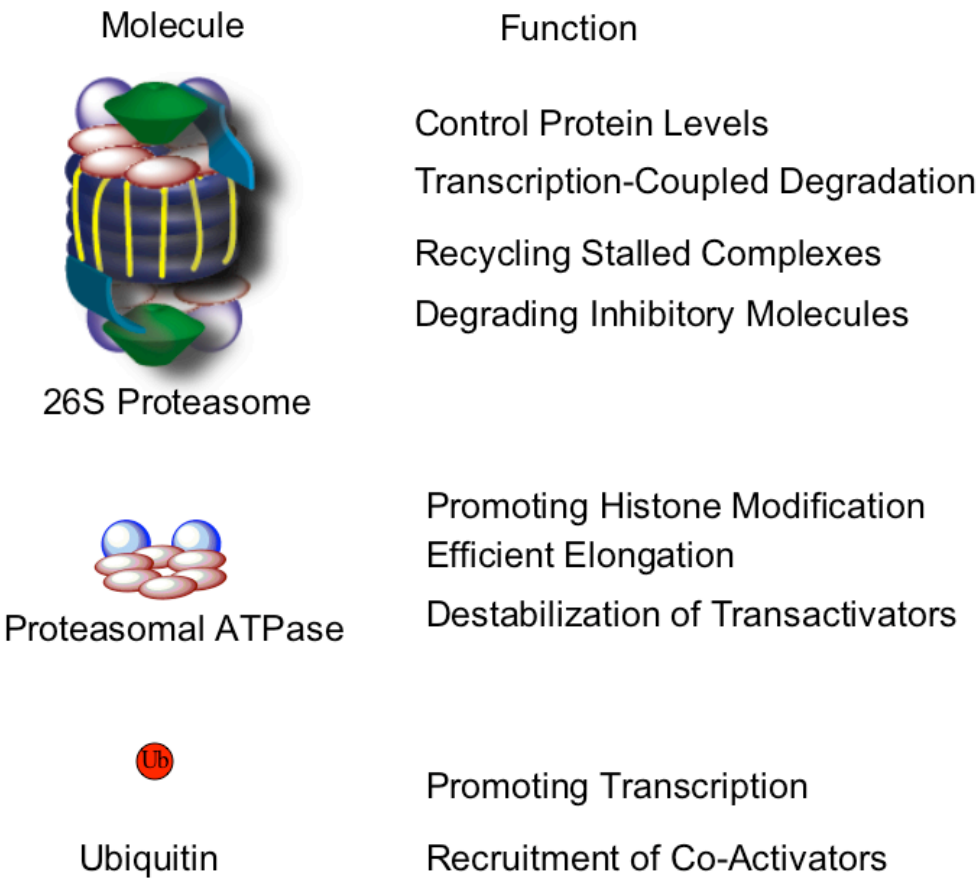

Figure 1-6 Role of the UPS in Transcription. The different components of the UPS and some of their roles in transcription are highlighted.

As mentioned previously, the truncation of the Gal4 AD at amino acid 853 (Gal4D) results in a transactivator that is deficient for activation of the *GAL* genes and Gal4D can be partially restored by mutation of two of the proteasomal ATPases. This suppression was originally thought to be because of increased Gal4D levels in a damaged proteasome mutant background, but this was not the case (97). The AD of Gal4 and the truncated AD of Gal4D both are able to bind

to the 26S proteasome. The interaction occurs between the AD and Rpt4/Sug2 and Rpt6/Sug1, the same two proteins that are suppressors of Gal4D when mutated (98). Interestingly, the AD of Gal4 dissociated the 26S proteasome when it bound (99). This in contrast to other 26S proteasome binding proteins, such as the UBL domain of Rad23, which bind and retain the entire 26S proteasome in pull-down assays. The AD of Gal4 retained only a subcomplex of the 26S proteasome including the 6 ATPases as well as Rpn1 and Rpn2, but not containing the 19S lid or CP. This complex, termed APIS, appears to be similar to the base of the RP, but full characterization of the complex has not been completed. The APIS complex, but not the remainder of the 26S proteasome, is recruited to the *GAL1* promoter upon induction of the gene, suggesting that this APIS complex is important for Gal4-mediated transcription.

The proteasomal ATPases are involved in recruiting co-activators to transactivators bound to DNA. The RP base of the 26S proteasome can promote interaction between the SAGA complex and Gal4 (100). The SAGA complex is important for transcriptional activity of Gal4 and contains a HAT domain to acetylate nucleosomes. The base of the RP increased the interaction of SAGA with DNA bound Gal4 and Gal4 bound to nucleosomes templates in an ATP dependent manner. The ability of the SAGA complex to acetylate histones was also stimulated by the RP demonstrating a role in targeting of the complex to transactivators as well as increasing its biochemical activity. The importance of

the proteasomal ATPases was also demonstrated *in vivo* by ChIP. Mutations in the Rpt6/Sug1 subunit (*sug1-25*, an ATPase domain mutation) reduced SAGA binding to the promoters dependent on Gal4 and another transcription factor Gcn5. Global as well as promoter specific actelyation was also decreased in the *sug1-25* background.

The proteasomal ATPases are also important *in vivo* for other chromatin modifications (101). Methylation of H3 is disrupted by mutations in the proteasomal ATPases, which normally associate with these genes. As a consequence of mutation of the proteasomal ATPases, gene silencing is disrupted. Interestingly, the ability of the proteasomal ATPases to promote H3 methylation is dependent on prior mono-ubiquitylation of H2B. Deletion of one of the enzymes responsible for mono-ubiquitylation of H2B, Rad6, is genetically lethal when combined with the *sug1-25* mutation. The *Δrad6* mutant or mutation of the mono-ubiquitylation site of H2B, *H2B-K123R*, results in reduced targeting of the Rpt4 or Rpt6 ATPase to the promoter region.

The proteasomal ATPases can influence transcription by methods that do not involve recruitment of co-activators. The proteasomal ATPases play an important role in stimulating efficient elongation. *In vitro* transcription assays done using Gal4-mediated templates with yeast lysates have shown a defect in full-length transcripts when the Rpt4 or Rpt6 proteins are crippled by temperature sensitive mutations or by antibodies raised against the Rpt6 protein (102,103).

This defect could be overcome by adding back purified RP to the reaction. The importance of a 26S proteasome subcomplex in promoting elongation is also seen in Tat-mediated HIV-1 transcription (104). In this case, Tat and a co-activator PAAF-1 recruit a 19S-like proteasome subcomplex to the HIV-1 promoter. Disruption of Tat or the proteasomal ATPases resulted in a defect of the RNAPII Holoenzyme complex to clear the promoter. In both cases it appears that the proteasomal ATPases are important for efficient elongation of transcription complexes. The advancement of transcription from preintiation complex on the promoter to an escaped elongating complex has been hypothesized to require reorganization of the complex to resolved 'tethering' protein-protein interactions (105). These rearrangements might be overcome by the chaperone-like activity of the proteasomal ATPases. Others have suggested that transcription of other genes in *S. cerevisiae*, such as the *CDC20* gene, also requires remodeling of complexes by the proteasome (106).

The original Gal4D suppressing mutation, *sug1-1* and *sug2-1*, were recessive, which suggests that these mutations function by loss of some negative effect of the proteasomal ATPases. The ability of the proteasomal ATPases to promote elongation, recruit co-activators, or promote proper translation of mRNA are not easily explained by a recessive function and the suppression of Gal4D remained a bit of a mystery. Recent evidence has demonstrated that the UPS does have a negative role in transcription. The proteasomal ATPases will remove or

destabilize a transactivator from DNA (107). Experiments *in vitro* demonstrated that this destabilization activity was ATP dependent, could be done with only the purified RP, and did not result in destruction or irreversible unfolding of the transactivator. When these experiments were repeated by first passing the transactivator-DNA complex through a HeLa nuclear extract in the presence of ATP the transactivator would be protected from destabilization by the proteasome. This protection from destabilization always correlated with mono-ubiquitylation of the transactivator, suggesting that this modification may be responsible for protection.

This correlation between protection from destabilization and mono-ubiquitylation is best demonstrated with a mutant form of Gal4 named Gap71. Gap71 contains point mutations in the DBD and is not able to promote transcription, although it was able to bind DNA about as well as Gal4 *in vitro* (108,109). Gap71 was not ubiquitylated in a HeLa nuclear extract and was stripped from DNA by the proteasomal ATPases (107). In yeast, Gap71 was able to occupy promoter sequences under non-inducing conditions where the AD is masked by interaction with Gal80, again demonstrating that Gap71 has no defect in DNA binding. However, when the AD of Gap71 was exposed by switching to activating conditions, Gap71 could no longer occupy DNA. The correlation between destabilization and the modification of the activator seems to hold true *in*

vivo as well as *in vitro*. The mechanism of protection by mono-ubiquitin from destabilization by the proteasomal ATPases is unknown.

Chemical Cross-linking as a Tool to Study Protein-Protein Interaction

An individual cell has several thousand different proteins expressed at any one time (110). These proteins must communicate with each other to carry out their respective functions. Often this communication occurs through interaction, whether in the context of signaling networks, transcriptional regulation, or a multi-protein machine (111). To understand communication in a cellular sense, one must be able to understand protein-protein interaction. There are many different methodologies used to detect these protein-protein interactions that vary from low-tech binary interaction data to atomic level detail structures of proteins or macromolecular complexes with each method having its own strengths and weaknesses.

Chemical cross-linking is a methodology that takes a non-covalent interaction and forms a covalent bond through several different types of chemistries to make the interaction permanent. This gives one the advantage of time, a transient interaction can be 'frozen' or captured for study at a more convenient time. Also, the covalent nature of the interaction can be manipulated to lock two interacting proteins together and separate out non-covalent

interactions. This can be especially useful when working with multi-protein complexes.

The most widely used type of cross-linker is the bifunctional cross-linker (Figure 1-7). These cross-linkers contain two reactive groups connected together by a linker arm of varying length. The reaction chemistries often target amines or thiols, which are present on the surface of proteins as lysines or cysteines, respectively. These simple cross-linkers are useful, but they have limitations. Often the chemistries employed work poorly in biological systems that have aqueous buffers with physiological pH values. They often suffer from sensitivity issues and require very large amounts of protein to detect an interaction. A cross-linked product is dependent on the appropriate reactive groups to be in reach of the linker arm and it is not uncommon to have false negatives. Unfortunately, bi-functional cross-linkers often suffer from false positives, possibly due to the large amounts of protein needed producing non-native interactions (112).

Additionally, there is no way to control or trigger the reaction. The cross-linkers are simply added to the complex or proteins under study. This makes study of transient or time-specific interactions difficult. One way to control cross-linking reactions is with photo-activatable groups. These molecules are inactive until they are triggered with light of the correct wavelength. This enables one to start a reaction or control the reactivity of the reaction by adding the light at certain times. These groups also may react with nucleophiles or incorporate into

C-H bonds. While these photo-active groups add some manner of control to the reaction, they still suffer from the limitations of bi-functional cross-linkers.

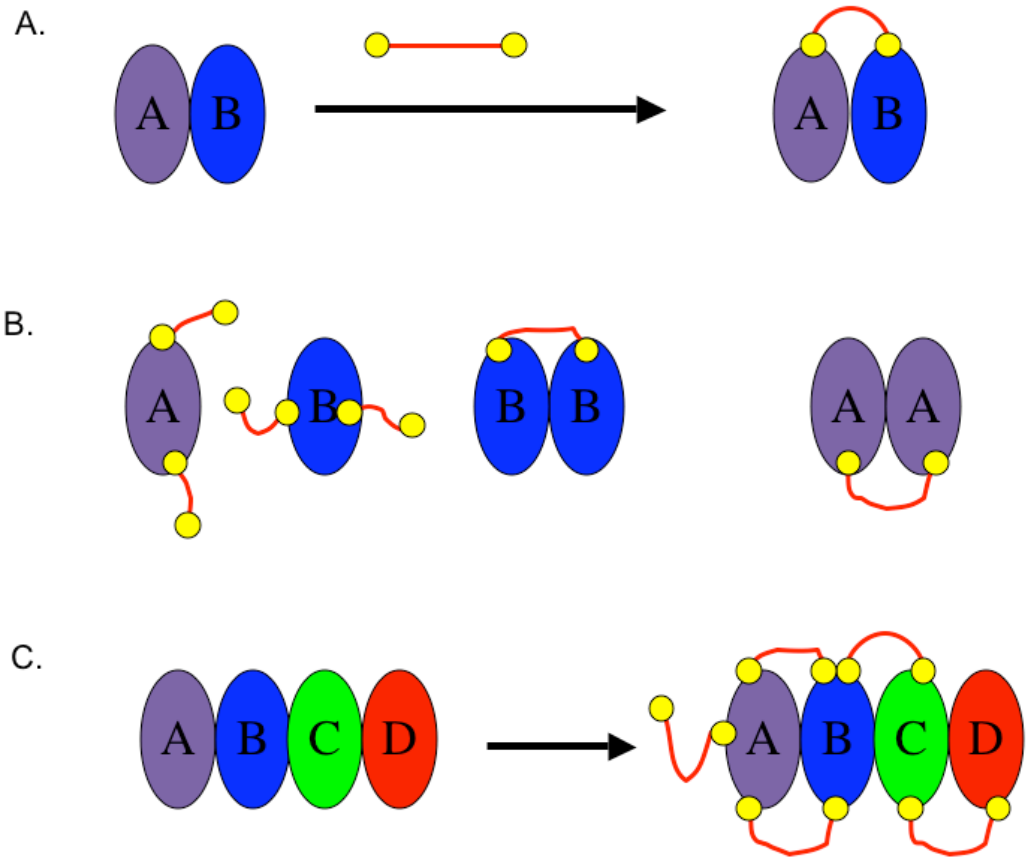

Figure 1-7 Bifunctional cross-linkers. A bifunctional cross-linking reagent ideally would form one cross-link between heterodimers (A), but lack of dynamic and spatial control makes this difficult (B). When one deals with multi-protein complexes this lack of control makes deconvoluting the interactions difficult (C).

One way around the problems is the use of 'zero-angstrom' oxidative cross-linkers. These types of chemistries oxidize proteins at aromatic residues, which cross-link to nearby aromatics. In the case of the $Ru(II)(bpy)_3^{2+}$- catalyzed

reactions used by our laboratory, these reaction were quick, had higher cross-linking yield, or only associated proteins would cross-link at the concentrations employed (113). The drawback of this technique is the difficulty in deciphering just what proteins have been cross-linked together in a complex solution (114). The cross-linked peptides are difficult to detect by MS. These reactions are difficult to control and often produce very high molecular weight species that are the result of multiple cross-links between different proteins.

To overcome these limitations of oxidative chemical cross-linking, we turned to 3,4 dihydroxyphenylalanine (DOPA) chemistry (115) (Figure 1-8). DOPA can be oxidized by periodate, which does not affect other amino acids, to produce an ortho-quinone. The ortho-quionone is attacked by nearby nucleophiles to cross-link the two proteins together. In contrast to the diffusible oxidative cross-linkers, the DOPA can be incorporated at a site of interest to control the cross-link by location and also limiting the amount of reactions that one molecule can make. The DOPA group was inserted into peptides and simple model systems between know binders were used to test its usefulness (116). Cross-link products were only formed between interacting proteins even in the context of cellular lysates demonstrating that templating of the interaction was required for the reaction. The use of biotin in the peptides gave a handle or tag used for detection. Additionally the cross-linked products could be enriched by using the biotin handle. The reaction was fast, on the order of seconds, after the

trigger was added. Several different amino acids, including cysteine, α-amines, lysine, and histidine, would serve as nucleophiles in the reaction. Finally, DOPA-mediated cross-linking produced detectable products at biological concentrations in biological amenable buffers. The reaction proceeded in aqueous buffers at physiological pH suggesting that this reaction would be useful for characterizing interactions in relevant conditions (117).

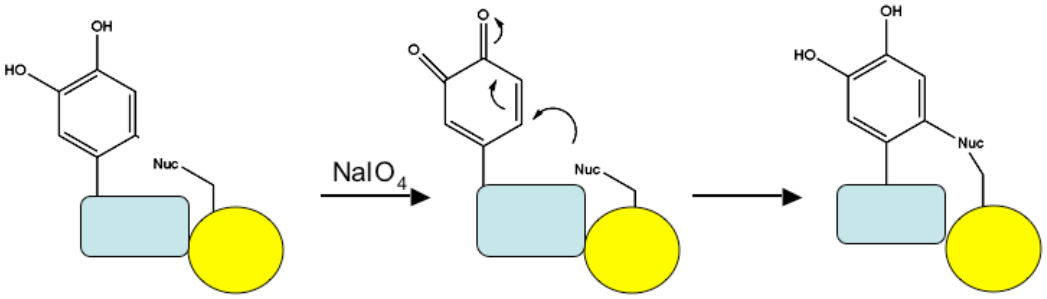

Figure 1-8 DOPA mediated chemical cross-linking. The DOPA group can be selectively oxidized by the addition of periodate. The resultant ortho-quinone can be attacked by nearby nucleophiles to form a covalent linkage between interacting proteins.

CHAPTER TWO

OXIDATIVE CHEMICAL CROSS-LINKING: A METHOD TO DETECT PROTEIN-PROTEIN INTERACTIONS IN THE CONTEXT OF MULTI-PROTEIN COMPLEXES.

SUMMARY

Most biological processes involve interactions between proteins. Ideally, one would like to study these interactions in their native contexts, but methodologies to do so are limited, especially in the case of interactions that occur in the context of very large multi-protein complexes. To overcome this, we have developed an oxidative chemical cross-linking methodology that is particularly well suited for study of interactions between small peptides or signaling molecules and proteins. This method allows the rapid identification of a receptor target in a multi-protein complex. The usefulness of this reaction is demonstrated on the previously characterized interaction between the activation domain of Gal4 and the proteasome.

The ability to monitor interactions between proteins often involves production of the protein as an artificial fusion, which in many cases may alter interactions. Cross-linking and label-transfer (the transfer of a tag from the

protein of interest to an interacting protein) strategies exist to study the protein in a native context. Unfortunately, these strategies often employ chemistries that are not compatible with biological buffers, labels that require special handling such as radioactivity, or cross-linkers that required covalent attachment of the cross-linking group to the target of interest. We have adapted our oxidative chemical cross-linking for use in label-transfer reactions with native proteins, using a modified FlAsH molecule and a six amino acid tag contained within the protein. We demonstrate the utility of this method using known interaction pairs and activation domain/proteasome interactions.

The work presented in this chapter was a collaborative effort between myself and Dr. Lyle Burdine and Dr. Bo Liu, both members of the Kodadek lab. Dr. Burdine and I contributed equally to the work demonstrating the usefulness of cross-linking to identify receptors on small peptides in multi-protein complexes. Dr. Burdine performed the experiment in Figure 2-2 and synthesized the peptides used in the reactions. The modification of the FlAsH molecule for cross-linking with proteins was first demonstrated by Dr. Liu and Dr. Burdine. Additionally, Dr. Liu was responsible for the synthesis and characterization of the FlAsH compound, the model in Figure 2-7, and the generation of many of the materials used. I originally constructed the biotin tag in the FlAsH DOPA cross-linker with Dr. Burdine and performed all other experiments shown.

INTRODUCTION

Protein-protein interactions are important in most biological processes. There are many methods with which to study these interactions. However in the case of multi-protein complexes, these methods either do not provide information about direct interactions or to do so requires removal of the proteins from the native complex. For instance, potential binding proteins from a multi-protein complex can be removed and tested individually for binding to a target protein. This method will tell you if an individual protein interacts with the target protein, but also exposes normally surfaces that are normally sequestered by the other members of the complex. This may lead to false positives. Chemical cross-linking can be used to provide information about direct interactions, but bifunctional cross-linkers suffer from difficulty in analysis of the products both due to multiple cross-linked products and also due to a lack on information about where the cross-linker incorporated into the target proteins. To address these limitations our laboratory has employed a cross-linking protocol based upon the reactivity of 3,4-Dihydroxyphenylalanine (DOPA) (116).

DOPA is unreactive in its reduced form, but it can be oxidized by the addition of periodate. The resultant electrophilic ortho-quinone reacts with nearby nucleophilic side chains to produce a covalent bond (118-120). The ability to trigger the reaction by altering the redox state allows one to control the

reaction. Previous work demonstrated that when DOPA was incorporated into small peptides it forms covalent products with direct binding partners in high-yield upon activation, but showed little reactivity to non-specific proteins even when they were present in excess (116). Additionally, the DOPA chemistry was efficient over a pH range of 6 to 8. Several different amino acid side chains, including lysine, histidine, and cysteine were shown to couple to the ortho-quinone intermediate (117). These results indicated that DOPA cross-linking chemistry would be useful for identifying interactions in complex biological samples. Additionally, the directed incorporation of the DOPA group prevents multiple cross-links between interacting proteins, a situation that can often produce complicated products that are difficult to analyze. To test the utility of DOPA-mediated chemical cross-linking to identify receptors in multi-protein complexes, we used the interaction between the activation domain of Gal4 and two known binding proteins in the 26S proteasome, Rpt4 and Rpt6 (55,99,121).

This chemistry has several advantages over other methods, highlighted above. However, while incorporating the DOPA residue in small molecules or peptides is fairly straightforward, it is more challenging to introduce the DOPA group into proteins of interest. Previously, most cross-linking and label-transfer protocols also require covalent modification of the protein of interest. Often this required genetic engineering of a specific amino acid (such as a cysteiene) into a non-critical but solvent exposed area of a protein and then adding the cross-

linking reagent with a reactive group that would form a covalent bond with the engineered amino acid (formation of a dithiol in this case). It is not hard for one to image that there would be more than one cysteiene available to react with the cross-linking reagent. To overcome these problems, we used non-covalent modification of our protein of interest using a molecule called FlAsH. FlAsH, a biarsenic fluoresciene derivative, binds specifically to a tetracystiene containing peptide tag fused to the protein of interest with high affinity (pM binding constant) (122). We hoped that modification of FlAsH with the DOPA cross-linking group and a biotin tag would provide an easy method for monitoring the interaction of a protein of interest with interacting proteins.

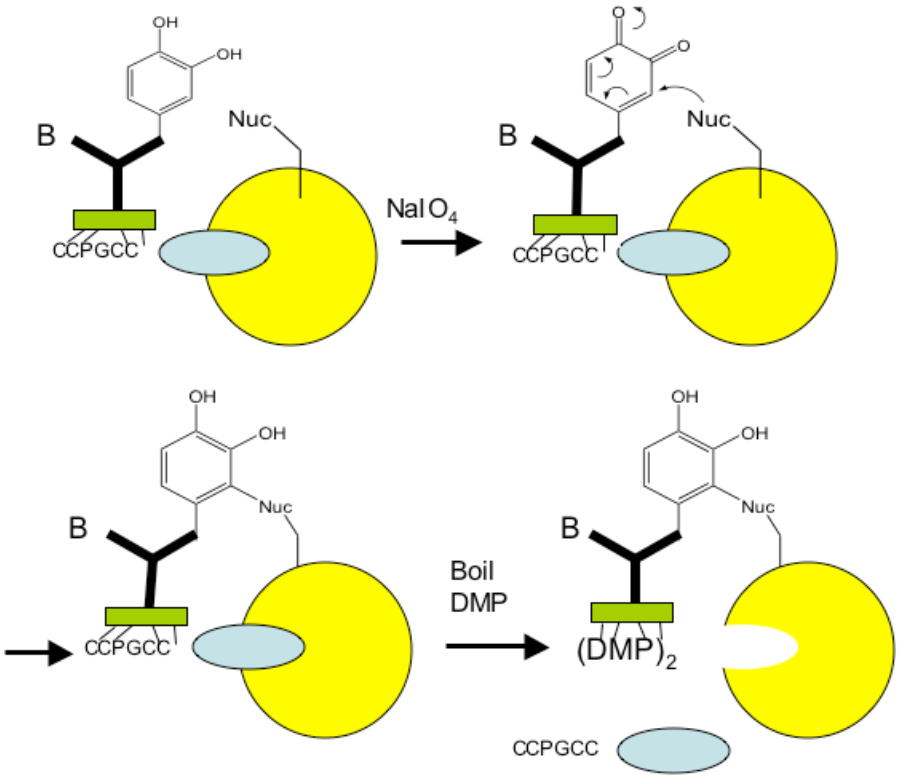

Figure 2- 1 The cross-linking and label transfer scheme.
See text for details.

MATERIALS AND METHODS

General Methods and Chemicals.

Fmoc-DOPA(acetonid)-OH and Fmoc-Glu(biotinyl-PEG)-OH were purchased from Novabiochem (San Diego, CA). All other Fmoc amino acid derivatives and resins were purchased from SynPep (Dublin, CA). Sodium Periodate and DOPAC (3,4-dihydroxyphenylacetic acid) were purchased from the Sigma-Aldrich Chemical Company (Milwaukee, WI) . Super Signal® West Pico

and Super Signal®West Dura kits were purchased from Pierce (Rockford, IL). Immobilon Transfer-P membrane was purchased from Millipore (Billerica, MA). Cross-linking reactions were analyzed by SDS-PAGE and western blotting using standard protocols. Peptide synthesis was performed on a Ranin Symphony 12 channel FMOC synthesizer. Analytical gradient reversed phase HPLC was performed on a Waters Breeze HPLC system with a VydacC18 analytical column. The flow rate was 1ml/min and detection was at 214 nm. Preparative HPLC was performed on the same instrument with a Vydac C18 preprative column. The flow rate was 10 ml/min. HPLC runs used linear gradients of 0.1% TFA and 90% acetonitrile plus 0.1% TFA. All of the synthesized peptides were analyzed by mass spectroscopy using a MALDI-Voyager DE Pro instrument.

4(5)-Carboxyfluorescein was purchased from Fluka. Mercury acetate and arsenic chloride (99.99 %) were from Sigma-Aldrich. Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-AEEA-OH), *O*-(7-azabenzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), and 1-hydroxy-7-azabenzotriazole (HoAt) were from Applied Biosystems. Fmoc-DOPA(acetonid)-OH and Fmoc-Glu(biotinyl-PEG)-OH were from NOVAbiochem. Rink Amide AM resin (0.57 mmol/g) was from NOVAbiochem. All other chemical reagents were of analytical grade, obtained from commercial suppliers, and used without further purification.

Peptide Synthesis

All peptides were synthesized on rink amide resin for cross-linking experiments. Importantly, each residue of the Gal4-AD was double-coupled to

drive up the final product yield. Furthermore, DOPAC was added as an N-terminal cap to the end of the this peptide as opposed to DOPA. Peptides were cleaved from the resin with a mixture of TFA:water:tri-isopropyl silane at ratio of 27:1.5:1.5. Crude peptides were precipitated and washed with anhydrous ether before being dissolved in 8M Guanidinium-HCL and purified over preprative HPLC. The pure peptides were characterized as desired products by MALDI-MS. After purification the peptides were resuspended in Buffer A (25mM Tris pH 7.4, 150mM NaCl, 5mM $MgCl_2$, 10% Glycerol) containing 1mM DTT aliquoted and stored at -80°C.

Gal-4 DOPA AD: DOPAC-TDQTAYNAFGITTGMFNTTTMDDVYNLPDDEDTPPNPKKEGGE-Biotin

Observed Mass: 5287.91 Predicted mass:5289.33

Gal-4 ND AD:

TDQTAYNAFGITTGMFNTTTMDDVYNLPDDEDTPPNPKKEGGE-Biotin

Observed Mass:5136.6 Predicted mass: 5138.33

Gal80-Binding Peptide: Biotin-EGG(DOPA)DQDMQNNTFDDLFWKEGHR

Observed Mass: 3045.04 Expected Mass: 3045.42

Control DOPA Peptide: Biotin-KG(DOPA)AHNRLIYMQD

Observed Mass: 1852.00 Expected Mass: 1850.32

Synthesis. 4(5)-Carboxy-[4', 5'-bis(acetoxymecuri)]fluorescein.

To a 500 mL round-bottom flask were added mercury acetate (2.0 g, 6.3 mmol) and glacial acetic acid (45 mL). The solution was heated to 50 °C before

4(5)-carboxyfluorescein (1.0 g, 2.7 mmol, dissolved in 150 mL of 2N NaOH) was added drop-wise in 1 h. The resulting solution was allowed to stir at room temperature for another hour. The red precipitate was collected by centrifugation, washed with water (100 mL × 2), and dried in vacuo for 3 days. The dry product was obtained as 1.8 g (76%) red powder, mp > 360 °C. ^{1}H NMR (300 MHz, D_2O, pD =13) δ 8.17 (s, 0.3H), 8.03 (d, J = 4.1 Hz, 0.3H), 7.91 (d, J = 3.9 Hz, 0.7H), 7.80 (d, J = 4.1 Hz 0.7H), 7.66 (s, 0.3H), 7.12 (d, J = 4.7 Hz, 1H), 7.06 (d, J = 4.5 Hz, 1H), 6.86 (d, J = 3.9 Hz, 0.7H), 6.66 (d, J = 4.7 Hz, 2H), 1.91 (s, 3H).

4(5)-Carboxyl-[4',5'-bis(1,3,2-dithioarsolan-2-yl)]fluorescein.

4(5)-Carboxy-[4', 5'-bis(acetoxymecuri)]fluorescein (2.0 g, 2.2 mmol) was mixed in anhydrous N-methyl-2-pyrrolidinone (33 mL) under argon, and arsenic chloride (3.2 mL, 38 mmol), palladium acetate (20 mg), and N,N-diisopropylethylamine (3.3 mL, 20 mmol) were added. (**Caution! Arsenic chloride is highly toxic. Take extra precaution when performing this step.**) The solution was allowed to stir at room temperature for 16 h before was poured into a stirred mixture (500 mL of acetone, 500 mL of 0.25 M, pH 7 phosphate buffer). 1,2-Ethanedithiol (6.0 mL) was added and the solution was stirred for 30 min. Chloroform (3 × 500 mL) was added and the layers separated. The combined organic layer was dried over sodium sulfate and concentrated. The red gooey residue was purified via silica gel chromatography. After the first orange band was eluted with 2 % methanol in chloroform, the elution solvent was

changed to 10% methanol in chloroform. The collected fractions gave a red residue after removal of the solvent. The red residue was dissolved in ethanol (15 mL), to which water was added until the solution became turbid. HCl (6N) was added to get an orange precipitate at pH 3.5, and the precipitate was collected by centrifugation. Drying under vacuum produced 0.85 g (54%) red gooey residue, mp115-116 °C dissociated. ^{1}H NMR (300 MHz, $CDCL_3$): δ 9.94 (S, 2H), 8.72 (s, 0.7H), 8.39 (d, J = 8.7 Hz, 0.7 H), 8.35 (d, J = 8.7 Hz, 0.3H), 8.07 (d, J = 8.6 Hz, 0.3H), 7.90 (s, 0.3H), 7.28 (d, J = 8.7 Hz, 0.7H), 6.61 (d, J = 8.7 Hz, 1.4H), 6.59 (d, J = 8.7 HZ, 0.6H), 6.52 (d, J = 8.7 Hz, 1.4H), 6.50 (d, J = 8.7 Hz, 0.6H), 3.80 – 3.45 (m, 8H). *N*-Methyl-2-pyrrolidinone coexists with the product. δ 3.41 (J = 7.0 Hz, 2H), 2.87 (S, 3H), 2.43 (J = 8.2 HZ), 2.04 (p, J = 6.9 Hz, J = 7.8 Hz, 2H). ESI MS calcd for $C_{25}H_{19}As_2O_7S_4^+$: 708.8446. Found: 708.8401.

3,4-Dihydroxyphenylalanine – Biotin – 4',5'-Bis(1,3,2-dithioarsolan-2-yl)fluorescein (DOPA-biotin-FlAsH, DBF).

Fmoc-DOPA(acetonid)-OH, Fmoc-Glu(biotinyl-PEG)-OH, and Fmoc-AEEA-OH were conjugated to Rink amide AM resin (5 μmol capacity) following standard solid-phase peptide synthesis protocol. 4(5)-Carboxyl-[4',5'-bis(1,3,2-dithioarsolan-2-nl)]fluorescein was conjugated to the resin following the same procedure except that the coupling solvent was N-methyl-2-pyrrolidinone (NMP) instead of dimethylforamide (DMF). The resin was washed with NMP (5 mL ∈ 4), dichloromethane (DCM, 5 mL ∈ 6), and dried under vacuum for 1 h. The dry

resin was treated in 300 μL of trifluoroacetic acid (TFA) / m-cresol (80:20 v/v) at room temperature for 80 min. After removal of the resin, ethyl ether (2 mL) was added to get an orange precipitate, which was collected by centrifugation and purified by HPLC on a C-18 analytical column. The yield was 25% based on the HPLC spectrum. 3,4-Dihydroxyphenylalanine – biotin – 4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein conjugate $[M+H]^+$ MALDI-TOF calcd: 1589.28. Found: 1589.24.

Plasmids and Protein Purification

His6-Gal80 protein was purified as described previously (123) from *E. coli* strain BL21. Affinity-purified 26S proteasome was purified from *S. cerevisiae* strain RJD1144 (*MATa* his3Δ200 leu2-3, 112 lys2-801 trpΔ63 PRE1FLAG::Ylplac211 (URA3) as described previously (124) with modifications (102). Actin was purchased from Sigma. The expression plasmid encoding the glutathione *S*-transferase – FlAsH receptor peptide (GST-FRP) fusion was constructed by annealing oligonucleotides (5'-*GA TCC* AAG TGC TGC GGC CCT TGC TGC GAC TGG T *A*-3' and 5'-*AG CTT* A CCA GTC GCA GCA AGG GCC GCA GCA CTT *G*-3'), and inserting the fragment into *BamH1/HindIII*-cleaved pGEX-2T-G80BP-A (123) plasmid (Kodadek lab stock pTK0216). The resultant plasmid was named pGEX-FRP. The same procedure was employed to construct the expression vector encoding the GST-FRP-G80BP fusion, except that the appropriate oligonucleotides were 5'-*GA TCC* TAT GAT

CAG GAT ATG CAG AAT AAT ACT TTT GAT GAT TTG TTT TGG AAG GAG GGG CAT CGG AAG TGC TGC GGC CCT TGC TGC GAC TGG T *A*-3' and 5'-*AG CTT* A CCA GTC GCA GCA AGG GCC GCA GCA CTT CCG ATG CCC CTC CTT CCA AAA CAA ATC ATC AAA AGT ATT ATT CTG CAT ATC CTG ATC ATA *G*-3'. The resultant plasmid was named pGEX-FRP-G80BP. To make the GST-G80BP-FRP fusion expression plasmid, a pair of synthetic oligonuleotides encoding FRP peptide (5'-*GA TCC* AAG TGC TGC GGC CCT TGC TGC GAC TGG G-3' and 5'-*GA TCC* CCA GTC GCA GCA AGG GCC GCA GCA CTT *G*-3') was annealed and inserted into *BamHI*-cleaved pGEX-2T-G80BP-A plasmid. The resultant plasmid was named pGEX-G80BP-FRP. The expression plasmid encoding protein GST-Gal4-(1-141)-VP16-FRP was constructed following the standard QuikChange Site-Directed Mutagenesis protocol from Stratagene using plasmid pGEX-GST-Gal4(1-141)VP16(78aa) (102) (Kodadek lab stock pTK0165) as the template and a pair of primers encoding FRP (5'- GGA ATT GAC GAG TAC TGC TGC CCG GGT TGC TGC TAG AAT TCA TCG TGA CTG -3' and 5'- CAG TCA CGA TGA ATT CTA GCA GCA ACC CGG GCA GCA GTA CTC GTC AAT TCC -3'). All proteins were purified from over-expressing *E. coli* BL21 (Stratagene) cells. The standard protocol for protein purification of GST-tagged proteins was employed in each case. Details are available upon request.

Antibodies and Western Blotting

26S proteasome antibodies were produced in house in rabbit. Mouse anti-GST monoclonal antibody (Santa Cruz), mouse α-Actin (Sigma), NeutrAvidin-HRP (Pierce), mouse α-His5 (Qiagen), goat α-rabbit-HRP (BioRad), and goat α-mouse-HRP (BioRad) were purchased. NeutrAvidin-HRP blots were done as previously described (4). All other antibodies were used in 4% milk/TBST using standard western blotting procedures at the following dilutions: Rabbit α-Rpt1 (1:2500), Rabbit α-Rpt3 (1:5000), Rabbit α-Rpt4 (1:5000), Rabbit α-Rpt5 (1:5000), Rabbit α-Rpt6 (1:4000), Mouse α-His5 (1:2500), Mouse α-Actin (1:1000), Goat αR-HRP (1:5000), and Goat α-R-HRP (1:5000).

Cross-linking Reactions

All cross-linking reactions were done in 1/2 Nuclear Extract Buffer (1/2 NEB) (10mM HEPES (pH 7.9), 10% Glycerol, 50mM KCl, 6.25mM $MgCl_2$, 0.1mM EDTA, 1mM DTT). 30μL reactions contained either 26S proteasome at 60nM or His6-Gal80 at 1μM with 1μM of the indicated peptide. Protein and peptide were incubated 10 minutes at room temperature until $NaIO_4$ was added at a final concentration of 5mM to start the reaction. After 1 minute, the cross-linking reaction was quenched with 6x protein loading buffer containing 100mM DTT. The samples were separated using standard SDS-PAGE. Cross-linking experiments in the presence of *E. coli* lysate were done using strain BL21. Cells pellets from 100mL cultures were resuspended in 1/2 NE buffer and Complete EDTA-free protease inhibitor tablets (Roche) and lysed by sonication. 1 μM

His6-Gal80 and 1 μM DOPA-Gal4 AD were added to 200 μg of *E. coli* lysate. Reactions were done as described above.

Competition Experiments

The competition experiments were done using Gal80 BP with either 26S proteasome or His6-Gal80. ND-Gal4 AD peptide or actin was added at the concentrations indicated and cross-linking was done as described above.

Identification of Cross-linked Product using 2D Gel Electrophoresis

Cross-linking was done as above, but a 5-fold larger volume (150μL) was employed, keeping the same final concentrations. Reactions were quenched in 100mM DTT and proteins were precipitated using 450μL of cold acetone for 2 hours at –20C. The precipitates were spun down for 10 minutes at 14, 000 rpm and supernatant removed. The pellet was resuspended in 125μL rehydration buffer (7M Urea, 2M thiourea, 4% CHAPS, 65mM DTT, and IPG buffer pH 3-10 (10μL IPG buffer:1mL rehydration buffer) (Amersham Pharmacia)) for 2 hours at room temperature. Samples were loaded on a pH3-10 IPG strip (Biorad) and separated according to pI on an IPGphor Isoelectric Focusing System (Amersham Pharmacia) according to the manufacture's instructions. After IEF, samples were equilibrated in equilibration buffer (50mM Tris (pH8.8) 6M Urea, 30% Glycerol, 2% SDS, 65mM DTT) for 15 minutes at room temperature and separated by SDS-PAGE.

Samples were analyzed by blotting as described above. After a blot the membrane was stripped with stripping buffer (62.5mM Tris (pH 7.4), 100mM DTT, 2% SDS) for 30 minutes at 55C. Membranes were checked with substrate to insure complete removal of previous antibody signal before next blot. Blot films were scanned and false colored in Adobe Photoshop. Blot pairs were aligned and merged to produce the overlay images.

Label Transfer Reactions.

Compound DBF was dissolved in a stock buffer (80 mM PIPES, pH 6.8, 2 mM $MgCl_2$, 1 mM EDTA, 5 mM β-mercaptoethanol, and 0.1 mM DTT) and stored at -80 °C. Individual GST fusion (1 μM) and compound DBF (0.1 μM) were mix in a cross-linking buffer (10mM HEPES, pH 7.8, 50 mM KCl, 10% Glycerol, 6.25 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM DTT). After 10 min at room temperature, His_6-Gal80 was added to a final concentration of 1 μM along with 50 μg of *E. coli* lysate. The total reaction volume was 30 μL. The proteins were allowed to bind for 10 min at room temperature and $NaIO_4$ was added at 5mM final concentration to start the cross-linking reaction. After 2 min the reaction was quenched with SDS loading buffer containing 100 mM DTT plus 2 mM BAL. Similar procedure was employed in label transfer reaction between 26S proteasome and GST-Gal4(1-141)-VP16-FRP except that the final 26S proteasome concentration was 100 nM.

Label Transfer Yield Measurement.

GST-FRP-G80BP (1 μM) was bound to 20 μl of prewashed Glutathione 4B Sepharose beads (Amersham) in cross-linking buffer for 30 min at 4 °C. An excess amount of DBF (≈ 1.5 μM) was added to the bead-protein complex and allowed to bind for 10 min at room temperature. Unbound DBF was washed away with the cross-linking buffer and an excess of His_6-Gal80 (≈ 1.5 μM) was added for 30 min at 4 °C to form a GST-FRP-G80BP –DBF – His_6-Gal80 complex. The unbound His_6-Gal80 was washed away and the cross-linking reaction was started with addition of 1 mM $NaIO_4$. After 2 min the proteins were eluted from the beads by addition of 10mM reduced Glutathione, 2mM BAL, and 0.1% SDS. The eluted proteins along with an excess *E. coli* lysate (50 μg proteins) were moved to a tube that contained Dynabeads M280 Streptavadin (Dynal). The proteins that were cross-linked and now contained a biotin tag were allowed to bind to the Dynabeads for 30 min. The unbound proteins were removed and saved as supernatant sample. The Dynabeads were washed once and the proteins were eluted in 5 × concentrated SDS loading buffer by boiling for 15 min.

Affinity Pull-down Assay.

The sepharose bead-bound GST-G80BP-FRP – DBF (or GST-FRP-G80BP – DBF) complex was formed as described above. The complex was incubated with His_6-Gal80 (≈ 1.6 μM) and 50 μg *E. coli* lysate for 30 min at 4 °C. After washing away unbound proteins with the cross-linking buffer, the proteins

were re-suspended in SDS loading buffer for gel analysis. The procedure was repeated without compound DBF.

RESULTS

Chemical Cross-linking of the Gal4-Activation Domain to Gal80

Standard solid phase peptide synthesis was employed to create a biotinylated, DOPA-containing Gal4 AD construct for use in the cross-linking experiments. To validate the utility of this reagent, we initially tested cross-linking of the DOPA-Gal4 AD to the Gal80 transcriptional repressor (Figure 2-2). DOPA-Gal4 AD (1 μM) was mixed with purified, recombinant His6-Gal80 protein (1 μM) and 5 mM of $NaIO_4$ was then added. After a 30 second incubation and quenching, the proteins were separated by SDS-PAGE gel and the AD-containing products were detected by probing the blot with NeutrAvidin-HRP (NA-HRP) (Figure 2-2 B). A biotinylated protein was detected that migrated more slowly that the 50 kDa standard, which corresponded to the expected DOPA-Gal4 AD-His6-Gal80 cross-linked product.

We then employed the biotinylated, DOPA-Gal80 binding peptide (DOPA-Gal80 BP) in the same experiment. As expected, DOPA-Gal80 BP also cross-linked to His6-Gal80, albeit with lower yield than the DOPA-Gal4 AD peptide. As a control, we then repeated the experiment with a DOPA peptide containing a sequence not known to bind to Gal-80, i.e DOPA-Control peptide

(Control in Figure 2-2). No cross-linking of this control peptide to His6-Gal80 was observed, demonstrating that specific binding is required for a cross-linked product to form. We conclude from these data that the DOPA-containing Gal4 AD and Gal80 BP are competent substrates for periodate-mediated cross-linking reactions.

To further probe the specificity of the DOPA-containing peptides for Gal80, we examined this reaction in the context of a crude *E.coli* extract containing thousands of proteins, none of which should interact with the Gal4 AD or the Gal80 BP specifically (Figure 2-2C). Lanes 1 and 2 of Figure 2-2C simply repeat the experiments done in Figure 2-2B, for comparative purposes. In lane 3, DOPA-Gal4 AD was present at a final concentration of 1μM in 200μg of cell extract from an *E. coli* strain expressing the His6-Gal80 protein (see lane 5 for a Commassie Blue-stained gel of this protein mixture). Upon addition of periodate, only two major biotin-containing products were detected in (Figure 2-2C, lane 3), one with the apparent molecular mass expected for the Gal4 AD/His6-Gal80 and another unknown product that was of lower apparent mass. No cross-linking was detected in the absence of periodate (Lane 4).

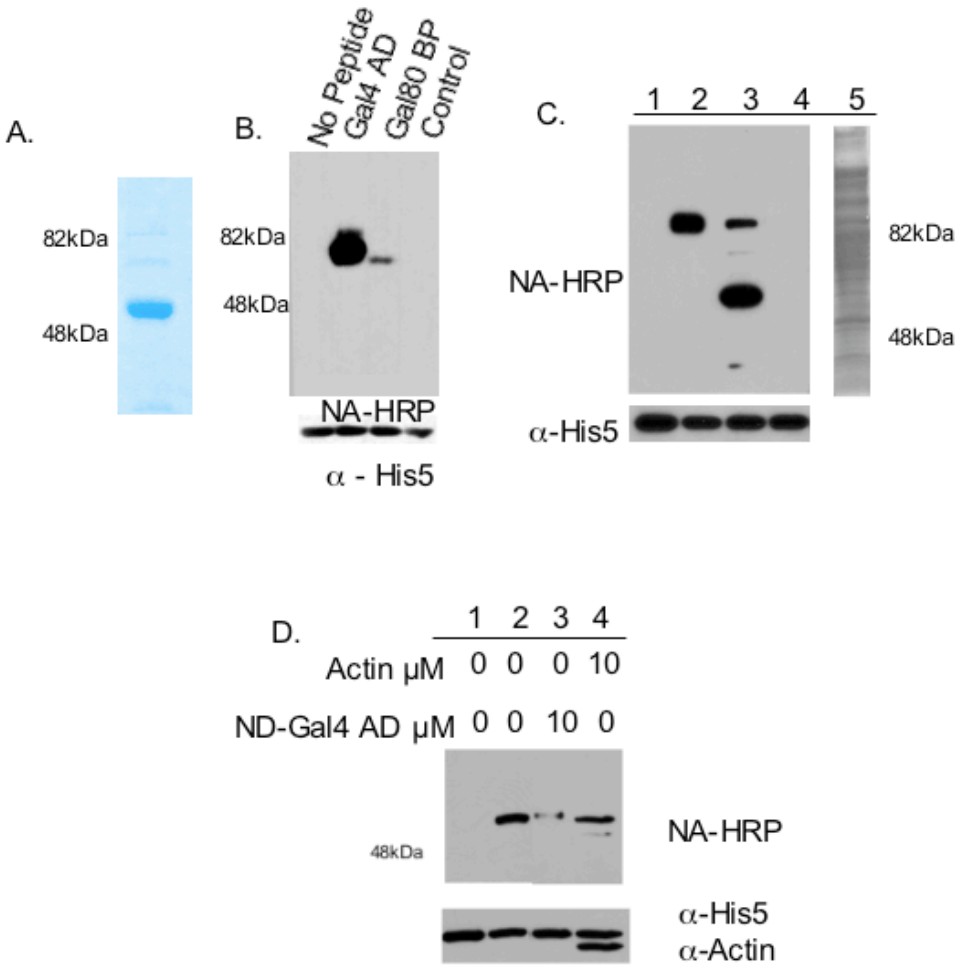

Figure 2- 2 Periodate Mediated Cross-linking of Peptides to Gal80. (A) Colloidal Blue stained SDS-PAGE of His6-Gal80. Apparent molecular weights are indicated. (B) NeutrAvidin (NA)-HRP was used to probe for the biotin tag incorporated in each peptide. The α-His5 blot shows the amount of the His6-Gal80 in each reaction. (C) Cross-linking between the DOPA-Gal4 AD peptide and His6-Gal80 protein in the presence of non-specific proteins. NA-HRP blot of a cross-linking reaction between 1 μM DOPA-Gal4 AD peptide and 1 μM His6-Gal80. The amount of the His6-Gal80 protein is indicated by the α-His5 blot of the reaction mix. (D) A NA-HRP blot shows the results of a cross-linking reaction between the DOPA-Gal80 BP and His6-Gal80 with the presence of specific competitor proteins. The amount of His6-Gal80 and actin are shown by the α-His5 and α-actin blots, respectively.

We then employed a competitive cross-linking experiment to ask if both peptides share a common binding interface on Gal80 through competition cross-linking experiments. Increasing amounts of a non-DOPA containing Gal4 AD peptide (ND-Gal4 AD) or actin were added to a reaction mix containing 1 μM of DOPA-Gal80 BP with 1 μM of His6-Gal80 protein in 1/2 NE buffer. Increasing amounts of ND-Gal4 AD decreased cross-linking between 1μM of Gal80 BP and His6-Gal80 as assayed by western blotting (Figure 2-2D Lanes 2-5). Addition of actin had no effect on the cross-linking reaction (Lane 6). The fact that the ND-Gal4 AD competed the DOPA-Gal80 BP-Gal80 cross-link, but the control protein actin did not, suggest that the native Gal4 AD and the Gal80 BP recognize overlapping surfaces of Gal80, though allosteric competition cannot be ruled out.

DOPA-Gal4 AD Cross-linking to the 26S proteasome

With the utility of these DOPA-containing peptides demonstrated from the Gal80 cross-linking studies described above, we turned to using this technique to study AD-proteasome interactions. In collaboration with the Johnston laboratory, we have shown that a sub-complex of the proteasome containing all six ATPases and probably other factors must be recruited to many activated promoters through direct interactions with transactivators (99). This study was directed towards identifying the specific AD-binding proteins in the proteasome.

1 μM of biotinylated, DOPA-containing peptide was equilibrated with 60 nM of 26S proteasome and the cross-linking reaction was triggered with periodate. Probing the blot with NA-HRP revealed one major cross-linked, Gal4 AD-containing product with an apparent mass of approximately 50kDa (Figure 2-3B). At this exposure, no Gal80 BP-containing product was apparent, but a longer exposure of the same blot (Figure 2-3C) shows that the Gal80 BP was incorporated into a cross-linked product of at approximately the same apparent mass, but in much lower yield. The control peptide did not cross-link to the 26S proteasome detectably.

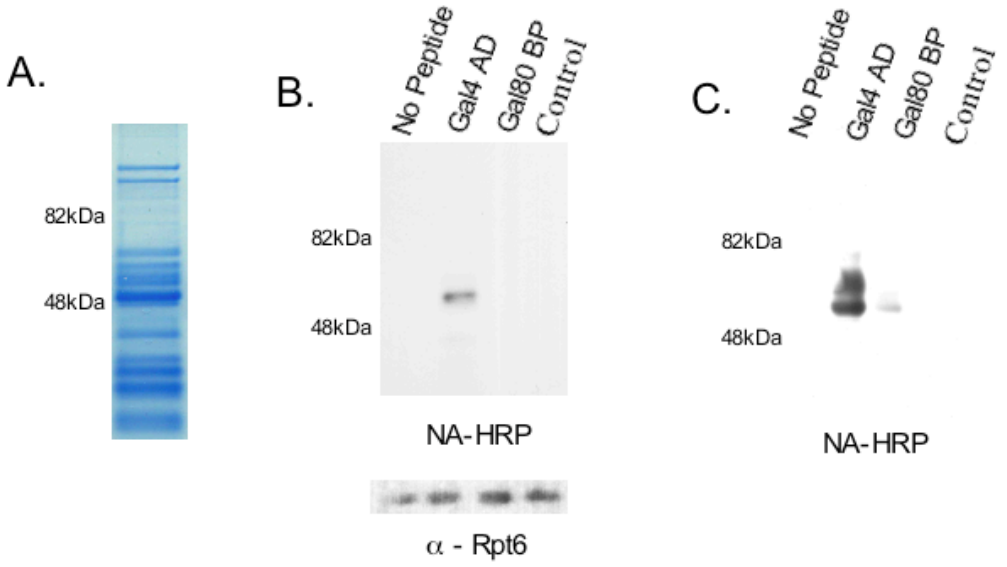

Figure 2- 3 Periodate Mediated Cross-linking of peptides to 26S proteasome. (A) Colloidal Blue stained SDS-PAGE of 26S proteasome. Apparent molecular weights are indicated. (B) NA-HRP blot of 1 μM peptides cross-linked to 60 nM 26S proteasome. The α-Rpt6 blot indicates the amount of 26S proteasome loaded. (C) A longer exposure of (B).

Identification of the Cross-linked Products

The products of both the DOPA-Gal80- and DOPA-Gal4 AD-26S proteasome cross-linking reactions had electrophoretic mobilities consistent with coupling of the activating peptides to one of the proteasomal ATPases, all of which have similar masses. We used 2D gel electrophoresis and western blotting to differentiate between these proteins and identify the major cross-linked species for both domains.

DOPA-Gal4 AD was cross-linked to the subunits of the 26S proteasome by mixing the peptide with the 26S proteasome and then performing a cross-linking reaction. The proteins were then separated by isoelectric focusing (IEF) using a pH 3-10 gradient, and separated in a second dimension by SDS-PAGE. A NA-HRP-probed blot (Figure 2-4A) showed one major cross-linked product was formed between DOPA-Gal4 AD and a 26S subunit of approximately 50kDa with a pI of 5.5. We then stripped and reprobed the blot with several antibodies against components of the 26S proteasome. The images in Figure 2-4B were false-colored blue for the cross-linked product (NA-HRP) and red for the 26S subunit. Overlaying these images (Figure 2-4B) unequivocally identified Rpt4/Sug2 as the 26S subunit that was cross-linked to the Gal4 AD peptide.

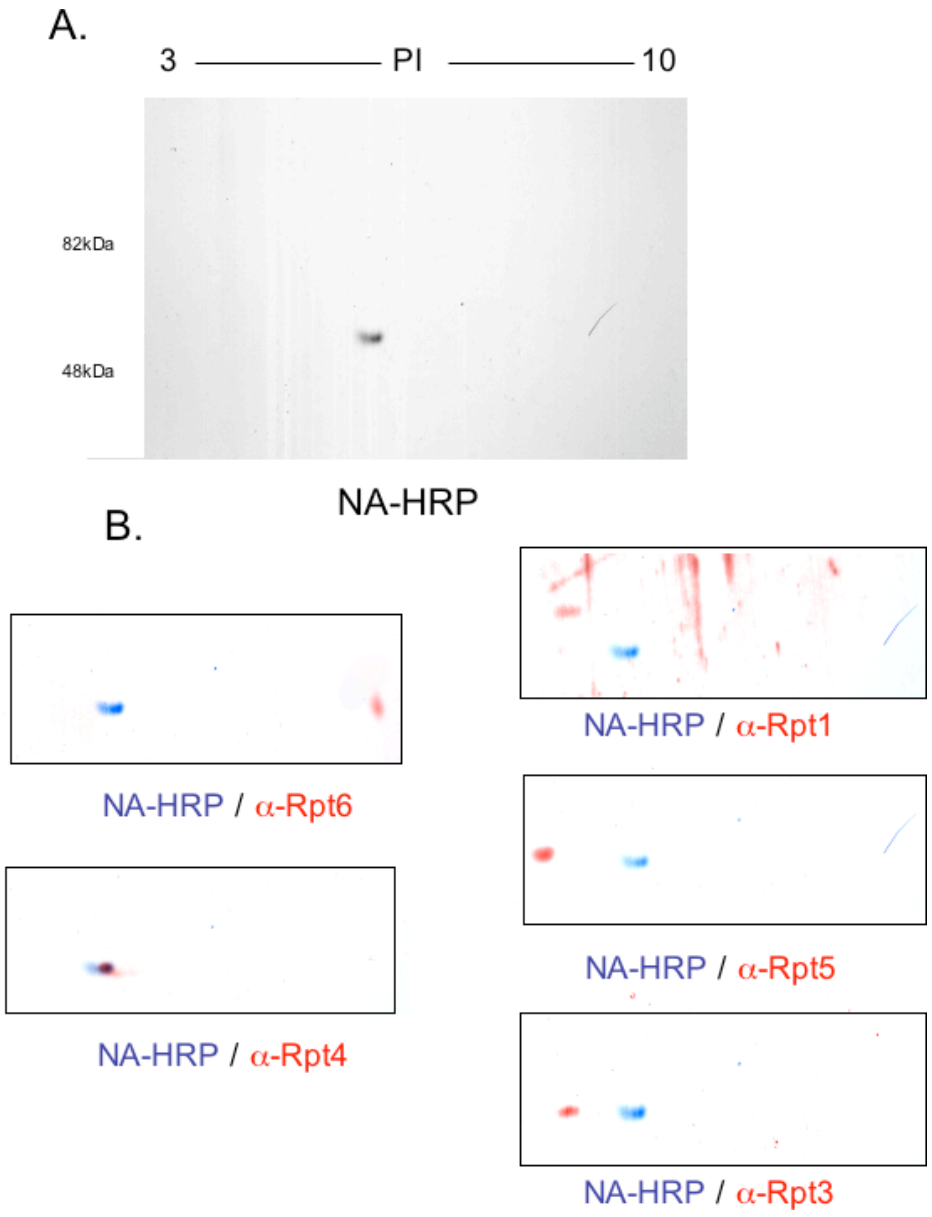

Figure 2- 4 Identification of Cross-linked Product of 26S proteasome and N-term DOPA-Gal4 AD peptide. A) Cross-linking reaction of DOPA-Gal4AD peptide and 26S proteasome was separated by 2D gel electrophoresis and blotted with NeutrAvidin-HRP. The blot shows the major cross-linked product of the reaction. (B) Western blots of cross-linked product and 26S subunits from membrane in (A) were overlayed. The NeutrAvidin-HRP blot was false colored blue and 26S subunit blots were false colored red. The 26S subunit is indicated under each overlay.

The chemical cross-linking and 2D mapping experiment was repeated to identify the cross-linked product between Gal80 BP and the proteasome. The NA-HRP blot (Figure 2-5A) showed a product with an apparent mass similar to that of the Gal4 AD-Rpt4 product, but a much more basic pI. Overlay of the cross-linked product (false-colored blue) and Western blots using antibodies against subunits of the 26S proteasome (false-colored red) (Figure 2-5B) identified Rpt6/Sug1 as the 26S subunit that forms a cross-linked product with Gal80 BP. No other proteasome subunits overlaid with the cross-linked products.

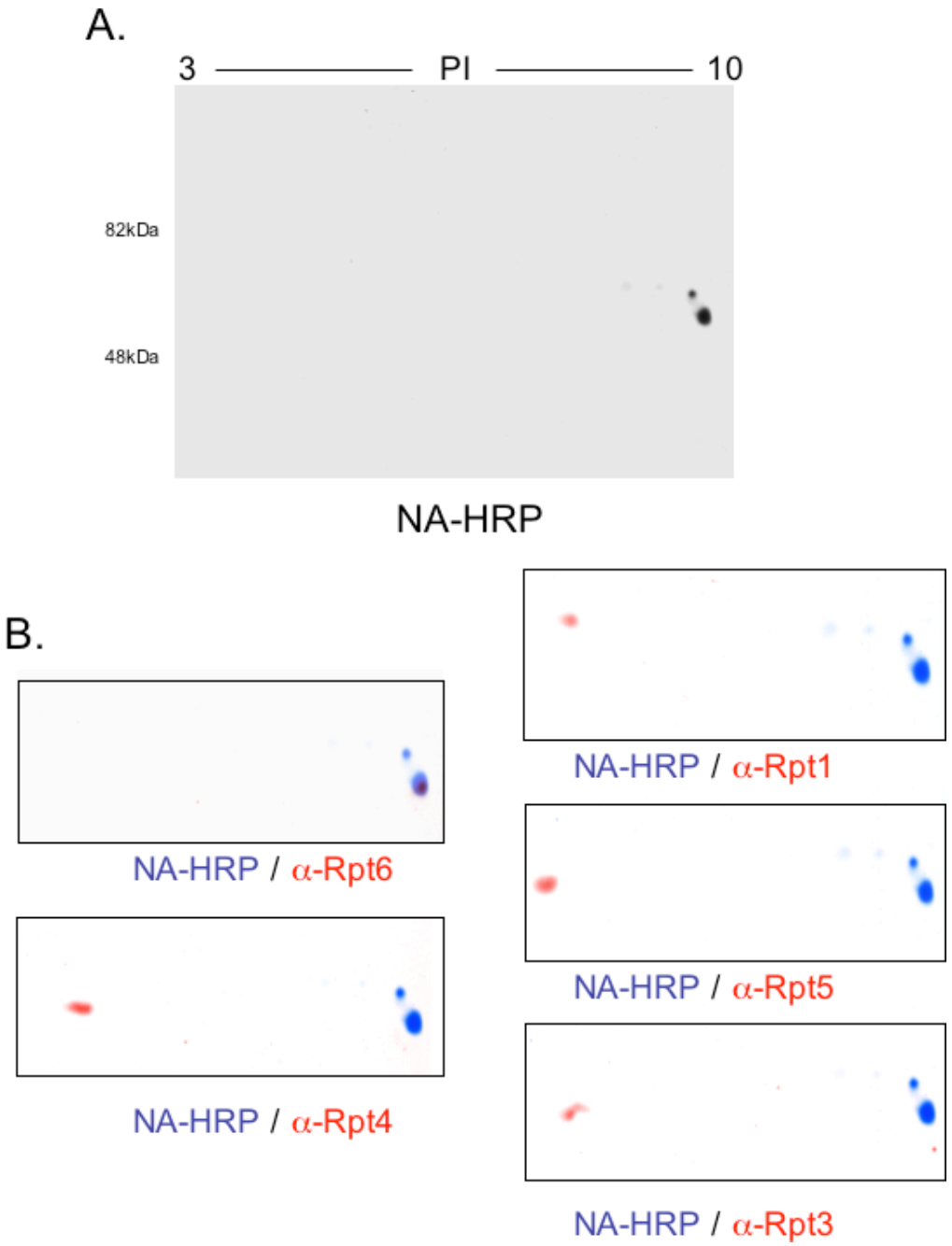

Figure 2- 5 Identification of Cross-linked Product of 26S proteasome and DOPA-Gal80 BP A) Cross-linking reaction of DOPA-Gal80 BP and 26S proteasome separated by 2D gel electrophoresis and blotted with NA-HRP. (B) Western blots of cross-linked product and 26S subunits from membrane in (A) were overlayed. The false coloring and overlaying were done as in figure 2-3B.

The finding that the DOPA-Gal4 AD and DOPA-Gal80 BP cross-link to different proteasomal ATPases was surprising since both recognize the same surface of Gal80 and therefore presumably have similar protein-binding preferences. Since there is genetic and biochemical evidence that Rpt6/Sug1 is also recognized by the Gal4 AD, we considered the possibility that both DOPA-containing peptides might contact Rpt4 and Rpt6, but that only one of these contacts resulted in a cross-linked product due to the position of the DOPA residue in the peptide. To test this model, we synthesized a Gal4 AD derivative containing the DOPA residue at the C-terminus and repeated the cross-linking experiments with the 26S proteasome. The NA-HRP blot (Figure 2-6A) now showed a product with an approximate molecular weight of 50kDa but with a much more basic pI than the cross-linked product created with the N-terminal DOPA Gal4-AD. Overlay of the cross-linked product and Western blots using antibodies against subunits of the 26S proteasome (Figure 2-6B) identified Rpt6/Sug1 as the 26S subunit that forms a product with the C-terminal DOPA Gal4-AD.

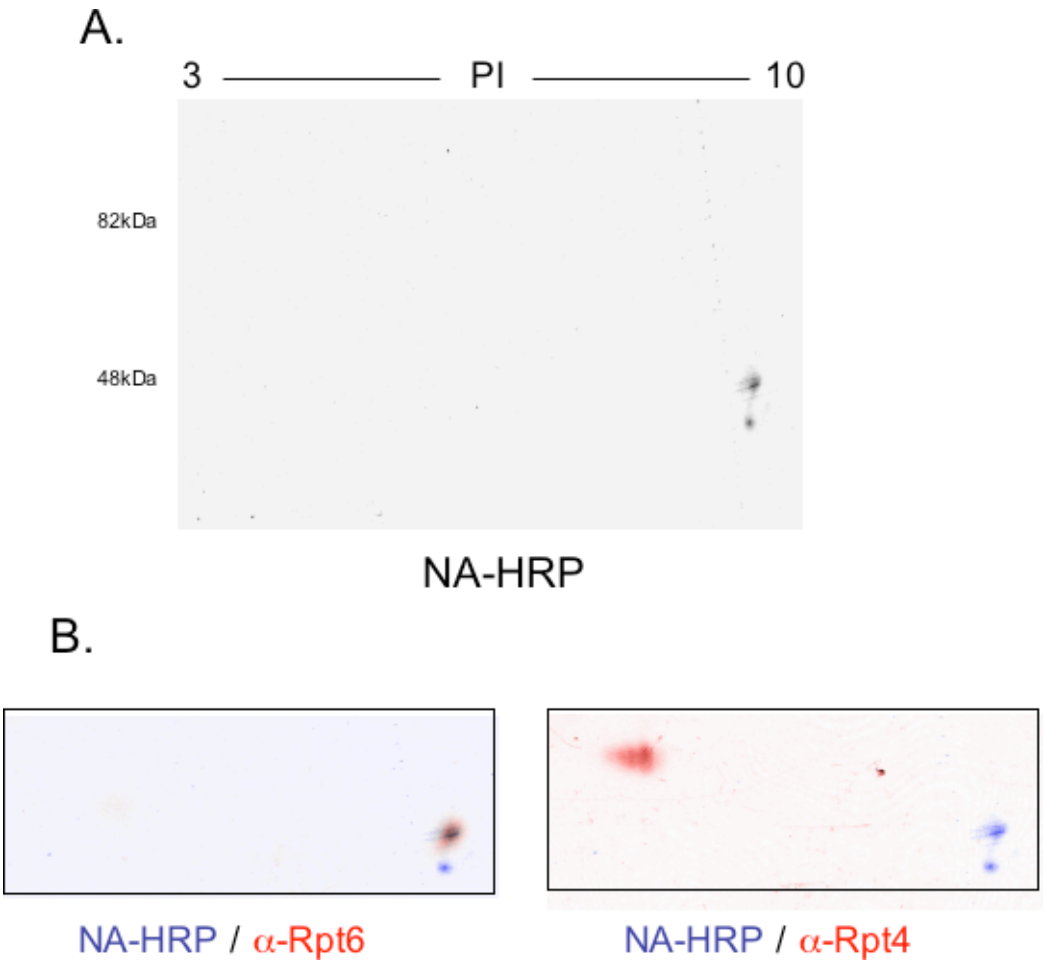

Figure 2- 6 Identification of Cross-linked Product of 26S proteasome and C-term DOPA-Gal4 AD peptide A) Cross-linking reaction of C-term DOPA-Gal4AD peptide and 26S proteasome was separated by 2D gel electrophoresis and blotted with NA-HRP. (B) Western blots of cross-linked product and 26S subunits from membrane in (A) were overlayed. The NA-HRP blot was false colored blue and 26S subunit blots were false colored red. The 26S subunit is indicated under each overlay.

To further confirm that the Gal80-BP and the Gal4-AD bind to the 26S proteasome in a similar fashion, we performed a competition experiment. A solution containing DOPA-Gal80 BP and 26S proteasome was equilibrated with

either increasing amounts of the ND-Gal4 AD or actin. The NA-HRP blot (Figure 2-7) of the products formed after periodate-triggered cross-linking showed that increasing amounts of ND-Gal4 AD peptide decreased the signal intensity of cross-linked product (Lanes 2-7). Addition of 20μM actin however, had no affect on the formation of cross-linked product (Lane 8). The α-Rpt6 and α-actin blots served as loading controls for the 26S proteasome and actin, respectively. Lane 1 shows the result of a chemical cross-linking reaction without the Gal80 BP. These results are consistent with a model in which both peptides bind Rpt4 and Rpt6, though we cannot completely rule out the alternative model where each peptide competes binding of the other by an allosteric mechanism.

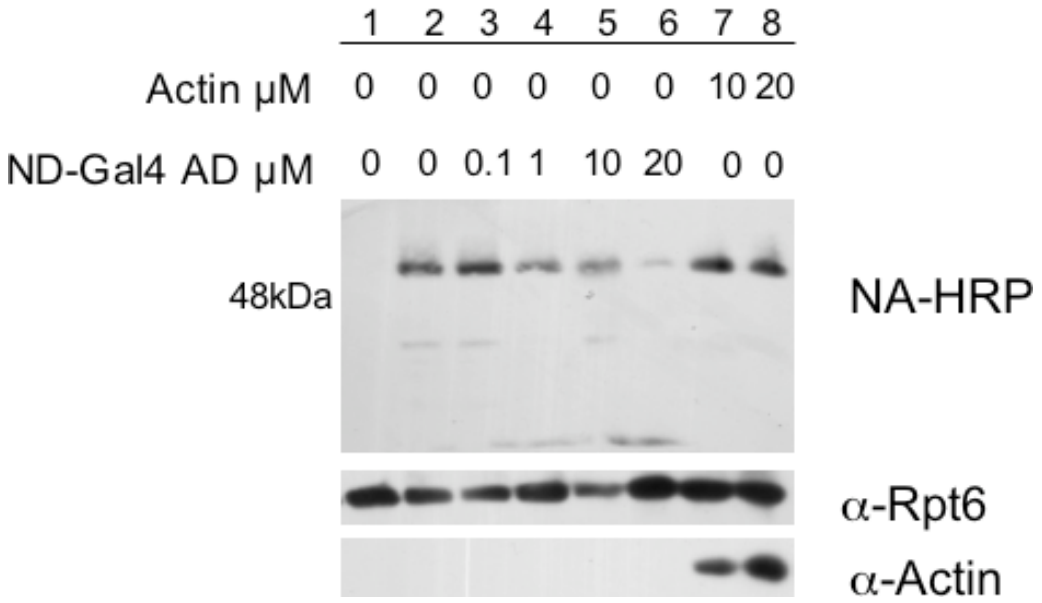

Figure 2- 7 The Gal4 AD and Gal80 BP compete for binding the proteasome

A NA-HRP blot shows the results of a cross-linking reaction between DOPA-Gal80 BP and the 26S proteasome in the presence of competitor proteins lacking a DOPA residue. Increasing amounts ND-Gal4 AD peptide reduced the amount of DOPA-Gal80 BP-Rpt6/Sug1cross-linked product (compare lane 2 and lane 6). Actin did not affect the cross-linking reaction (compare lane 2 and lane 8). α-Rpt6 and α-Actin Western blots serve as controls for the amount of 26S proteasome and actin, respectively.

The FlAsH Based System

As shown above, the incorporation of the DOPA cross-linking group was useful for identifying interacting partners of peptides. While this methodology could be used for peptides or small-molecules the approach above would not be amenable to proteins. We have adapted our delivery of the DOPA cross-linking reagent and report a new type of label transfer system that eliminates the need for covalent modification of the protein of interest (see Figure 2-8). It involves tagging the "bait" protein with a tetracystiene-containing peptide (CCPGCC, here called FRP for FlAsH Receptor Peptide) that binds with high affinity and specificity to a biarsenical derivative of fluorescein called FlAsH (122). We have synthesized a FlAsH derivative that also contains a biotin tag and the DOPA cross-linking residue (DBF). This reagent allows for non-covalent attachment of the DOPA cross-linking group to a protein of interest for use in identifying interacting partners. After cross-linking, the FlAsH-FRP complex can be dissociated by boiling in an excess of dithiol, resulting in transfer of the biotin label to nearby partner proteins.

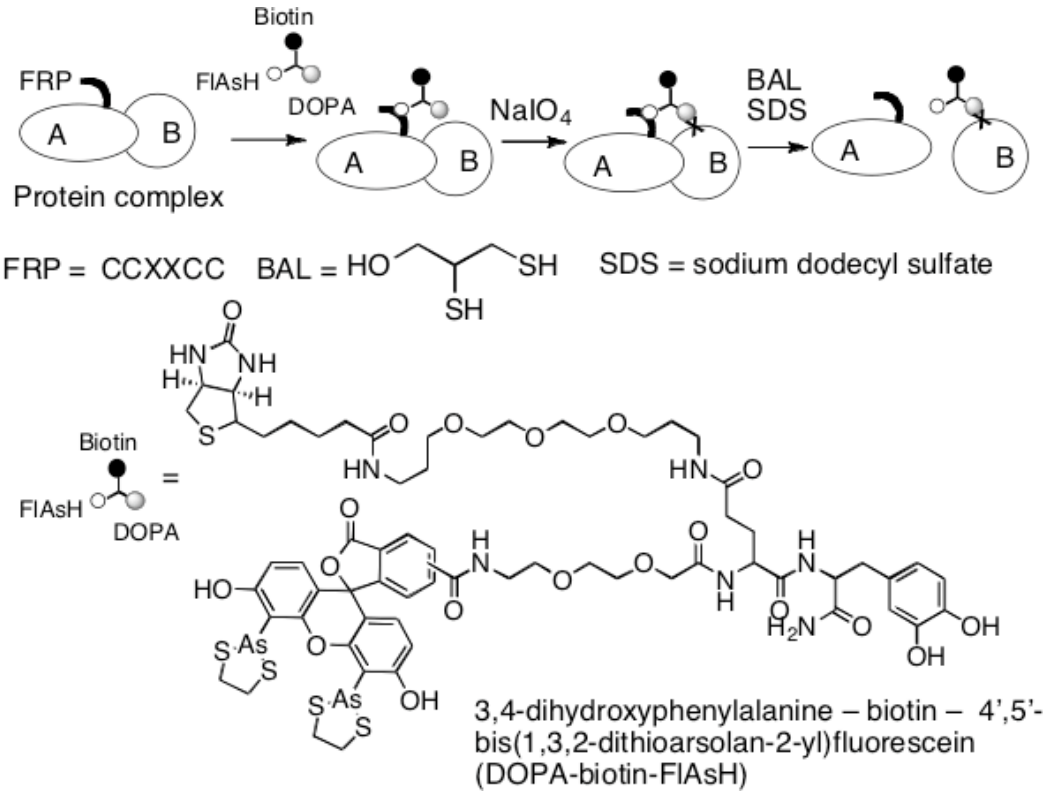

Figure 2- 8 The biotin-DOPA-FlAsH Reagent and Cross-linking Scheme. The reagents and scheme used for cross-linking proteins to interacting partners.

Interactions Between Known Binding Partners

We first assessed the efficiency of this new label transfer reaction in a relatively simple system, by examining the interaction of a Glutathione-*S*-Transferase-Gal80-binding-peptide (GST-G80BP) fusion protein to the His_6-Gal80 protein. The 20-residue G80BP peptide (123), selected by phage display, binds His_6-Gal80 with a K_D of ≈ 300 nM, a value typical of many moderate affinity protein-protein interactions. An FRP site was inserted either between GST and G80BP or at the C-terminal end of the protein and the resultant constructs

(GST-FRP-G80BP and GST-G80BP-FRP) were expressed and purified. Two control GST fusions lacking either the G80BP (GST-FRP) or the FRP (GST-G80BP) were also prepared. Each GST fusion (1 μM) was incubated with compound the DOPA-biotin-FlAsH conjugate (DBF; see Figure 2-8) (0.1 μM) and His_6-Gal80 protein (1 μM) in an excess *E. coli* lysate such that the GST fusion and Gal80 proteins represented less than 4% of the total protein mass. Sodium periodate (5 mM) was then added to trigger the cross-linking reaction. After two minutes, the reaction was quenched with buffer containing 100 mM dithiolthreitol (DTT) and 2 mM British anti-Lewisite (BAL; 2,3-dimercaptopropanol). The samples were boiled, subjected to SDS-PAGE and the gels were blotted with NeutrAvidin-HRP conjugate to detect biotinylated proteins.

Strong bands representing biotinylated proteins with a molecular mass corresponding to the GST fusion protein was observed in all cases where the fusion included the FRP tag (lanes 3, 5, 6, 7, 9 and 10, Figure 2-9, top panel). This represents self-labeling through reaction of the FRP-tethered DBA with a nucleophilic residue in the fusion protein. When the GST protein lacked the FRP tag, little or no self-labeling was observed (lane 4 and 8, top panel, Figure 2-9).

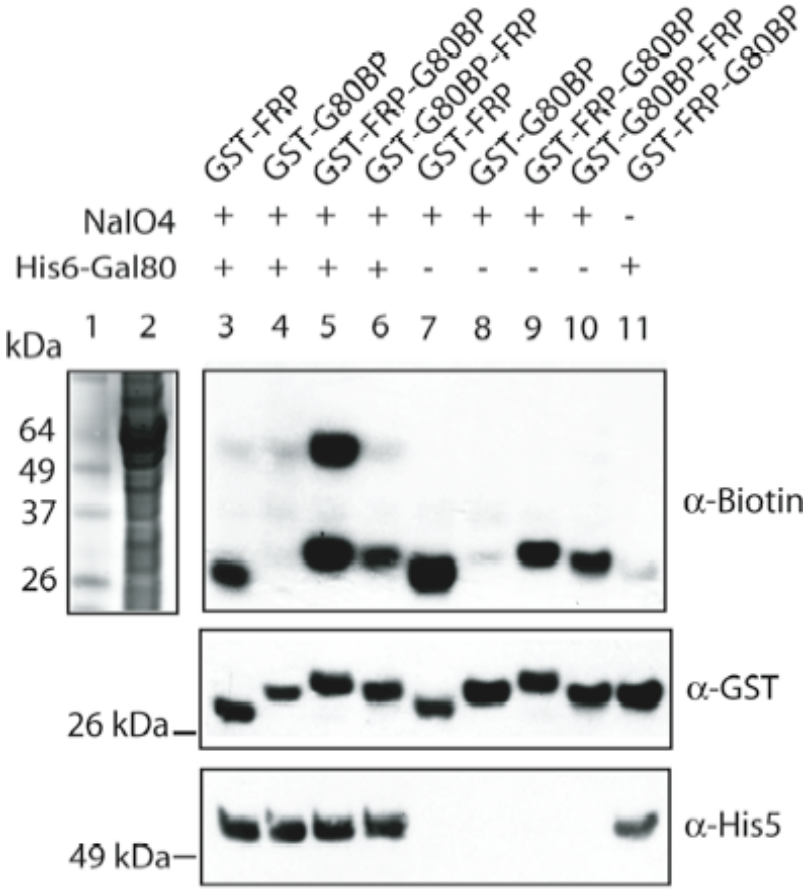

Figure 2- 9 DBF transfer from GST-FRP-G80BP to His_6-Gal80. Lane 1: Protein standards. Lane 2: *E. coli* lysate containing reaction mixture stained by Coomassie blue. Lane 3-6: Reaction mixtures with His_6-Gal80. Lane 7-10: Reaction mixtures without His_6-Gal80. Lane11: Same as lane 3 but without $NaIO_4$ induction.

In the solution containing both GST-FRP-G80BP and His_6-Gal80, addition of periodate resulted in the production of a distinct band with an apparent mass of ≈ 60 kDa (lane 5, top panel, Figure 2-9). This band represents biotinylated His_6-Gal80, as demonstrated by blotting with an anti-His5 antibody (bottom panel, Figure 2-8). This band was absent when His_6-Gal80 (lane 9, top panel, Figure 2-

9) or $NaIO_4$ (lane 11, top panel) was excluded from the reaction. Also, note that in all of the reactions, although the GST fusion protein and Gal80 constitute less than 4% of the total protein present, there is no evidence for non-specific biotinylation of the bacterial proteins. These results are consistent with specific label transfer of the DBF compound from GST-FRP-G80BP to His6-Gal80 upon periodate oxidation and boiling in dithiol.

The cross-linking yield of the reaction was also estimated. The GST-FRP-G80BP was immobilized on agarose beads and then loaded with the DBF reagent. This protein was saturated with an excess of His_t-Gal80 to force full complex formation. Unbound His_6-Gal80 was washed away and the cross-linking was triggered and quenched. Proteins were eluted from the beads by addition of 10 mM reduced glutathione, 2 mM BAL, and 0.1% SDS. The mixture was then incubated with Streptavidin beads along with an excess *E. coli* lysate (50 μg proteins) that was used to eliminate nonspecific protein binding on beads. The His_6-Gal80 protein on the Streptavidin beads represented the label transfer product that was tagged by DBF and the His_6-Gal80 protein remained in the supernatant of Streptavidin beads enrichment represented the un-reacted GST-FRP-Gal80BP – bound His_6-Gal80. By measuring the amount of His_6-Gal80 retained on the beads we estimated the yield of the label transfer reaction as at least 85% (see Figure 2-10), far higher than is normally observed for similar reactions using photo-activated label transfer agents (18).

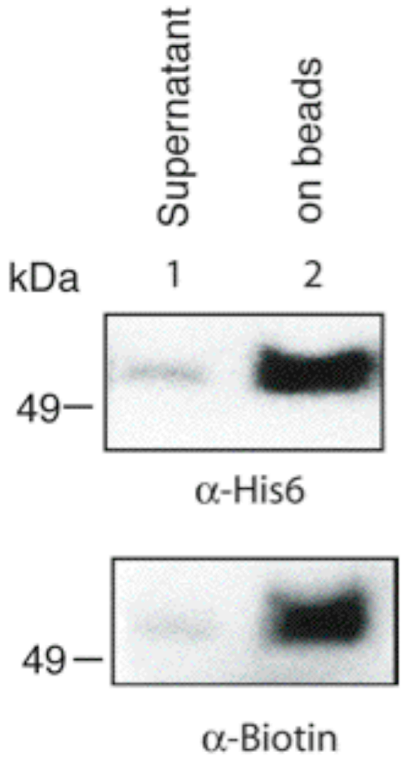

Figure 2- 10 Yield of DBF-mediated label transfer from GST-FRP-G80BP to His_6-Gal80. A GST-FRP-G80BP – DBF – Gal80 complex was formed by incubating glutathione beads in 1 μM GST-FRP-G80BP, 1.5 μM DBF, and 1.5 μM His_6-Gal80. Lane 1: Western blotting of the total supernatant of the Streptavidin beads enrichment. Lane 2: Western blotting of proteins eluted from the Streptavidin beads.

Unexpectedly, when GST-G80BP-FRP was incubated with His_6-Gal80 and periodate, little or no label transfer to His_6-Gal80 was detected (lane 6, top panel, Figure 2-9). Glutathione Sepharose-bound GST-G80BP-FRP or GST-FRP-G80BP fusion was incubated in *E. coli* lysate containing His_6-Gal80 protein (1.6

μM, comprised about 5 % of the total protein mass). The column was washed thoroughly, and the bound proteins were then eluted with SDS protein loading buffer. These "pull-down" assays confirmed that both GST-FRP-G80BP and GST-G80BP-FRP fusions bound to His_6-Gal80 with similar affinities and that DBF binding to the two GST fusions did not compromise the protein-protein interactions (Figure 2-11). Thus, the lack of product is a result of low cross-linking yield. Given the requirement for templating of the DOPA-derived ortho-quinone intermediate and a reactive nucleophile(116,117), we speculate that placing the FRP tag on the C-terminal side positions the bound DBF reagent beyond the reach of an appropriately nucleophilic Gal80 residue.

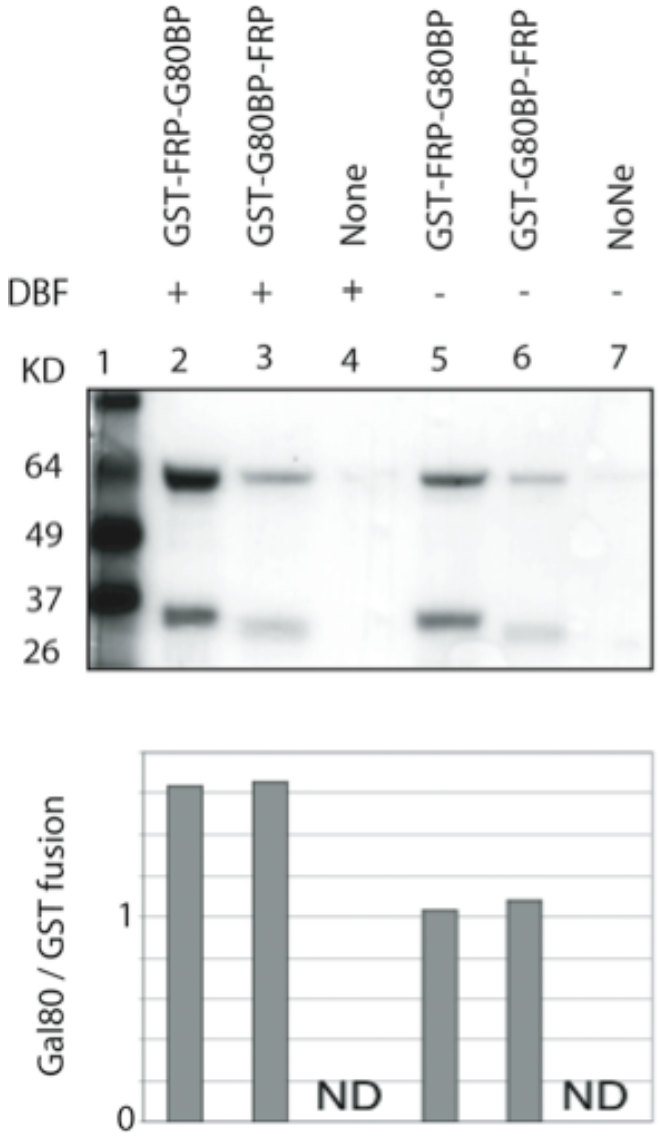

Figure 2- 11 His_6-Gal80 protein pull-down assay with glutathione bead – bound GST-FRP-G80BP and GST-G80BP-FRP. Lane 1: Protein standards. Lane 2-3: Coomassie Blue staining after SDS-PAGE of reaction mixture with compound DBF and GST fusions. Lane 4: Coomassie Blue staining after SDS-PAGE of reaction mixture with compound DBF but without GST fusions. Lane 5-7: Same as lane 2-4 but without compound DBF.

FlAsH for use with Multi-Protein Complexes.

We next attempted to apply this method to a more complex system. Acidic activation domains from transactivators bind directly to proteins in the 19S regulatory particle of the 26S proteasome (54,98,99) and that this interaction is important for efficient transcription of many eukaryotic genes(73). Above, we demonstrated that DOPA-mediated chemical cross-linking was a useful method for identifying the proteasomal subunits that interact with a peptide that corresponds to the Gal4 AD. As a test of the FlAsH based system we set out to determine the direct binding partners of the potent viral VP16 AD (125). The fusion protein GST-Gal4-(1-147)-VP16-FRP was incubated with DBF, 26S proteasome and $NaIO_4$, followed by boiling in dithiol to effect label transfer. SDS-PAGE followed by blotting with NeutrAvidin-HRP revealed that two proteins, in addition to the FRP-tagged activator, were labeled by DBF (labeled as **a** and **b**, left panel, Figure 2-12). These two bands were absent when 26S proteasome or periodate was omitted (lanes 3 and 4, left panel, Figure 2-12). Western blotting using anti-Sug1/Rpt6 antibody confirmed that band **a** was Sug1/Rpt6 (right top panel, Figure 2-12), one of the six AAA-ATPases located in the 19S regulatory particle of the proteasome (72). This protein has previously been shown to be a target of several acidic activators (98). This result argues that the VP16 AD-Sug1/Rpt6 interaction is indeed physiologically relevant and likely

to be a general target for many acidic activators. Based on the mobility of band **b**, it likely represented Rpn11(114,126). The important point is that incubation of an FRP-tagged protein with a very large macromolecular complex selectively labels only two proteins, arguing that these are nearest neighbors to the C-terminus of the VP16AD.

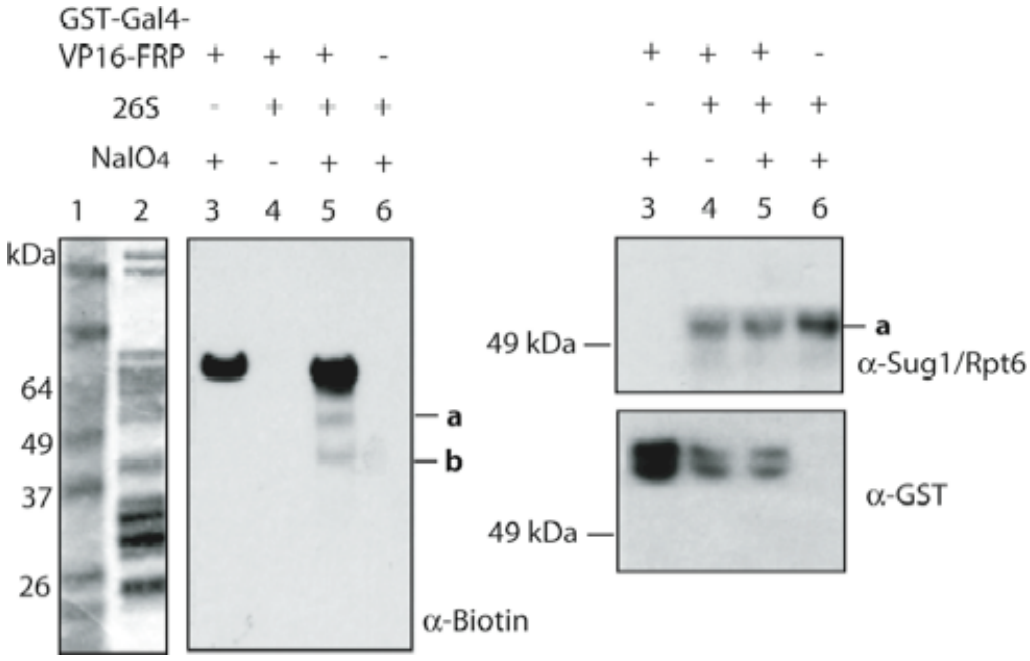

Figure 2- 12 DBF transfer from GST-Gal4(1-147)-VP16-FRP to 26S proteasome. Lane 1: Protein standards. Lane 2: 26S proteasome stained by Coomassie blue. Lane 3-6: Label transfer reactions probed by Western blotting. Blots with antibodies raised against biotin, Sug1/Rpt6, and GST are indicated.

DISCUSSION

We recently developed chemistry by which molecules containing a catechol (ortho-dihydroxybenzene) unit could be cross-linked efficiently to their protein receptor with little or no coupling to non-receptor proteins, even in complex solutions. The catechol is oxidized to a reactive ortho-quinone by sodium periodate under conditions where native protein residues are unaffected, allowing selective coupling of the catechol-containing molecule to the protein receptor, often in high yield. The catechol unit is easily incorporated in to peptides and small protein domains by chemical synthesis using DOPA as a tyrosine surrogate. DOPA has also been incorporated into bacterially expressed proteins using the appropriate orthogonal tRNA-aminoacyl/tRNA synthetase pair (127). In this study, we apply this methodology to understanding the molecular interactions between acidic transcriptional activation domains and the proteasome. Genetic and biochemical data have indicated an interaction between AD and the Rpt4 and Rpt 6 proteins. However, these interactions had not been demonstrated in the context of the native multi-protein complex.

The 34 residue native Gal4 AD, with a C-terminal biotin tag and an N-terminal DOPA substituent (see methods), was made by solid-phase synthesis. For comparative purposes, we also created a biotinylated, DOPA-containing derivative of a 20 residue Gal80-biding peptide. This peptide has been shown

previously to act as an activation domain when expressed in yeast and mammalian cells when fused to the Gal4 DNA-binding domain. As shown in Figure 2-2, both DOPA-containing peptides cross-link specifically to Gal80 when treated briefly with periodate, demonstrating their effectiveness as probes for activator-binding proteins.

Periodate-mediated cross-linking of the DOPA-containing peptides to immunopurified 26S proteasome resulted in highly specific coupling Rpt4/Sug2 and Rpt6/Sug1. Which were the previously implicated targets of the Gal4 AD based on other methods. Rpt6/Sug1 has been implicated as a target of many activators and the periodate-mediated cross-linking of the Gal80 BP to this protein identifies this Rpt6 as a target of this "artificial AD" as well.

We then attempted to adapt the DOPA chemistry such that it could be used in the context of polypeptides too large to synthesize by normal methods. To do this, the previously described interaction of the FlAsH molecule with tetra-cysteiene motifs was used. FlAsH was modified to include both the DOPA cross-linking group as well as a biotin tag. Using two different systems, we were able to demonstrate that this cross-linking and label transfer strategy would be useful for identification of the interaction partner of a target protein. While it unlikely the DBF reagent will be of utility in cells as a cross-linking reagent, we feel that the specificity of and ease of loading the target protein make this molecule useful for characterization of interactions in complicated mixtures and lysates.

In summary, we have used periodate-triggered cross-linking of DOPA-containing peptides that correspond to acidic transactivation domains to identify their direct binding partners in the proteasome. Additionally, this work demonstrates the utility of a novel label transfer system that does not require covalent modification of the protein of interest and that employs an efficient oxidative cross-linking reaction. We anticipate that these techniques will be of utility in the characterization of protein-protein interactions in the context large, multi-protein assemblies and characterization of protein-protein interactions in their native environments.

CHAPTER THREE

ACTIVATION DOMAIN-DEPENDENT MONO-UBIQUITYLATION OF GAL4 PROTEIN IS ESSENTIAL FOR PROMOTER BINDING *IN VIVO*

SUMMARY

The *Saccharomyces cerevisiae* Gal4 protein is a paradigmatic transcriptional activator containing a C-terminal acidic activation domain (AD) of 34 amino acids. A mutation that results in the truncation of about two-thirds of the Gal4AD (*gal4D*), results in a crippled protein with only 3% the activity of the wild-type activator. We show here while the Gal4D protein is not intrinsically deficient in DNA binding, it is nonetheless unable to stably occupy *GAL* promoters *in vivo*. This is shown to be due to the activity of the proteasomal ATPases, including Sug1/Rpt6, which bind to Gal4D via the remainder of the AD and strip it off of DNA. A mutation that suppresses the Gal4D "no growth on galactose" phenotype represses the stripping activity of the ATPase complex, but not other activities. We further demonstrate that Gal4D is hypersensitive to this stripping activity due to its failure to be mono-ubiquitylated efficiently *in vivo* and *in vitro*. Evidence is presented that the piece of the AD that is deleted in Gal4D protein is likely a recognition element for the E3 ubiquitin ligase that modifies

Gal4. These data argue that acidic ADs are comprised of at least two small peptide sub-domains, one of which is responsible for activator mono-ubiquitylation and another that interacts with the proteasomal ATPases, coactivators and other transcription factors. This study validates the physiological importance of Gal4 mono-ubiquitylation and clarifies that its major role is to protect the activator from being destabilized by the proteasomal ATPases.

Agnes Delahodde and Fernando Gonzalez originally worked on trying to explain the suppression of *gal4D* by *sug1-1* and *sug2-1*. Dr. Delahodde was the first to demonstrate that *sug1-1* restored occupancy of Gal4D on the *GAL1* promoter *in vivo* under inducing conditions. The result in Figure 3-1 is my recreation of her experiment. The pull-down experiment in Figure 3-6D was performed by Dr. Gonzalez.

INTRODUCTION

The best-studied intersection of the UPS and nucleic acid metabolism is RNA polymerase II transcription. The first hint of such a link was the finding that specific alleles of *SUG1* and *SUG2 (sug1-1* and *sug2-1)*, genes that encode two of the proteasomal ATPases (Rpt 4 and Rpt6), could rescue the activity of a *S. cerevisiae* Gal4 transactivator derivative lacking about two-thirds of the C-terminal activation domain (Gal4D) (47,70). Subsequent experiments demonstrated that this effect could not be explained by altered proteolysis of Gal4D (97). The activation domains (ADs) of Gal4 bind directly to Sug1/Rpt6 and Sug2/Rpt4 and extract from the proteasome a complex that we coined APIS, which includes the six ATPases, Rpn1, Rpn2 and perhaps other associated proteins, but not the 20S core proteasome or the "lid" sub-unit of the 19S RP (98,99).

There are now several known functions of the proteasomal ATPases, highlighted in Chapter 1, but none explained the ability of the *sug1-1* and *sug2-1* alleles to suppress the "no growth on galactose" phenotype of *gal4D*. These mutations were clearly recessive (70), which is difficult to reconcile with the requirement of Sug1 protein activity for elongation. Instead, it seems more likely that the *sug1-1* and *sug2-1* mutations eliminated some repressive activity of the wild type complex to which Gal4D was hypersensitive.

We recently reported a new activity of the proteasomal ATPases that provides a possible rationalization for these genetic data (107). When a transactivator-DNA complex is exposed to the RP, it is rapidly and reversibly dissociated. Direct interactions between the ATPases and the AD of the transactivator are essential since RP does not affect the stability of a protein-DNA complex lacking an AD. These new data, however, provided a conundrum. How do wild-type activators resist this potent stripping activity of the RP and remain associated with the promoter to drive high-level transcription? A detailed study of the effect of the RP on GST-Gal4-VP16-promoter complexes suggested that the answer lies in the post-translational modification of the Gal4 DNA-binding domain (DBD). When exposed to a HeLa nuclear extract, all of the DNA-bound activator is mono-ubiquitylated (107) and this form of the protein is insensitive to the destabilization activity of the RP.

In this chapter, we address the physiological relevance of ATPase-mediated destabilization of activator-DNA complexes and the role of mono-ubiquitylation in blocking this activity to understand the *gal4D* phenotype and its suppression by *sug1-1* and *sug2-1*. First, we monitor the ability of the Gal4D protein to occupy Gal4-dependent genes *in vivo* and the ability of the *sug1-1* mutation to modulate occupancy. Moreover, we show that a Gal4D derivative is not ubiquitylated efficiently *in vitro* and evidence is presented that this is the case *in vivo* as well. We show the *sug1-1* mutation cripples the destabilization activity

of the proteasome, but not other functions of the ATPase, and largely restores the ability of the Gal4D protein to occupy DNA *in vivo* even though it is not mono-ubiquitylated. The extensive correlation between the genetic and biochemical data argue strongly that the molecular basis of the *gal4D* phenotype is that truncation of the AD cripples activator mono-ubiquitylation and that this, in turn, renders the Gal4D•promoter complexes hypersensitive to disruption by the proteasomal ATPases. Finally, we demonstrate that the piece of the Gal4 AD that is lacking in Gal4D can compete activator mono-ubiquitylation *in trans*, arguing that it is probably a docking site for the E3 ubiquitin ligase complex that targets Gal4. These data, in addition to further validating the physiological relevance of Gal4 mono-ubiquitylation, also reveal a previously unsuspected sub-domain structure of the Gal4 acidic AD, comprised of a short peptide (12 residues) that is capable of acting as a classical AD and a 22 amino acid piece that is essential for activator mono-ubiquitylation.

MATERIALS AND METHODS

Yeast strains, plasmids and proteins.

Pre1-FLAG tagged 26S and 19S have been described (124). A set of congenic strains derived from W303 (MAT a *leu2-3 112, his3-115, trp1-1, can1-100, ade2-1, ura3-1*) were used in this study. For generation of *SUG* wild type

and mutant proteasome containing a T7 tagged *SUG* the following strains were used: Sc507 (T7-*SUG1*) and Sc654 (W303 T7-*sug1-1*), (described in (97)). For chromatin immunoprecipitation (ChIP) experiments, yeast strains Sc726 (*SUG1 gal4::HIS3*), Sc728 (*sug1-1 gal4::HIS3*), Sc732 (*sug1-20 gal4:HIS3*), Sc736 (*sug2-1 gal4:HIS3*), and Sc738 (*sug2-13 gal4:HIS3*) as previously described (97) were used. Yeast strains used for ChIP experiments were transformed with single copy plasmids (derived from pSB32) expressing either wild type *Gal4* (pSJR263) or *gal4D* (pSJR261). In each case the encoded proteins were tagged at their N-termini with three tandem copies of the T7 epitope tag (Novagen). Genetic fusion of ubiquitin to S10 tagged Gal4D in the pSB32 vector was done by removing Ub from GST-Ub-Gap71-VP16 (107) using a NcoI digest and inserting Ub into the NcoI site at the start codon of the T7 tag. For all these constructs, the *GAL4* gene was expressed from its own promoter. Pull downs to detect ubiquitylated Gal4 were done in Sc726 using a pSB32 plasmid with a His9 tag. Alpha-Galactosidase assays were done using Sc244 (strain 21) (a *gal4-2, Gal80, ura3-52, leu2-3 112, ade1, MEL1*) transformed with the pSB32 plasmids mentioned above. The transformed strains were grown in complete medium lacking leucine, to select for the plasmids, with raffinose as the carbon source. The use of raffinose ensured that there was no selection for suppressors of *gal4D*. Induction of the *GAL* genes was performed by adding galactose directly to the medium (2% final concentration).

26S and 19S proteasome were purified using a FLAG affinity tag as described (124) with modifications (102). The *sug1-1* 26S proteasome and congenic wild-type 26S proteasome were purified as in the FLAG affinity tag case except using T7 agarose (Novagen) and T7 peptide, followed by a Mono-Q (Amersham) column using a 20mM – 1M NaCl gradient over 30 column volumes, which eluted proteasome at ~ 450mM NaCl. GST-Gal4(1-147)-VP16, GST-Gal4(1-147), GST-Gal4 AD, GST-Gal4D AD, GST-UBL, His6-SUMO, His6-Gal80, and His6-Ub were purified from *E. coli* BL21 cells as described (102). The mini-Cla Gal4 and Gal4D constructs have been described previously (128). Antibodies against PentaHis (Qiagen), Ubiquitin (Dako Cytomation), GST (Santa Cruz Biotech.), NeutrAvidin-HRP (Pierce), and HRP conjugated secondary antibodies (Biorad) were used according to instructions. The α-Rpt6, α-Rpt4, α-Rpt1, α-Gal4 N-terminus, α-Gal6, and α-20S antibodies were produced in rabbit. α-Rpn1 and α-Rpn2 were produced in mouse.

Destabilization of Activator-DNA complexes by the proteasome

The destabilization assay has been previously described (107). In these assays the following changes were made. The activator-DNA complex was added at a 60 nM final concentration of activator-DNA complex to the reaction mix containing 25 nM 26S proteasome, 3 mM ATP, and 1 μM non-biotinylated DNA containing Gal4 binding sites in TR reaction buffer (10mM HEPES (ph 7.8) 50mM KCL, 6.25mM $MgCl_2$, 0.1mM EDTA, 1mM DTT, 2% Glucose (v/v)).

Ubiquitylation reaction

The mono-ubiquitylation of GST-Gal4-VP16 has been described (107). The protocol here was similar, but the stated mini-Cla versions were used in place of GST-Gal4-VP16. The non-ubiquitylated controls were done exactly as stated, but 3 U of hexokinase were used and the ATP was omitted. At the end of the reaction, 6M Urea was added to denature everything and TALON (Clontech) affinity resin was used to pull down the proteins covalently linked to His6-ubiquitin.

Chromatin Immunoprecipitation (ChIP) assay

ChIP assays were performed according to the protocol described (99). Cells were treated grown in raffinose containing medium, with galactose added to 2% to induce the *GAL* genes. Induction was carried out for 30 minutes prior to addition of formaldehyde. Immunoprecipitations were carried out using anti-Gal4 N-terminal antibody as previously described in Gonzalez *et al*. or the anti-ubiquitin antibody.

For gel-based analysis of ChIPs, 5 μl of the final product was used in a standard PCR reaction with the indicated primer. Reactions were run on a 1% agarose gel, scanned, and analyzed by densitometry to produce the graphs in figure 1. Quantitative PCR of precipitated chromatin was performed using an iCycler Thermal Cycler and the IQ SYBR Green Supermix, (2x mix containing 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 U/ml iTaq DNA

polymerase, 6 mM MgCl2, SYBR Green I, 20 nM fluorescein, stabilizers (Biorad, Hercules, CA)). Relative enrichment of specific DNA was calculated by comparing products derived from primers against the *GAL7* promoter from the precipitated samples with the specific antibody and an unspecific control antibody. Data were graphed as a percent of the total DNA from each chromatin sample.

***GAL1-10* Promoter ChIP**	
UAS F	GTGGAAATGTAAAGAGCCCC
UAS R	CTTTATTGTTCGGAGCAGT
TATA F	CAACCATAGGATGATAATGCG
TATA R	CTTCTTCTGAATGAGATTTAGTC
***GAL7* Promoter ChIP**	
GAL7 F	TGCTCTGCATAATAATGCCC
GAL7 R	TTGCTTTGCCTCTCCTTTTG

Table 3- 1 Primers used in ChIP analysis

RT-PCR

Total RNA was isolated from 10 mL of cells OD_{600} 0.6-0.8 after addition of galactose. Cells were centrifuged 5 minutes at 3000 g in a Sorvall RT7 centrifuge with a RTH-750 swing bucket rotor. Cells were washed with PBS and centrifuged as before. Cell pellets were frozen in liquid nitrogen and stored at -80 C. Cell pellets were resuspended in 400 μL water and 400 μL water – saturated phenol was added and vortexed 1 min. The mixture was incubated at 65°C for 45 minutes. The aqueous layer was removed and extracted with water – saturated

phenol followed by chloroform. RNA was treated with RQ1 DNase (Promega corp., Madison, WI) for 1 hour. RNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform. The RNA was precipitated by adding 40 μL 3M NaOAc pH 5.3 and 1 mL 95% EtOH. RNA quantity was measured by measuring OD_{260}. 1 μg of Total RNA was used to make cDNA using the Stratascript first strand cDNA synthesis kit (Stratagene, La Jolla, CA) and oligo dT. CDNA was measured by quantitative PCR as above using *GAL1* and *ACTI* primers. The ratio of *GAL1*/*ACT1* from three samples was averaged and graphed.

RNA Analysis	
GAL1 F	CTCTGTTTGCGGTGAGGAAG
GAL1 R	ACCTTTATTCGTGCTCGATCC
ACT1 F	CCTACGTTGGTGATGAAGCT
ACT 1 R	GTCAGTCAAATCTCTACCGG

Table 3- 2 Primers used for RNA analysis

Alpha-Galactosidase Assays

Alpha-Galactosidase assays have been described (129). Briefly, Sc244 with the pSB32 vectors containing Gal4 and Gal4 mutants were grown in synthetic complete lacking leucine with raffinose as the carbon source. At an OD 600nm of 0.6 galactose was added to 2% final. After a 45 minute induction, the cells were pelleted and lysed by bead disruption. 50 μg of total protein was used for each assay and the nM min^{-1} μg^{-1} of p-Nitrophenyl produced was determined.

The mean and standard error of the mean was graphed with the activity from Gal4 set as 100%.

Pull-downs to detect ubiquitylated Gal4

Pull-downs to detect ubiquitylated Gal4 from yeast were done in Sc726 transformed with an empty pSB32 vector or one containing a His9 tagged *Gal4* behind the native promoter. 4L of cells were grown in synthetic complete lacking Leu with 2% glucose to OD600 of 0.6 or in 2% raffinose to OD600 of 0.6 and then induced with 2% galactose for 1h. Cells were collected by centrifugation (4000 rpm, 10 min, 4C), flash frozen, and stored at –80C. Pellets were resuspended in 50mM Tris (7.6), 6M Urea, 0.1% Triton X-100, with 20μM MG132 (Sigma), Complete Protease Inhibitor tab (Roche), 5μM Ubiquitin aldehyde (Boston Biochem) and disrupted with bead-beating. The soluble fraction was collected by centrifugation (17K rpm, 20 min. 4C) and bound to TALON resin (Clontech) for 2h at 4C with rotation. Beads were collected, washed briefly, and bound proteins were eluted with SDS loading buffer for analysis by western blot.

GST Fusion Protein Retention Assays.

5 μg GST, GST-Gal4 AD (GST-AD), or GST-Gal4D AD (GST-Gal4D), while on agarose beads, was incubated with the yeast whole cell extracts in MTB (50mM HEPES, pH 7.5 100mM potassium acetate, 5mM magnesium acetate, 1mM EGTA, 0.1 mM DTT 10% glycerol, 0.1% NP40) that also contained 10mg

ml^{-1} soluble E. coli lysate as a non-specific competitor. Glutathione beads and labeled proteins were incubated at 4°C with gentle agitation for 1 hour. Following incubation, the beads were centrifuged (13k rpm, 1 minute), and washed 2 times in 1.5 ml MTB. After the final wash, the entire supernatant was carefully removed and 20µl SDS loading buffer was added to the beads. The beads were then boiled for 5 minutes and loaded onto a 10% SDS-PAGE gel. The gels were transferred to PVDF and membranes were then used for measurement of retained protein by western blot analysis. Inputs represent 10% of total input.

RESULTS

The *gal4D* Mutation Results in a Defect in Promoter Occupancy *in vivo*

The recent finding that the proteasomal ATPases could antagonize transcription factor-DNA binding led us to employ chromatin immunoprecipitation (ChIP) to test the ability of Gal4D to occupy *GAL* promoters *in vivo*. Yeast cells deleted for *GAL4* were transformed with single copy vectors expressing Gal4 or Gal4D from the native *GAL4* promoter. In galactose medium, Gal4 was resident on the UAS region of the *GAL1/10* promoter, but not on the *GAL1* core promoter, as expected (Figure 3-1A and B). However, the Gal4D protein was not able to occupy its binding site under the same conditions in the wild-type *SUG1* background. The failure of Gal4D to occupy its binding site is not a defect in the intrinsic DNA binding activity of the protein. *In vitro*,

constructs containing the DNA binding domain and activation domain of Gal4D have been shown previously to exhibit an affinity for *GAL* promoter-containing DNAs similar to that of the wild-type Gal4 (52).

If the inability of Gal4D to occupy *GAL* promoters under inducing conditions is indeed the molecular basis of the no growth on galactose phenotype, then the prediction is that the suppressing *sug* alleles, but not non-suppressing alleles, should reconstitute Gal4D-promoter interactions. This was tested by ChIP in a *sug1-1* or *sug1-20* background (Figure 3-1B). Compared to the wild-type *SUG1* background, the levels of Gal4D bound to the promoter were higher in the *sug1-1* strain (50% of Gal4 levels), while the binding of wild-type Gal4 to the *GAL1/10* promoter was similar in both strains. In contrast, the non-suppressing mutation *sug1-20* did not restore the ability of Gal4D to bind to the promoter (Figure 3-1B). A similar result was obtained when the experiment was repeated in the *sug2-1* (suppressing) and *sug2-13* (non-suppressing) backgrounds (data not shown). We conclude that the molecular basis of Gal4D dysfunction is likely due to an antagonistic effect of the proteasomal ATPase complex on Gal4D-promoter binding to which wild-type Gal4 is immune, or at least far less sensitive.

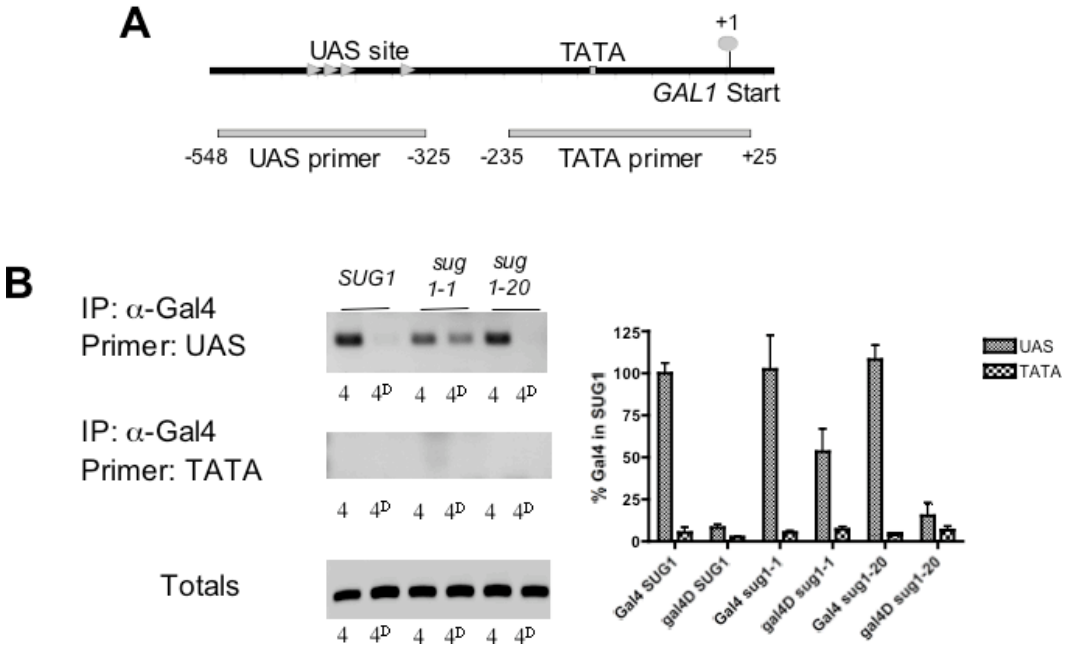

Figure 3- 1 Effect of *sug1-1* mutation of Gal4D promoter occupancy *in vivo*
A. Schematic of the *GAL1* promoter region. The Gal4 binding site (UAS), TATA box (TATA) and start site are indicated. The regions amplified by the two different primers sets are indicated in grey below the gene. B. A chromatin immunoprecipitation performed against mid-log cells after a one hour induction with galactose. An antibody raised against the N-terminus was used to precipitate Gal4 or Gal4D in the strain background indicated above the figure. PCR with the UAS primer shows DNA precipitated from the Gal4 binding site and the TATA primer shows DNA from the start site. Totals represent a PCR with the UAS primer from chromatin isolated but not precipitated. The graph at right presents the average and SEM of densitometry analysis of three experiments with the level of Gal4 in SUG1 set as 100%.

Gal4D is Ubiquitylated Inefficiently

We next examined the extent to which Gal4D is mono-ubiquitylated, since previous *in vitro* and *in vivo* experiments have indicated that this modification is essential for Gal4-promoter complexes to resist being dissociated by the

proteasomal ATPases (107). Moreover, mono-ubiquitylation of Gal4-VP16 *in vitro* is dependent on the presence of an activation domain. Since Gal4D lacks about the two-thirds of the native Gal4 AD (Figure 3-2B), inefficient ubiquitylation of Gal4D seemed a reasonable model to explain its promoter-binding properties *in vivo*.

Due to the difficulty of purifying and conducting biochemical experiments with native, full-length Gal4 we turned to the "mini-Cla" version of Gal4 and Gal4D (Fig 3-2) (128). This protein contains the native DBD and AD of Gal4, but lacks a central segment encoded by DNA flanked by ClaI sites. In *S. cerevisiae* the mini-Cla Gal4 is a potent activator that responds to the same repressive and activating signals as full-length Gal4 and thus serves as an excellent substitute for the native activator in biochemical experiments.

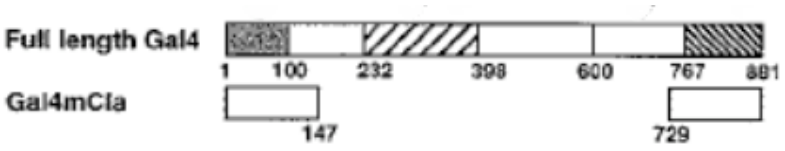

Figure 3- 2 Schematic of mini-Cla Gal4
See text for details.

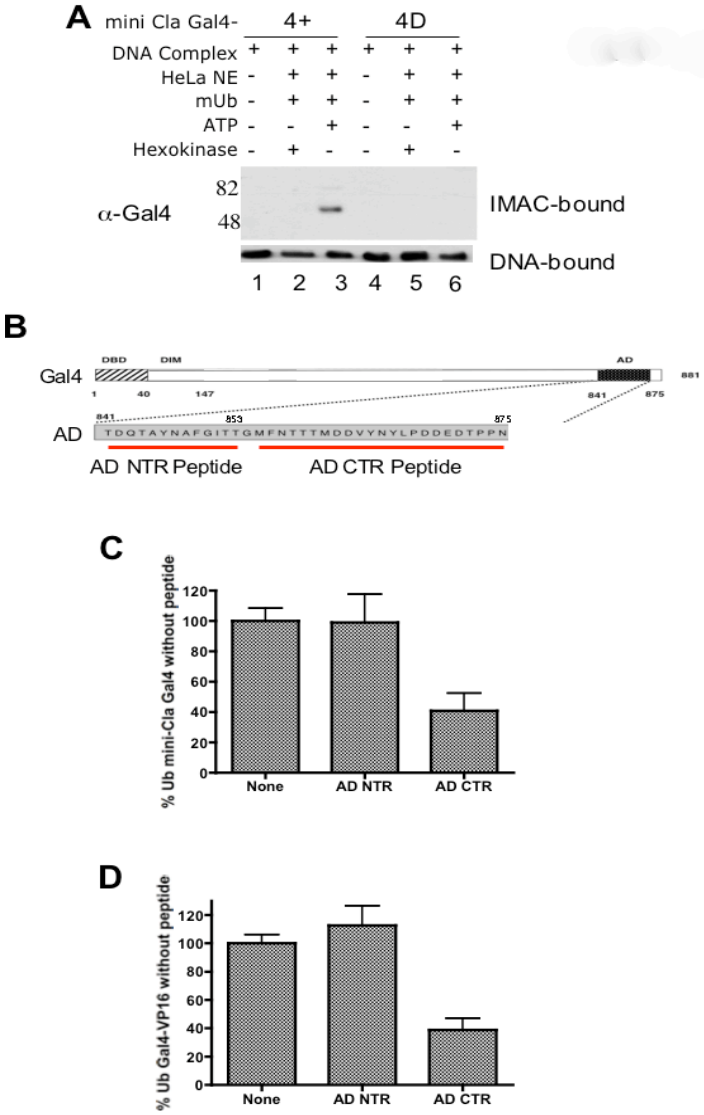

Figure 3- 3 Gal4D mono-ubiquitylation. A. Mini-Cla versions of Gal4 (lane 1 –3) and Gal4D (lane 4 – 6) were tested in a ubiquitylation reaction in a solution supplemented with His6-tagged Ub. Ubiquitylated proteins were pulled down by IMAC under denaturing conditions. B. Schematic of the Gal4AD. A close up view of the region that contains the AD (AA 841-881) is shown. The sequences of the peptides made are indicated under the sequence. C. Ubiquitylation experiment as in A, but with addition of the indicated peptide at 10 μM. Graph of the average and SEM with the amount of ubiquitylated product recovered with no peptide added set at 100%. D. As in C, but GST-Gal4-VP16 is used in place of mini-Cla Gal4.

We employed a previously reported assay to monitor the mono-ubiquitylation of mini-Cla Gal4 and mini-Cla Gal4D *in vitro* (107). The activators were first bound to immobilized *GAL* promoter-containing DNA. After exposure to HeLa nuclear extract (HNE), ATP and His6-Ub (or not), 6M urea was added and the solution was subjected to immobilized metal affinity chromatography (IMAC) to isolate proteins appended covalently to His6-Ub. As shown in Figure 3-3A, lane 3, an anti-Gal4 reactive band was observed in the IMAC-retained fraction when mini-Cla Gal4 was subjected to these conditions. This band was also reactive with anti-ubiquitin antibody (data not shown) demonstrating the ubiquitylation of the mini-Cla Gal4. Depletion of ATP with hexokinase prevented the modification of the activator (lane 2), as expected. When the same experiment was repeated with mini-Cla Gal4D, no anti-Gal4 reactive bands were detected in the IMAC-associated fraction (lane 6). The lack of recovered mini-Cla Gal4D was not due to the inability of the protein to bind to the UAS region in the template DNA. Mini-Cla Gal4D was able to bind DNA as well as mini-Cla Gal4 prior to the addition of the HeLa NE (Figure 3-3A, lower panel). This provides further evidence that Gal4D does not have an intrinsic defect in its ability to bind DNA. We conclude that mini-Cla Gal4D is not mono-ubiquitylated efficiently *in vitro*.

Identification of a Sub-domain of the AD Required for Mono-ubiquitylation

This previous result argues that the C-terminal two-thirds of the native Gal4 AD is essential for activator mono-ubiquitylation. One model is that these residues interact with the (as yet unknown) E3 ligase complex that targets Gal4. To test this idea directly, peptides corresponding to the N-terminal 12 residues of the Gal4 AD that remain intact in Gal4D (residues 841-853, called the AD N-terminal region (NTR) peptide) or the C-terminal piece of the AD missing from Gal4D (residues 855-875, called the AD C-terminal region (CTR) peptide) were synthesized to use in an ubiquitylation assay (Figure 3-3B). If the region of the AD missing from *gal4D* (AD CTR peptide) contacts the ubiquitylation machinery, addition of an excess of the peptide would inhibit ubiquitylation of the activator by competing the AD of the protein for the ubiquitylation machinery. Addition of the AD NTR peptide to an ubiquitylation reaction at 10 μM (a ~30-fold excess over DNA bound activator) had no effect on the amount of ubiquitylated mini-Cla Gal4 recovered (Figure 3-3C). In contrast, addition of the AD CTR peptide reduced the amount of ubiquitylated mini-Cla Gal4 recovered by 60% (Figure 3-3C). The AD CTR peptide, but not the AD NTR peptide, also reduced the amount of ubiquitylated Gal4-VP16 protein recovered (Figure 3-3D) arguing that the Gal4 AD and the VP16 AD must have similar properties relative to stimulating activator ubiquitylation.

Ubiquitylation of Gal4 *in vivo*

Considerable efforts directed towards the direct biochemical observation of mono-ubiquitylated Gal4 in yeast cells were inconclusive. For example, we attempted to probe the native ubiquitin state of Gal4 using a Δ*gal4* strain that has been transformed with the empty pSB32 vector (Vector) or a vector expressing the His9-tagged Gal4 under the control of the *GAL4* promoter. The His9 tag allows for lysis of the cells and recovery of the protein by IMAC under denaturing conditions to limit the activity of proteases and deubiquitylases. Cells were grown to mid-exponential phase in either glucose or raffinose then induced with galactose for one hour prior to lysis under denaturing conditions in the presence of inhibitors of the proteasome, proteases, and deubiquitylation enzymes. As shown in Figure 3-4, western blot analysis of the IMAC-retained fraction showed a band at the expected apparent molecular weight that was reactive anti-Gal4 antibodies (lane 4). Blots with anti-ubiquitin antibodies suggest that the same band contains ubiquitin. This band was not detected when the cells were grown in glucose (lane 3) or in the cells grown in galactose not expressing Gal4 (lane 2) suggesting one major ubiquitylated form of Gal4 under inducing conditions. However, the quality of these blots is not good enough to demonstrate unequivocally that most or all of the Gal4 is mono-ubiquitylated. We note that, to the best of our knowledge, there are no reported examples of mono-ubiquitylated

transcription factors isolated from a yeast whole cell extract, perhaps attesting to technical difficulties with this experiment.

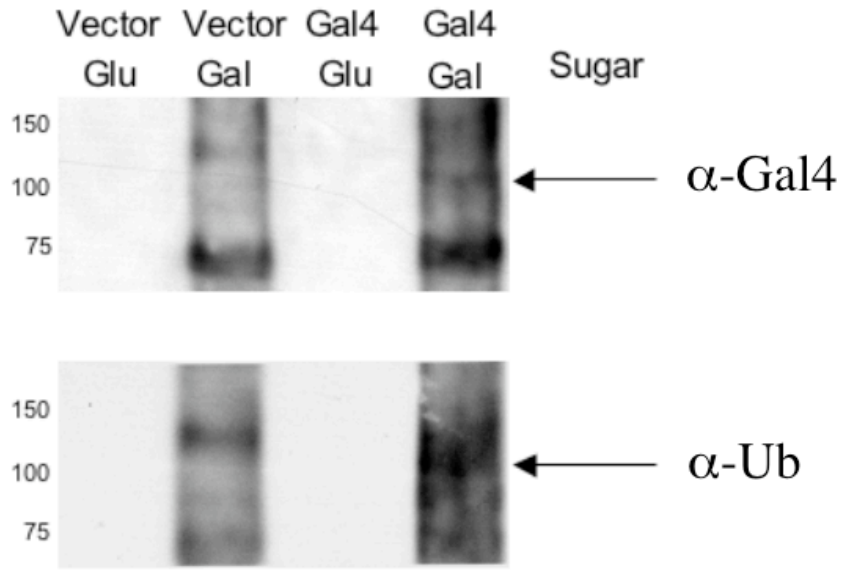

Figure 3- 4 Evidence that Gal4 is ubiquitylated *in vivo*. A strain deleted for *Gal4* was transformed with an empty single copy plasmid or one expressing His9-tagged Gal4 from its own promoter. His9-tagged Gal4 was isolated in denaturing conditions from yeast grown in glucose (lane 3) or galactose (lane 4) using an IMAC pull-down from the lysate. The strain expressing Gal4 grown in galactose (lane 5) shows an anti-Gal4 and anti-Ub reactive band that is not detectable when grown in glucose (lane 3) or in the strain transformed with the empty plasmid (lane 1 and 2).

Therefore, to assess the physiological relevance of Gal4 mono-ubiquitylation we employed ChIP assays to determine whether promoter-bound Gal4 is ubiquitylated in vivo. Cells were grown to mid-log phase in raffinose and

then induced with galactose for one hour. ChIP assays were performed with antibodies directed against Gal4 or against ubiquitin and the amount of DNA recovered from the *GAL7* UAS_G was quantified by quantitative PCR (qPCR). Similar to the results shown in Figure 3-1B, Gal4 was able to occupy the promoter in the *SUG1*, *sug1-1*, and *sug1-20* strains (Figure 3-5A). In the *SUG1* or non-suppressing (*sug1-20*) strains, Gal4D did not occupy the promoter. In the *sug1-1* background, Gal4D occupancy was increased to ~75% the level observed for Gal4 in the same background. The partial, but not complete, recovery of occupancy of Gal4D promoter binding in the *sug1-1* strain correlates with the partial recovery of activity of *GAL* gene expression in this background seen previously (70).

The presence of ubiquitin on the *GAL7 UAS* region was probed using the same chromatin samples, but substituting an anti-ubiquitin antibody for the anti-Gal4 antibody and again quantifying the results by qPCR (Figure 3-5B). An ubiquitin-dependent signal was seen when Gal4 was bound to the promoter in all cases. Of course, no Gal4- or ubiquitin-dependent signal was observed in *SUG1* or *sug1-20* strains that express Gal4D protein, as expected. These data are consistent with the idea that Gal4 is mono-ubiquitylated but cannot exclude the possibility that one or more different proteins bound to the *GAL7* UAS region are ubiquitylated in a Gal4-dependent fashion. However, the ChIP data obtained in the *sug1-1* strain expressing Gal4D shed considerable light on this issue.

Whereas Gal4D binding to the UAS is reconstituted in the *sug1-1* background, no ubiquitin-dependent signal was observed from the same chromatin sample. Taken together, these experiments argue strongly that although Gal4D is resident on the promoter in the *sug1-1* strain, it is not ubiquitylated. This, in turn, strongly suggests that the ChIP signal observed in cells containing wild-type Gal4 is due to ubiquitylated Gal4 itself. If the ubiquitin-dependent ChIP signal were due to an ubiquitylated histone or some other transcription factor, then one would expect to also observe this in the *gal4D/sug1-1* strain, since *GAL* transcription is quite active.

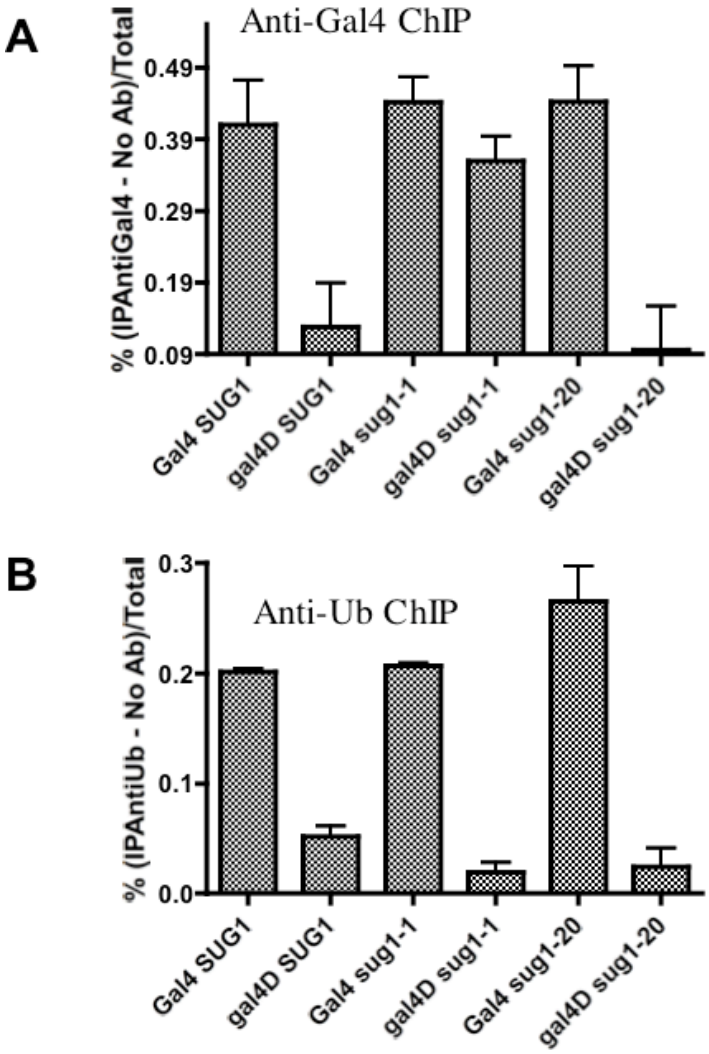

Figure 3- 5 Gal4 ubiquitylation *in vivo.* A. A ChIP assay was performed on cells activated with galactose and the amount of DNA precipitated from the UAS_G region of *Gal7* was quantitated by qPCR. Gal4 or Gal4D was expressed from the native Gal4 promoter from a single copy plasmid. The graph shows the average amount of DNA precipitated by the anti-Gal4 antibody after subtracting out the no antibody background as a percent of the total chromatin sample from 3 experiments. B. Experiment performed as in A, but the anti-ubiquitin antibody was used in place of anti-Gal4 antibody.

Gal4D function can be Rescued by Genetic Fusion of Ubiquitin

The biochemical experiments shown in Figure 3-3A and the *in vivo* results in Figure 3-5 argue that the reason Gal4D is deficient in promoter occupancy is that the activator is not mono-ubiquitylated efficiently. To test this model further, we asked if genetic fusion of a mono-ubiquitin to the N-terminus of Gal4D would rescue its activity. Genetic fusion of mono-ubiquitin restores partial activity of other proteins in yeast that are not efficiently mono-ubiquitylated due to either the absence of the cognate E3 ligase or mutations in the activator (93,107). As seen above, Gal4D cannot occupy the UAS_G in a *SUG1* strain (Figure 3-1B and 3-5A). In contrast, ChIP analysis revealed that expression of a fusion protein in which ubiquitin is fused to the N-terminus of Gal4D resulted in occupancy of the promoter at 50% of the wild-type Gal4 level, as determined by qPCR (Figure 3-6A). Consistent with the recovery of promoter occupancy, the Ub-Gal4D fusion protein drove *GAL1* transcription to ≈ 40% of the level observed in cells containing wild-type Gal4 (Figure 3-6B). Fusion of mono-ubiquitin to Gal4D also increased α-galactosidase activity, the product of the *MEL1* gene (Figure 3-6C, compare columns 3 and 4). Thus, as anticipated by the biochemical model, the defect in Gal4D can be partially overcome by fusion of mono-ubiquitin to Gal4D, arguing that natural mono-ubiquitylation is important for Gal4 function.

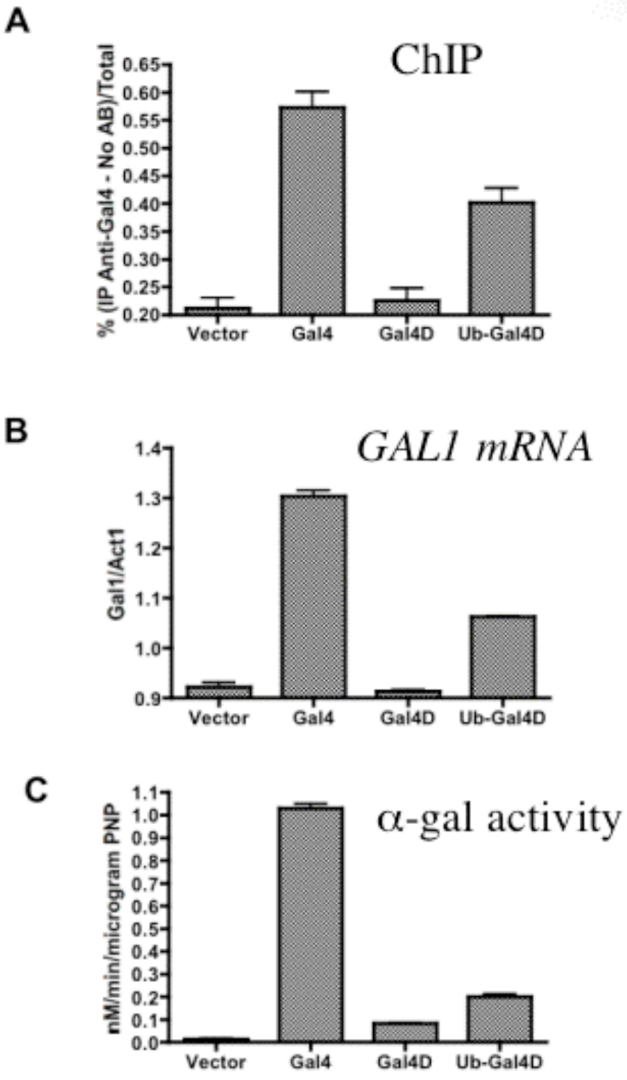

Figure 3- 6 Fusion of mono-ubiquitin to Gal4D. A. A single copy plasmid expressing *gal4D* from its own promoter had ubiquitin inserted N-terminally in-frame. ChIPs under activating conditions with the anti-Gal4 antibody were performed as in figure 3-1A and quantitated by qPCR. B. The amount of *GAL1* mRNA produced by the indicated activator was measured by qPCR. RNA was isolated from activated cells and reversed transcribed into cDNA. The graph is the average of the replicates of the ratio of *GAL1*/*ACT1*. C. An α-galactosidase activity assay. The average nM of PNP produced from alpha-galactose-PNP per minute per microgram of cell lysate for three experiments is shown.

Decreased "stripping" Activity of Sug1-1-containing Proteasomes

The previous experiments demonstrate the importance of mono-ubiquitin to Gal4 function and support the model that Gal4D cannot occupy promoters because of a defect in ubiquitylation that makes it hypersensitive to destabilization. We hypothesized that the *sug1-1* mutation suppresses this defect because this mutation decreases the destabilization activity of the proteasomal ATPases. In this model, the reduced destabilization activity allows Gal4D to occupy promoters even without the protective effect of mono-ubiquitylation.

To test this directly, 26S proteasome was purified from either wild-type cells or cells that contained the *sug1-1* mutation and the destabilization activity was measured. We previously reported a simple *in vitro* assay to monitor the ability of the proteasomal ATPases to destabilize activator-DNA complexes (107), which is shown schematically in Figure 3-7. An excess of mini-Cla Gal4D was bound to 60 nmoles of biotinylated DNA containing five Gal4 binding sites (300 nM of binding sites) immobilized on a StreptAvadin agarose bead. The mini-Cla Gal4D-DNA complex was exposed to 25 nmoles of highly purified 26S yeast proteasome in the presence of 1mM ATP and a 15-fold excess of soluble DNA containing Gal4 binding sites. The bead bound DNA and associated proteins were isolated after the incubation, washed, and probed by SDS-PAGE

and Western blotting to monitor the amount of mini-Cla Gal4D still associated with the immobilized DNA.

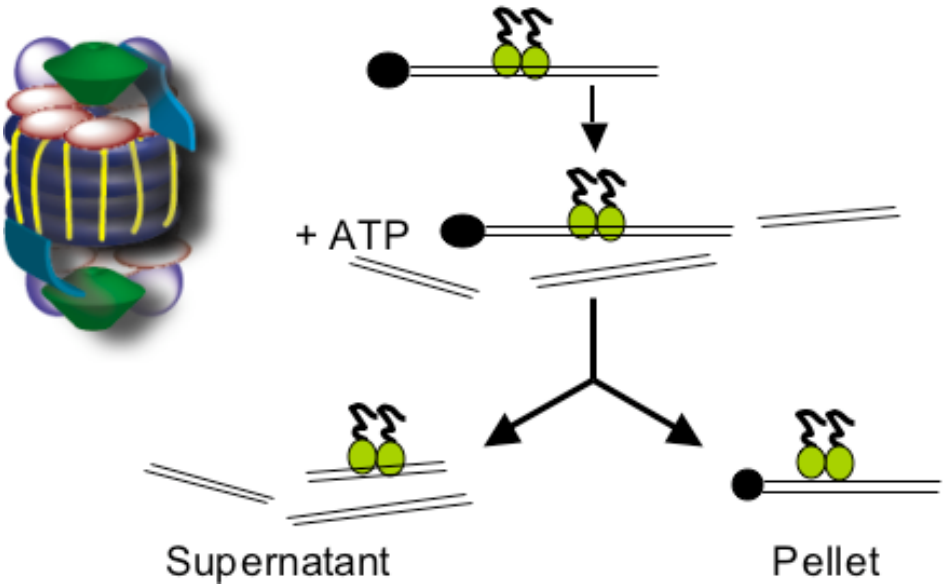

Figure 3- 7 The destabilization assay.
See text for details.

The wild-type 26S proteasome removed the majority of the mini-Cla Gal4D from the DNA (Figure 3-8A) in the destabilization assay. In stark contrast, when Sug1-1-containing 26S proteasome was used most of the mini-Cla Gal4D was retained on the bead-bound DNA. We conclude that the *sug1-1* mutation indeed attenuates the stripping activity of the proteasomal ATPases.

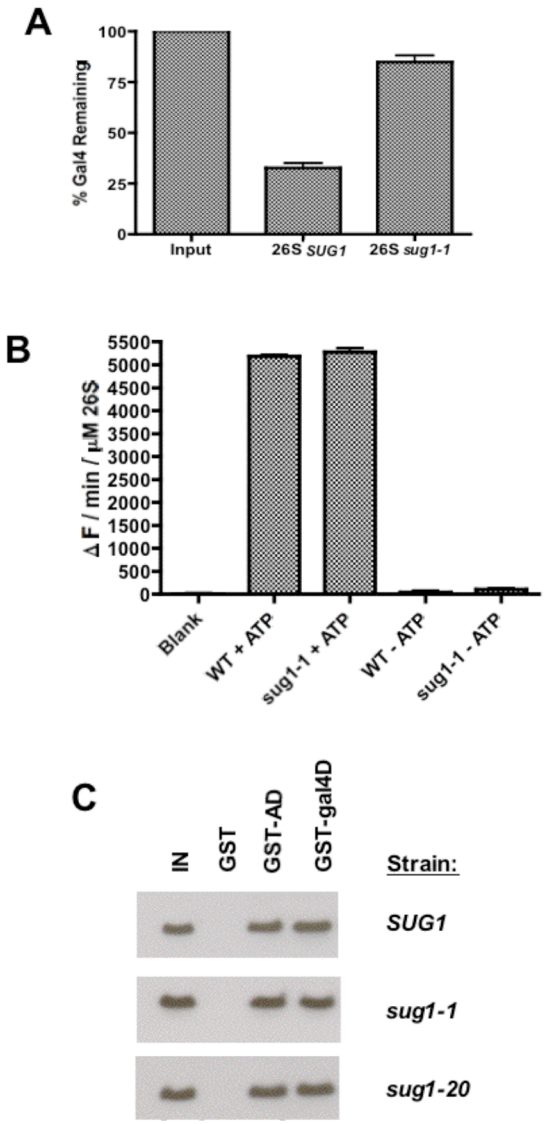

Figure 3- 8 *sug1-1* containing 26S destabilization activity. A. A destabilization assay was used to test the destabilization activity of *sug1-1* 26S. The graph shows the mean % mini-Cla Gal4D remaining on DNA and SEM of three independent experiments. B. The chymotryptic-like peptidolysis activity of the proteasome was measured. The graph represents the average from 3 experiments with the SEM as the error. C. Yeast whole cell lysates from each of the *SUG* strains listed were used as the input in GST-protein retention assays with wild type Gal4 AD (GST-AD) or gal4D (GST-Gal4D). APIS retained by the immobilized activation domains was visualized by western blot analysis in which a biotinylated anti-Sug2 antibody was used as the probe.

Two other functions were checked to ensure that this result was not simply due to poor activity of the Sug1-1 proteasome preparation in general. First, the peptidolysis activity of either wild-type proteasome or proteasome containing Sug1-1 was compared. Cleavage of the pro-fluorescent peptide by the chymotryptic-like activity of the proteasome results in a fluorescent signal that can be monitored by a spectrophotometrically. In order for the peptide to enter into the cavity of the proteasome efficiently, the ATPases must hold open a "flap" that otherwise blocks substrate access, thus providing an independent assay for proteasome function (130). Both wild type and Sug1-1 containing proteasome peptidolysis activity was stimulated by the addition of ATP to similar levels (Figure 3-7B). Second, Sug1 interacts directly with the Gal4 AD, as does Sug2 (98,99) (and Chapter 2). To determine if the Sug1-1-containing ATPase complex can bind to the Gal4 AD, the ADs of wild-type Gal4 and Gal4D (Figure 3-3B) fused to GST were used as the bait in pull-down assays with yeast lysates made from strains carrying different *sug* mutations. The results (Fig. 3-8C) show that the Gal4 AD binds equally well to ATPase complexes containing Sug1, Sug1-1 or Sug1-20 proteins, validating that the Sug1-1 protein is active. Interestingly, we also found that all three ATPase complexes bind equally well to the truncated AD present in Gal4D, demonstrating directly that this region (NTR; see Figure 3-3B) is fully capable of binding the ATPase complex. We conclude that the ATPase

complex derived from Sug1-1-containing proteasomes is specifically deficient in destabilizing activator•promoter complexes but is not generally inactive.

DISCUSSION

The discovery of destabilization of activator-DNA complexes by the proteasomal ATPases caused us to revisit the truncation mutant of Gal4, Gal4D, and suppression of that no growth on galactose phenotype by the *sug1-1* or *sug2-1* mutations. The mechanistic underpinnings of these genetic observations have never been explained adequately. This work set out to test if Gal4D was hypersensitive to destabilization, and if the suppressing *sug* alleles reduced the destabilization activity. We found that although Gal4D was able to bind to the UAS_G *in vitro* (Fig. 3-3A), it was unable to occupy promoters under inducing conditions *in vivo* (galactose-containing media) unless the yeast strain carried a suppressing *sug1-1* mutation (Figure 3-1B and 3-5A). Furthermore, it had been reported previously that the *gal4D* phenotype can also be partially suppressed by forcing occupancy by massive over expression of the protein (47) consistent with a defect in DNA occupancy. Finally, biochemical analysis of the stripping activity of proteasomes isolated from *sug1-1* yeast showed that this mutation indeed down-regulates this activity (Figure 3-8A). Taken together, these results argue that the molecular basis of the *gal4D* phenotype is almost certainly hypersensitivity to destabilization by the proteasomal ATPase complex.

Why is Gal4D hypersensitive to this stripping activity of the ATPase complex? We showed that a derivative of Gal4D, called mini-Cla Gal4D is not mono-ubiquitylated efficiently *in vitro* (Figure 3-3A). Moreover, ChIP analysis of Gal4 and Gal4D promoter binding *in vivo* strongly suggested that the wild-type activator is ubiquitylated, but that Gal4D is not. This is based on the finding that even when Gal4D is resident on the *GAL* promoter (in a *sug1-1* strain) immunoprecipitation with an anti-Ubiquitin antibody fails to enrich the *GAL* promoters. In contrast, when wild-type Gal4 is resident on the promoter, strong co-immunoprecipitation of *GAL* promoters with ubiquitin was observed (Figure 3-5). Taking into account these results and our previously reported work (107), we believe that mono-ubiquitylation acts to protect the activator from the destabilization activity of the ATPase complex and that the inability of Gal4D to be mono-ubiquitylated is the root cause of its poor activity in *vivo*. This is further supported by the fact that genetic fusion of mono-ubiquitin to Gal4D significantly rescues its activity even in a *SUG1* strain. These ideas are incorporated into the model shown in Figure 3-9.

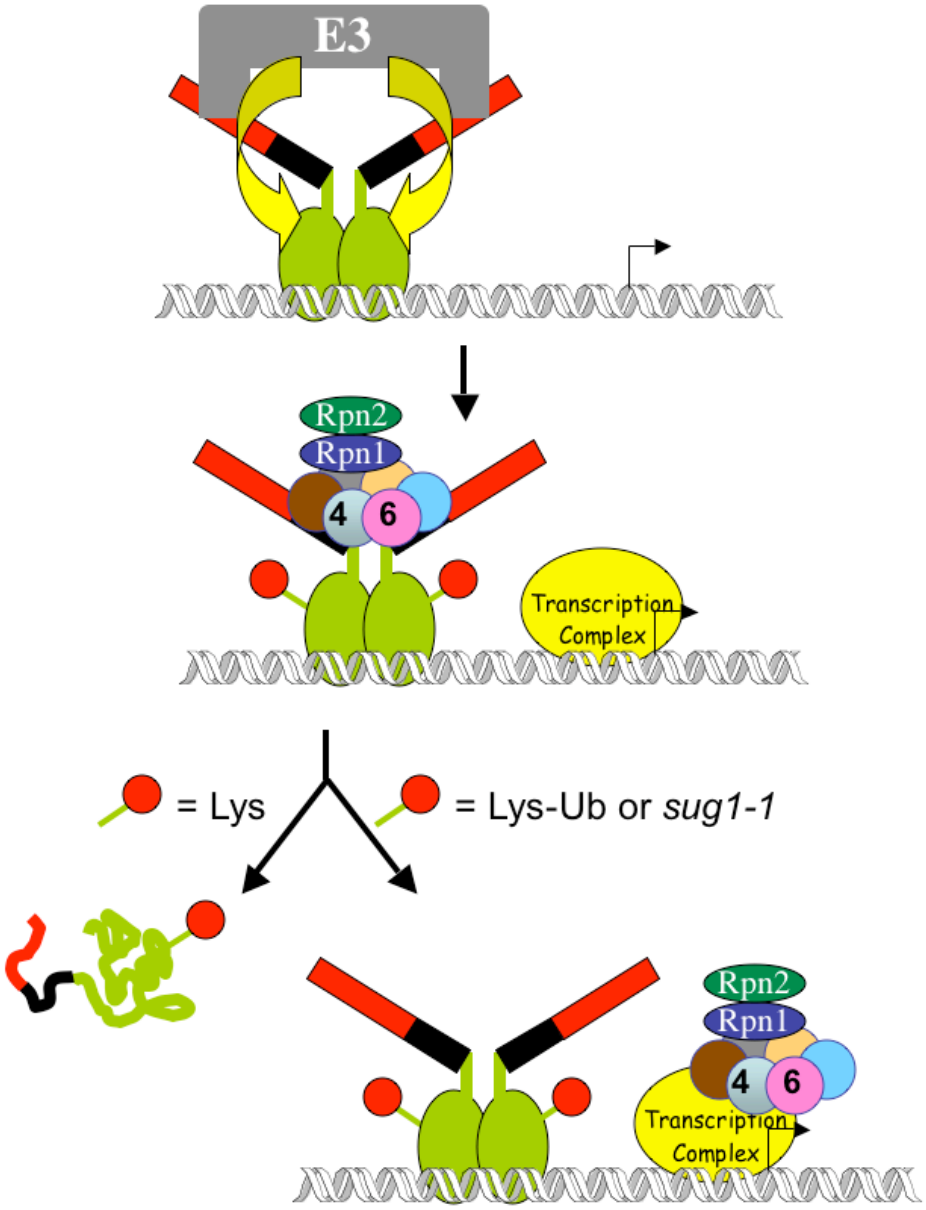
E3
Rpn2
Rpn1
4
6
Transcription
Complex
= Lys
= Lys-Ub or sug1-1
Rpn2
Rpn1
4
6
Transcription
Complex

Figure 3- 9 A model for activator mono-ubiquitylation promoting function. The sub-domains of the Gal4 activation domain determine the ability of Gal4 to occupy DNA and promote transcription. The AD CTR (red line, upper panel) is responsible for recruiting the ubiquitylation machinery, which mono-ubiquitylates the activator in the DNA binding domain. The AD NTR (black line, upper panel) recruits the transcriptional machinery and binds to the proteasomal ATPases Rpt4 and Rpt6. Gal4 is able to recruit the ubiquitylation machinery (upper panel), which leads to the mono-ubiquitylation that protects it from destabilization due to the interaction with the proteasomal ATPases (middle panel). This allows Gal4 to promote transcription (lower panel, right side). Gal4D lacks the AD CTR and the lack of mono-ubiquitylation allows it to be destabilized from DNA as a consequence of interaction with the proteasomal ATPases and prevents transcription (lower panel, left side). However, mutations that reduce destabilization activity (such as *sug1-1*) allow Gal4D to occupy DNA and promote transcription even in the absence of mono-ubiquitylation.

The data reported here and previous studies of Gal4 truncation mutants also reveal important and interesting new insights into the sub-domain structure of acidic activation domains. Gal4 (1-841), which lacks the 34 amino acid AD entirely (Figure. 1-4), is completely inactive in any strain background even when over expressed (70). Gal4D (Gal4 (1-853)) contains an additional 12 amino acids, but lacks 22 residues of the classical 34 residue Gal4 AD (residues 854-873; note that the residues in native Gal4 that are C-terminal to the AD (874-881) are not important for activity (51,131,132)). Gal4D is a potent activator when its promoter occupancy is rescued by suppressing mutations in *SUG1* or *SUG2* or by over expression, displaying about 60% the activity of wild-type Gal4 (i.e., ≈ 600-fold activation of *GAL1*) (47,70). This shows that the 12 amino acid peptide

present in Gal4D, but absent in Gal4 (1-841), is the core activation domain and that it must be competent to interact with coactivators, Sug1/Rpt6, Sug2/Rpt4 (this study) and whatever other factors are critical targets for activated transcription. The fact that Gal4D is not mono-ubiquitylated efficiently, whereas Gal4 is, shows that the 22 amino acid C-terminal region of the AD, is largely, if not completely, involved in mediating ubiquitylation of the activator. There have been previous reports of the partial overlap of ADs and degrons in activators such as Myc (78), but this finding is somewhat different in that the C-terminal region of the Gal4 AD signals mono-ubiquitylation rather than K48-linked poly-ubiquitylation and thus is not a degron.

Moreover, we found that a synthetic peptide containing the 22 residues of the Gal4 AD CTR (Figure 3-3B, MFNTTTMDDVVNYLPDDEDTPP) significantly inhibited mini-Cla Gal4 and Gal4-VP16 ubiquitylation in HNE when added in excess (Figure 3-3C and D). In contrast, a synthetic peptide containing the 13 N-terminal residues of the AD present in Gal4D (TDQTAYNAFGITT) did not affect the efficiency of ubiquitylation. We propose that this peptide is likely to be a recognition site for the E3 ligase protein(s) that target mono-ubiquitin to Gal4. More work will be required to determine the residues in the CTR peptide required for this activity, but once this is accomplished the Gal4 CTR AD peptide and a suitable inactive mutant might be valuable tools for the affinity purification of the ligase, which has so far eluded identification.

Another important insight provided by these data is that the dramatic difference between the activity of Gal4 and Gal4D in the *SUG1* background (>30-fold) and the modest difference in activity in the *sug1-1* strain (<2-fold) argues that the major effect, by far, of Gal4 mono-ubiquitylation is to protect the activator from ATPase-mediated stripping. Any effect of this modification downstream of that event must not contribute more than a small degree to the overall activity of the activator. However, this conclusion may or may not be general for other activators, a point that will obviously require more investigation to address. For example, there is a report that mono-ubiquitylation of the artificial activator LexA-VP16 results in more efficient recruitment of the elongation factor P-TEFb (133).

The lack of detectable ubiquitylation of Gal4D provides a reasonable explanation for its lack of activity in the context of a model that incorporates destabilization by the proteasomal ATPases. This claim would be strengthened if one could force the ubiquitylation of Gal4D and demonstrate that modification would rescue activity. The identification of the ligase responsible is still under investigation so we turned to other methods. Genetic fusion of ubiquitin has been used for other activators to argue for the importance of the modification (93) (107). A genetic fusion of mono-ubiquitin to Gal4D will partially rescue DNA occupancy and transcriptional activity of Gal4D *in vivo* (Figure 3-5). These data

support the model that mono-ubiquitylation of Gal4 is required for DNA occupancy and activity.

In summary, we have deduced the molecular basis of the defect in the truncation mutant Gal4D as well as its rescue by the *sug1-1* mutation. These experiments have revealed a novel sub-domain structure of the Gal4 AD and shed important new light on the process of activator mono-ubiquitylation.

CHAPTER FOUR

PHYSICAL AND FUNCTIONAL INTERACTIONS OF MONO-UBIQUITYLATED TRANSACTIVATORS WITH THE PROTEASOME

SUMMARY

Destabilization of activator-DNA complexes by the proteasomal ATPases can inhibit transcription by limiting activator interaction with DNA. Modification of the activator by mono-ubiquitylation protects it from the destabilization activity. In this chapter, we probe the mechanism of this protective effect. Using novel label transfer and chemical cross-linking techniques, we show that ubiquitin contacts the ATPase complex directly, apparently via Rpn1 and/or Rpt1. This interaction results in the dissociation of the activation domain-ATPase complex via an allosteric process. Mutational analysis of ubiquitin implicates the hydrophobic patch as an important surface for the protective effect of ubiquitin *in vivo*. A model is proposed in which activator mono-ubiquitylation serves to limit the lifetime of the activator-ATPase complex interaction and thus the ability of the ATPases to unfold the activator and dissociate the protein-DNA complex.

Dr. Anwarul Ferdous was the first to demonstrate that mono-ubiquitin added *in trans* would inhibit the destabilization activity of the proteasomal

ATPases. Drs. Lyle Burdine and Bo Liu assisted in preparation of the DBF cross-linking reagent. The experiments shown in this chapter are my own.

INTRODUCTION

We recently demonstrated that a transactivator-DNA complex is rapidly and reversibly dissociated in an ATP-dependent manner by the proteasomal ATPases via a mechanism that does not involve proteolysis (107). Direct interactions between the ATPases and the activation domain (AD) of the transactivator are essential. How do wild-type activators resist this potent activity of the proteasomal ATPases and remain associated with the promoter long enough to drive high-level transcription? Although some activators may undergo rapid exchange with their DNA-binding sites *in vivo* (134), others, including HSF (135), HIF-1 (136), and Gal4 (137), do not. The answer, at least in the case of Gal4, lies in the post-translational modification of the Gal4 DNA-binding domain (DBD). When exposed to a HeLa nuclear extract, all of the DNA-bound activator is phosphorylated (Ferdous et al., unpublished data) and mono-ubiquitylated (Ub) (107) and this form of the protein is insensitive to the destabilization activity of the 19S RP. In Chapter Three demonstrated that the lack of ubiquitylation seems to explain the defect in Gal4D. Additionally, we showed the physiological relevance of the ubiquitin modification by demonstrating that genetic fusion of mono-Ub to Gal4D will partially restore occupancy and activity *in vivo* (Archer et al. *JBC* In Press, and Chapter Three).

It is not clear how ubiquitin might manifest its effect. Clearly, ubiquitin promotes DNA occupancy of the transactivator and we hypothesized this occurred because mono-ubiquitin prevented proteasomal-mediated destabilization. As noted in the Introduction, for other activators such as CTIIA, mono-ubiquitylation correlates with increased interaction with co-activators (95). It is unclear if this is also true for Gal4. The identification of such a co-activator and how its recruitment could prevent destabilization are also unknown.

Another possibility is that mono-ubiquitin interacts with either the activation domain of Gal4 or a proteasome subunit to prevent destabilization. The AD/proteasome interaction is required for destabilization so disrupting this interaction would inhibit that activity (107). However, at first glance, previous experiments do not support this idea. While there is strong evidence that mono-ubiquitin is attached covalently to Gal4, there is no evidence that the mono-ubiquitin interacts with the AD of Gal4. Cross-linking experiments performed by Pickart and co-workers have indicated that mono-ubiquitin does not cross-link with the any of the subunits of the 26S proteasome but they were able to easily detect cross-linked products formed between subunits of the 26S proteasome and tetra-ubiquitin chains (18). What about interaction with a proteasomal subunit in the context of the RP? The AD of Gal4 is known to dissociate the 26S proteasome into a smaller subcomplexes and interact with the subcomplex that contains the base of the RP and well as Rpn1 and Rpn2, but not the lid of the RP

or the CP (99). We thought that testing the mono-ubiquitin/RP interaction would be a test of the utility of the DBF reagent cross-linking and label-transfer protocol first explained in Chapter Two.

Here, we set out to determine the mechanism by which ubiquitin protects activators from destabilization. First, we show that activator mono-ubiquitylation is indeed the key to stabilizing the activator-promoter complex. Second, we apply the DBF cross-linking reagent and demonstrate that mono-Ub interacts directly with the APIS complex or the 19S RP, but not the intact 26S proteasome, via the Rpt1 and/or Rpn1 proteins. This interaction results in dissociation of the AD-ATPase contacts, apparently via an allosteric mechanism. Finally, we identify a surface feature of ubiquitin that accounts for the specificity of the inhibition effect.

MATERIALS AND METHODS

Materials.

CCPGCC-Ub was constructed by including the DNA encoding the amino acid sequence CCPGCC into the 5' primer of a pair of PCR primers (see below) designed for an Ub expression plasmid. The PCR product was digested with HindIII and KpnI and inserted into the pT7-FLAG-1 vector (Sigma P 1118). This fusion protein was expressed and purified according to manufacture's directions (Sigma). Ubiquitin mutants fused to Gal4D were constructed by site directed

mutagenesis (using a Quickchange kit (Qiagen)) of the ubiquitin S10 tagged Gal4D in the pSB32 vector described in Chapter Three (see below). These proteins were extracted from the pSB32 vector with the restriction enzymes BamHI and EcoRI and inserted into the YEp351 multi-copy vector by standard procedures.

	GCGGAAGCTTTGCTGCCCGGGTTGCTGCG
CCPGCC-Ub F	CGGGAATTTTTGTCAAGACAC
CCPGCC-Ub R	GGGGTACCCCCACCCCTCAA CCTCAAGACAAGGTG
Ubl44A F	CCAGACCAGCAAAGATTGGCTTTTGCCGGTAAGCAACTAG
Ubl44A R	CTAGTTGCTTACCGGCAAAAGCCAATCTTTGCTGGTCTGG
UbD58A F	GGTAGAACGCTGTCGGCCTACAATATTCAAAAGG
UbD58A R	CCTTTTGAATATTGTAGGCCGACAGCGTTCTACC

Table 4- 1 Primers used for cloning

26S proteasome and the 19S RP were purified using a FLAG affinity tag as described (124) with modifications (102). GST-Gal4(1-147)-VP16, GST-Gal4(1-147), GST-Gal4 AD, GST-UBL, His6-SUMO, His6-Gal80, and His6-Ub were purified from *E. coli* BL21 cells as described (102). K48 linked tetra-Ub chains were from Boston Biochem. Antibodies against PentaHis (Qiagen), Ubiquitin (Dako Cytomation), GST (Santa Cruz Biotech.), NeutrAvidin-HRP (Pierce), and HRP conjugated secondary antibodies (Biorad) were used according to instructions. The α-Rpt6, α-Rpt4, α-Rpt1, α-Gal4 N-terminus, α-Gal6, and α-20S antibodies were produced in rabbit. α-Rpn1 and α-Rpn2 were produced in

mouse. The DBF cross-linking reagent (138) and the DOPA-Gal4 AD peptide have been described (98).

Destabilization of Activator-DNA complexes by the proteasome

The destabilization assay was conducted as described previously (107) with the following changes. The activator-DNA complex was preformed and added to the reaction mix at a 60 nM final concentration of activator-DNA complex. The remainder of the reaction mix contained 25 nM 26S proteasome, 3 mM ATP, and 1 μM non-biotinylated DNA containing Gal4 binding sites in TR reaction buffer (10mM HEPES (ph 7.8) 50mM KCL, 6.25mM $MgCl_2$, 0.1mM EDTA, 1mM DTT, 2% Glucose (v/v)). The reaction was treated with 3 units of Hexokinase without ATP or 100nM MG132 for Figure 1B. Antibody inhibition was done by addition of 0.8 μg of the purified antibody indicated. Destabilization in the presence of mono-Ub, tetra-Ub, or SUMO was done by adding the indicated amount of protein to the reaction mix immediately before addition of the activator-DNA complex.

Ubiquitylation reaction

The mono-ubiquitylation of GST-Gal4-VP16 has been described (107). The non-ubiquitylated controls were done exactly as stated, but 3 Units of hexokinase were used and the ATP was omitted.

Pull-downs

The GST-protein/proteasome pull-downs were performed by mixing 1 μM of GST fusion protein with 50 nM 26S proteasome in TR reaction buffer. After a 30 minute incubation at 4C, glutathione agarose beads (Amersham) were added for 30 minutes. The beads were washed with TR reaction buffer and resuspended in SDS loading buffer for Western blot analysis. The GST-protein/His6-Ub experiments were performed as above but contained 10 μM His6-Ub or His6-Gal80.

Pull-downs to detect ubiquitylated GST-Gal4-VP16/proteasome interactions were done by incubating the activator-DNA complex after the ubiquitylation reaction with 110 nM 26S proteasome in 30μL TR buffer containing 3U hexokinase. After 30 minutes at room temperature the pellets were washed extensively with TR buffer. The pellets were resuspended with SDS loading buffer and run on SDS-PAGE for Western blot analysis.

Cross-linking reactions

Cross-linking to detect DOPA-Gal4 AD/proteasome interactions have been described (98,116). The proteins indicated in the figure were added to the reaction mix at the same time as peptide addition. Cross-linking to detect mono-Ub/proteasome interaction was done as described (138) with the following changes. 110 nM 26S or 19S proteasome was mixed with 10 μM of the CCPGCC-Ub in TR reaction buffer. After 10 minutes, 10 μM of the DBF was

added and incubated for 10 minutes in the dark. The reaction was triggered with 5 mM $NaIO_4^-$ and, after 2 minutes, SDS loading buffer containing 100 mM 2,3 dimercapto-1-propanol (DMP or British Anti-Lewisite BAL) was added to quench the reaction and transfer the bFD label to the cross-linked partner. Experiments that included GST-Gal4-VP16 to dissociate 26S proteasome were done as above, but 110 nM GST-Gal4-VP16 was added to the proteasome for 15 minutes at RT before adding mono-Ub and completing the reaction. Identification of the mono-Ub interacting proteins in the proteasome was done using a 5-fold increase of the above reaction as described (98,116).

Peptidolysis and ATPase assays

The peptidolysis activity of the 26S was tested using the fluorescent substrate Suc-LLVY-AMC (Bachem). 50 nM of substrate was mixed with 7 nM 26S proteasome and 3 mM ATP in Pep Buffer (50 mM Tris (pH 8.0) and 20 μM mM BME). The change in fluorescence (ex:360nm, em:465nm) was monitored for 30 minutes at RT. The indicated proteins were added at 10 μM and MG132 was added a 100 nM. The graphs in Figure 4-4B and Figure 4-8A show the average change in fluorescence over 30 minutes from 3 measurements with one standard deviation of the mean as error. Experiments done in the presence of $NaIO_4$ were done as above with the addition of 5mM $NaIO_4$.

The measurement of ATP hydrolysis by coupling the production of ADP to the oxidation of NADH has been described (139). The absorbance at 340 nM

of 5 nM proteasome in the reaction mix was monitored. GST or mono-Ub was included at 10 μM in the indicated lanes. The change in absorbance was converted to nmoles of ATP hydrolyzed per minute for 3 measurements and graphed with one standard deviation of the mean as error.

Inhibition of the destabilization activity of the proteasome

The destabilization assay was done as stated above. 10 μM of the DOPA-Gal4 AD peptide (DOPAC-TDQTAYNAFGITTGMFNTTTMDDVYNLPDDEDTPPNPKKEGGE-Biotin) or the Control DOPA peptide (Biotin-KG(DOPA)AHNRLIYMQD) (98) were added to the proteasome prior to addition of the activator-DNA complex. The graph in Figure 4-10 shows the mean and standard error of the mean for 3 experiments.

Expression level of the Ubiquitin-Gal4D proteins

A ΔGal4 strain (Sc726) was transformed with Yep351 multi-copy plasmid encoding the indicated Ub-Gal4D fusion protein. A 500mL culture of these strains was grown to mid-log phase in complete media lacking leucine with raffinose as the carbon source. Galactose was added and 2 hours later the cells were collected by centrifugation. A 1mL aliquot was saved to measure the cell density at 600nm and an equal amount of cells were resuspended in 50 microliters of water and 1X SDS loading buffer. The cells were subjected to three cycles of freeze/thaw and cell debris was spun down at 14K for 10 minutes. The lysate was loaded on gel and subject to SDS PAGE and western blotting with an anti-Gal4

N-terminal antibody.

RESULTS

Ub Antagonizes Proteasome-Mediated Destabilization *In Trans*

To begin to probe the mechanistic basis of mono-ubiquitin to protect transactivators from destabilization, we first addressed whether mono-ubiquitin is responsible for the protective effect. The ability of a mono-ubiquitin fusion to rescue Gal4D (Archer *et al. JBC* In Press) suggests this is true, but it was difficult to exclude other modifications in the *in vivo* system. If monomeric ubiquitin were solely responsible for the protection, it might be possible to observe the inhibition of destabilization by adding Ub to a reaction containing non-ubiquitylated activator-DNA complex. We previously reported a simple *in vitro* assay to monitor the ability of the proteasomal ATPases to destabilize activator-DNA complexes (107), which is shown schematically in Figure 3-6. As shown in Figure 4-1 the destabilization activity of the purified proteasome (lane 2) was inhibited strongly by adding mono-Ub *in trans* to concentrations of 10 μM (lane 3). Note that this experiment did not involve the use of HeLa NE and thus cannot involve the attachment of the Ub to the Gal4 DBD, which was confirmed by Western blot analysis using an anti-Ub antibody (not shown). This result argues that it is indeed the Ub moiety that is key to blocking proteasome-mediated destabilization. Mono-Ub inhibited destabilization with an IC_{50} of approximately 0.7 μM (Figure

4-2A and 4-2B), which was a ~ 30-fold molar excess of mono-Ub to 26S proteasome. Remarkably, the addition of K48 linked tetra-Ub chains (pUb, Figure 4-1, lane 4) did not result in stabilization of the activator-DNA complex, demonstrating a striking difference between the properties of these different forms of the same protein. In addition, when the highly homologous small Ub-like modifier (SUMO) protein (lane 5) was added *in trans* at 10 μM, it had little effect on the destabilization activity of the proteasome. We conclude that interaction of monomeric Ub with either the proteasome or the activator is responsible for stabilization of the activator-DNA complex in the presence of the proteasome.

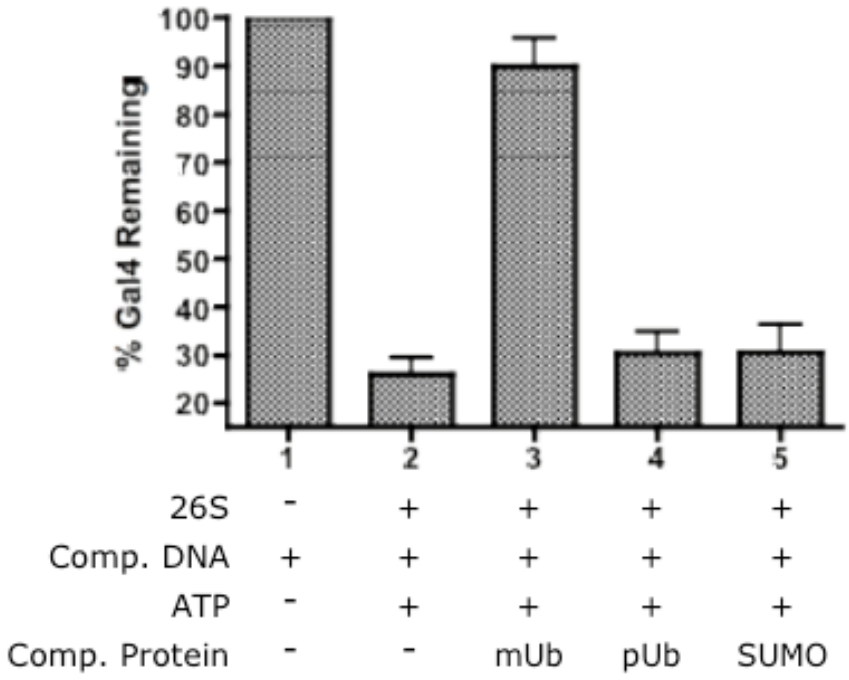

Figure 4- 1 Destabilization of Activator-DNA complexes by the 26S proteasome in the presence of ubiquitin and related proteins. Destabilization of activator-DNA complexes by proteasome with a 15 minutes pre-incubation with 10 μM mono-Ub (lane 3), 10 μM K48 linked tetra-Ub (lane 4), or SUMO (lane 5). The amount of Gal4-VP16 retained by DNA was measured in three experiments by Western blot and normalized with lane 1 being set as 100%. The average amount remaining and the SEM are graphed.

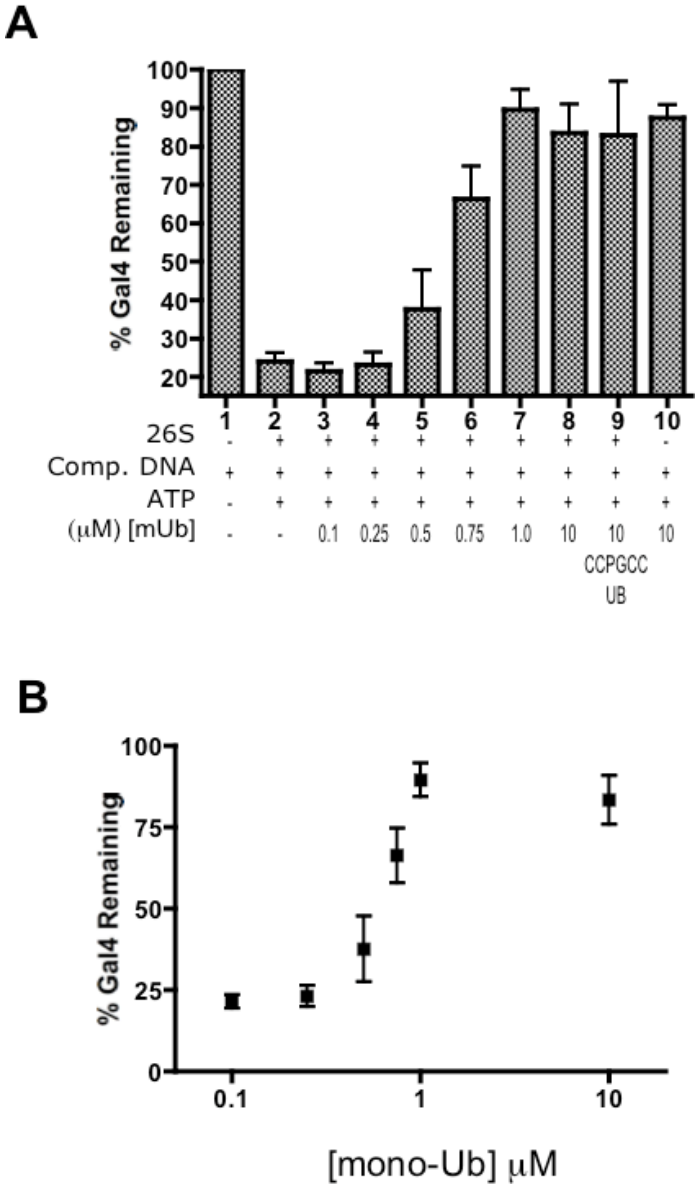

Figure 4- 2 Effect of free ubiquitin of the destabilization of Activator-DNA complexes by the 26S proteasome A. Titration of His6-mono-Ub *in trans* into a destabilization assay. The GST-Gal4-VP16 removed from DNA by the 26S proteasome in the presence of 0 – 10 μM His6-mono-Ub was monitored by Western blot and graphed as in Fig 4-1. Inhibition with 10 μM CCPGCC-Ub (lane 9) is the same as His6-mono-Ub. B. Destabilization assay was performed in the presence of increasing concentration of mono-Ub. The amount of Gal4-VP16 remaining was measured as in 4-1 and graphed as a function of mono-Ub concentration.

The differences in the effectiveness with which mono-ubiquitin and the other proteins inhibit destabilization are striking. Structurally, SUMO and ubiquitin are similar. Both proteins are about the same size and display similar structural folds (140,141). However, SUMO, while having many hydrophobic residues displayed on the surface, lacks the 'hydrophobic patch' centered around isoleucine 44 on ubiquitin. This patch has been implicated as being important for many of ubiquitin's functions and mutation of residues in this patch will disrupt these functions (36,37). As mentioned in the Introduction, the hydrophobic patch is buried at the interface between the monomers in K48-linked ubiquitin. The lack of activity of these proteins that do not have the exposed hydrophobic patch suggested that it is important for inhibition of destabilization. To test this, the destabilization assay was repeated with the addition of K63-linked tetra ubiquitin (Figure 4-3). This linkage results in an open confirmation that is akin to four copies of ubiquitin monomers, quite different from the closed and tightly packed quantanary structure of K48-linked tetra-ubiquitin. K63-linked tetra-ubiquitin was as effective as mono-ubiquitin at inhibiting destabilization by the proteasome, arguing that the hydrophobic patch is important for this function.

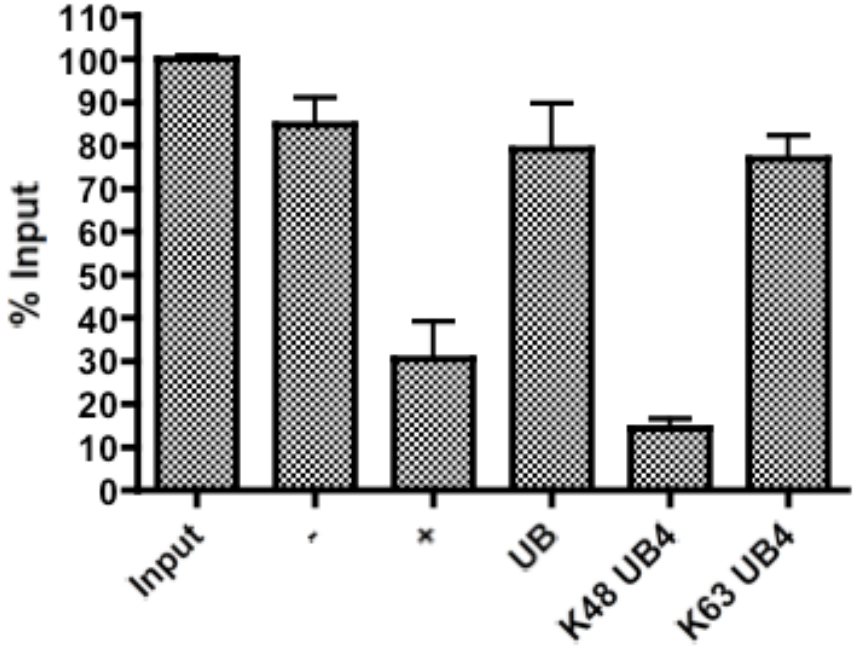

Figure 4- 3 Effect of exposure of the hydrophobic patch of ubiquitin in inhibiting destabilization. A destabilization assay was performed as in Figure 4-1. The indicated form of ubiquitin was added at a final concentration of10 micromolar. The results were normalized to the amount of Gal4-VP16 in the input.

Ub binds directly to the 19S RP

To distinguish whether the activator or the proteasome is the target of free Ub, we turned to a novel type of cross-linking/label transfer chemistry developed recently in our laboratory (138). In this method (see Figure 4-4A), the protein of interest, in this case Ub, is tagged with a short peptide sequence (CCPGCC) that

binds tightly and specifically to a biarsenical fluorescein derivative called FlAsH (142,143). We synthesized a derivative of FlAsH tethered to a biotin moiety and a dihydroxyphenylalanine (DOPA) unit via linker arms. The catechol ring of the DOPA sub-unit can be oxidized to an ortho-quinone selectively with sodium periodate under conditions that do not affect proteasome function (Figure 4-4B). The quinone is a reactive electrophile that will couple covalently with nearby nucleophilic residues on a receptor protein (Figure 4-4). Extensive control experiments have revealed that cross-linking occurs only when the DOPA-containing molecule and the receptor are associated stably (116,117). When the cross-linked products are boiled in a dithiol-containing buffer prior to electrophoresis, the FlAsH-CCPGCC association is disrupted, resulting in transfer of the biotinylated FlAsH-DOPA reagent to the receptor protein (Figure 4-4). We employed this method to determine if Ub binds directly to the proteasome or Gal4-VP16.

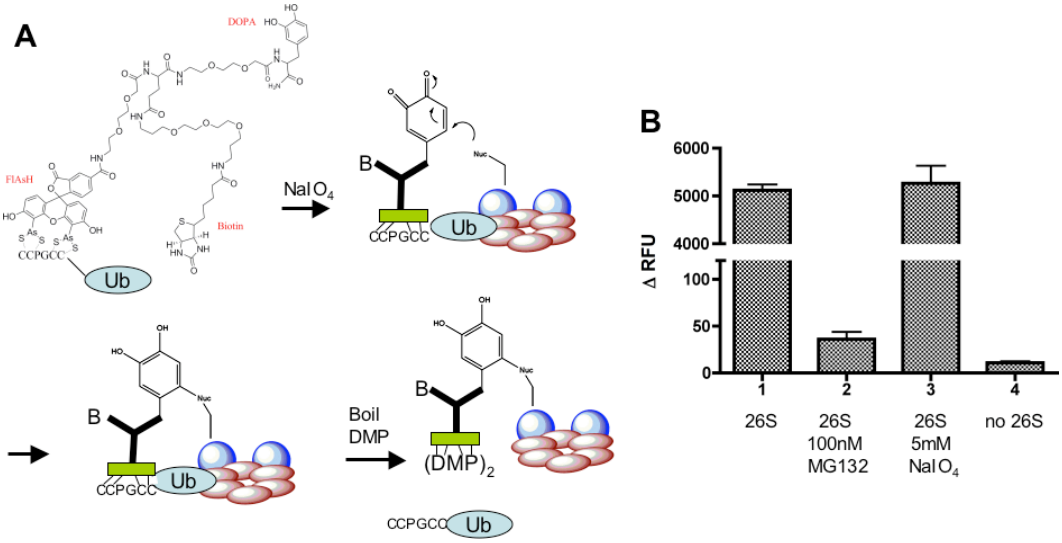

Figure 4- 4 The DBF cross-linking and label transfer scheme A. The scheme used to find ubiquitin interacting proteins. See text for details. B. Periodate does not inhibit 26S proteasome function. The ability of the proteasome to cleave a pro-flourescent peptide was monitored to determine if periodate altered proteasome function. The change in the relative fluorescent units (RFU) were graphed.

As shown in Figure 4-5A, when 10 μM CCPGCC-Ub and biotinylated FlAsH-DOPA (DBF) were incubated with the 26S proteasome and sodium periodate, no significant cross-linking was detected by SDS PAGE analysis followed by Western blotting with a NeutrAvidin-HRP conjugate. This is in agreement with previous experiments. Despite the lack of cross-linking to 26S proteasome, CCPGCC-Ub inhibited destabilization *in trans* equally as well as His6-Ub (Figure 4-2A compare Lane 8 vs. 9). However, significant cross-linking and label transfer was observed when the same experiment was carried out with the 19S RP rather than the full 26S proteasome (Figure 4-5A, compare lanes 1

and 2). At least two major biotinylated bands were observable, one of approximately 55 kDa and a larger species that migrated slightly above the 110 kDa marker. The ability of Ub to cross-link with the 19S proteasome but not the 26S proteasome suggests that the Ub interaction site is obscured in the full 26S proteasome. The Gal4 AD will bind to Rpt4 and Rpt6, removing a proteasomal sub-complex and dissociating the proteasome (99). Adding Gal4-VP16 to the cross-linking reaction allowed CCPGCC-Ub to form the same cross-linked products with the 26S proteasome (Figure 4-5B compare lane 1 and 2). When CCPGCC-Ub, DBF, and periodate were mixed with GST-Gal4-VP16 in the absence of any proteasomal proteins, no labeled bands were observed (see Figure 4-5B, lane 3). Thus, the cross-linking/label transfer data indicate an Ub-19S RP interaction, but provide no evidence for direct interaction with the activator.

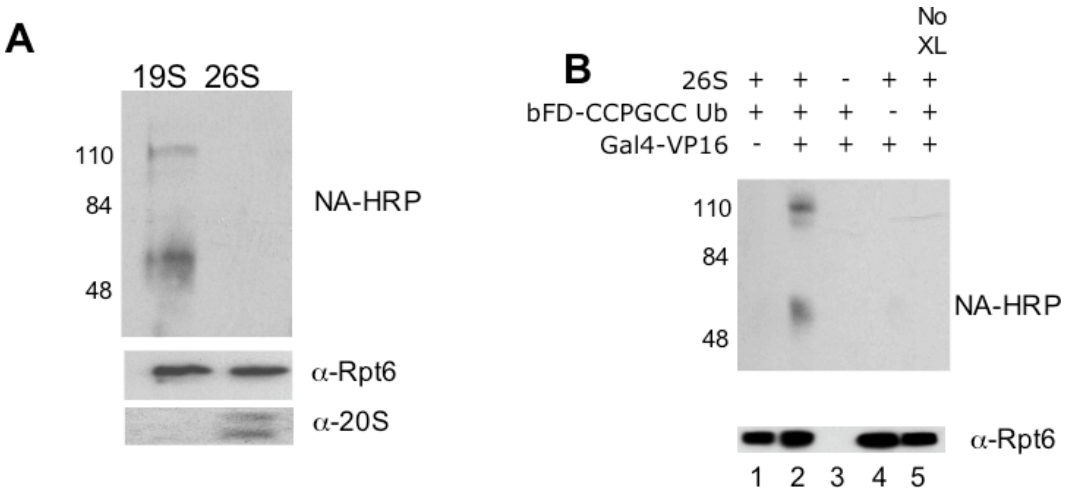

Figure 4- 5 Mono-Ub cross-links to subunits of the 19S proteasome. A. Oxidative cross-linking with the bFD diffusible cross-linker and CCPGCC-Ub was used to test if mono-Ub interacts with the 26S or 19S proteasome. 110 nM of Proteasome was incubated with 10 μM of mono-Ub and bFD.. The NA-HRP blot shows labeled proteins and antibodies raised against Rpt6 and 20S show loading of proteasome in the reaction. B. 26S proteasome was incubated for 15 minutes with or without 110 nM GST-Gal4-VP16 to dissociate the proteasome and then cross-linking with mono-Ub was repeated as in B. Leaving out 26S proteasome, CCPGCC-Ub, or periodate resulted in no specific cross-linked proteins (lanes 3-5, respectively).

Identification of Ub-proximal proteins in the 19S RP

We next turned to identification of the proteins that acquired biotin in the cross-linking/label transfer reaction (Figure 4-5B, lane 2). The high molecular weight region of a gel run for a longer period of time to achieve maximal separation of the larger products showed an intense band running just above the 110 kDa marker and a less intense band just below the marker (left panel, Figure 4-6). These bands are approximately the size expected for Rpn1 (upper band) and

Rpn2 (lower band), respectively. These assignments were confirmed by aligning the NeutrAvidin-HRP blots with Western blots using antibodies raised against the Rpn1 and Rpn2 subunits. The other major band(s) displayed an apparent mass of slightly above 50 kDa (Figure 4-5). Several proteins in the 19S RP, including all six ATPases, have similar gel mobilities in this region. Therefore, the cross-linking and label transfer products were analyzed by two-dimensional electrophoresis using isoelectric focusing in the first dimension and SDS-PAGE in the second (Figure 4-6B). The biotin-labeled protein has an isoelectric point of 5.3 and a mass of ~ 55 kDa, suggesting that it is the Rpt1 subunit (114). To confirm this assignment, the membrane was stripped and re-probed with antibodies raised against Rpt1 and Rpt4, the latter being the 19S protein closest in molecular mass and isoelectric point to Rpt1. The false-colored overlays of the NeutrAvidin-HRP blot with the Western blots against the proteasomal subunits are shown in the lower panels of Figure 4-6B. Note that Rpt1, but not Rpt4, showed an overlap with NA-HRP signal.

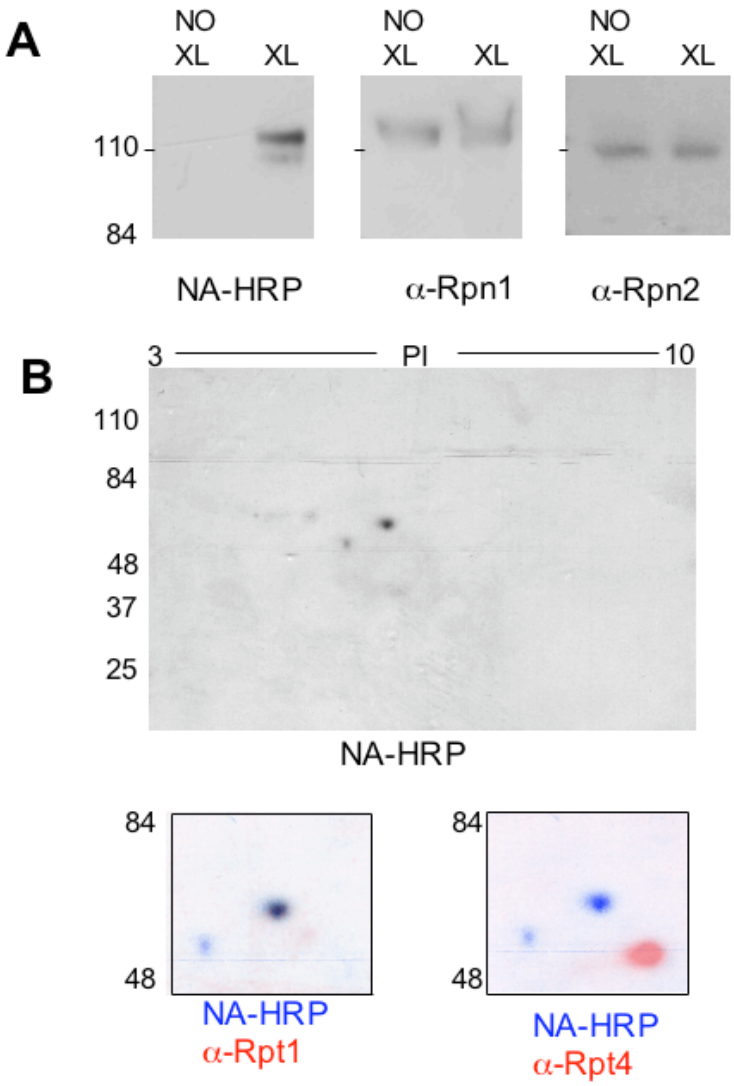

Figure 4- 6 Mono-Ub labels subunits of the proteasome. A. Mono-Ub/26S proteasome cross-linking was repeated to identify the proteins labeled by interaction with mono-Ub. The upper portion of an SDS-PAGE gel was probed with the indicated antibodies and aligned. The 110 kDa marker is noted on the left hand side of each blot. B. After cross-linking, the sample was separated by isoelectric point and then by mobility in a SDS-PAGE gel. The upper panel shows the biotin labeled proteins in the gel. The lower 2 panels are false colored overlays of a portion of the membrane probed with the indicated antibodies.

As final confirmation of the ATPase identification, 6 different *E. coli* strains expressing the His6 tagged version of each individual, recombinant ATPase was used in a cross linking and label transfer reaction (Figure 4-7). After performing the reaction in the cellular lysate a gel was run and probed with NA-HRP. The only ATPase to show any reactivity was Rpt1 (Figure 4-7 upper panel) even though all 6 ATPases were present at roughly similar levels (lower panel). This demonstrates clearly that the major labeled ATPase is Rpt1. We conclude that mono-Ub binds to the RP at or near the Rpn1 and Rpt1 subunits. Importantly, there is no evidence for binding of CCPGCC-Ub to Rpt4 or Rpt6 (Sug2 and Sug1, respectively), which are the direct binding partners of acidic ADs (98). Based on models of the 19S architecture, these proteins are predicted to be on the opposite side of the ATPase ring (144).

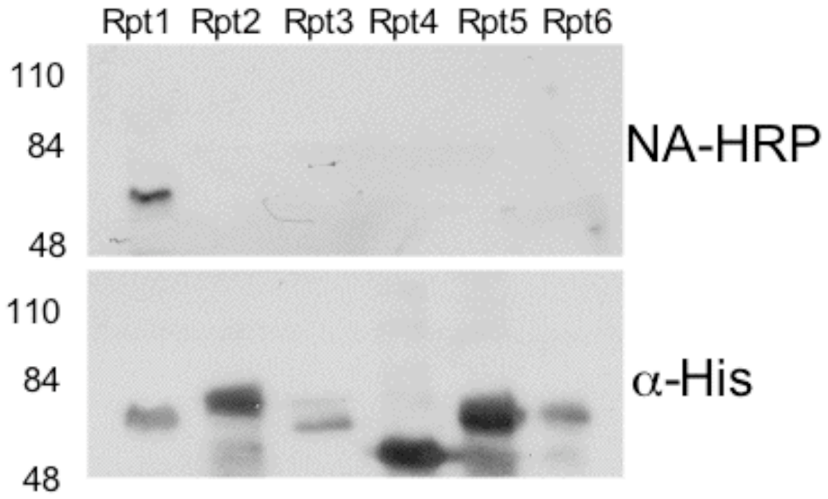

Figure 4- 7 Mono-Ub interaction with recombinant ATPases of the proteasome. Lysates from *E. coli* expressing the indicated protein were subject to a cross-linking reaction. The NA-HRP blot shows the labeled protein and the anti-His blot shows the amount of each of the His-tagged ATPases.

Ubiquitylated Gal4-VP16 binds the proteasomal ATPases weakly

The experiments described above support a model in which the proteasomal ATPase-catalyzed destabilization of activator-DNA complexes is inhibited by Ub *in trans* through direct Ub-RP interactions. This could be the result of a general Ub-mediated down-regulation of RP activity. However, we found that mono-Ub had little or no effect on either the RP-dependent peptidolysis activity of the proteasome or, perhaps more importantly, the ATPase activity of the RP itself (Figure 4-8).

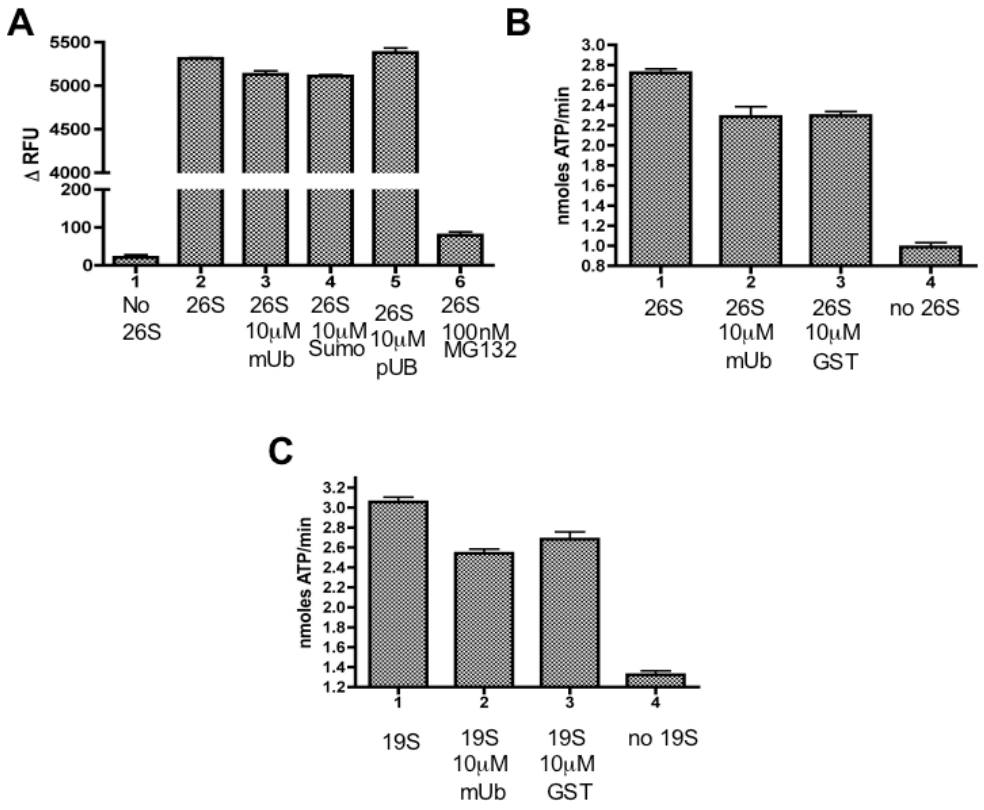

Figure 4- 8 Mono-Ub and other activities of the 26S proteasome A. The chymotryptic-like peptidolysis activity of the proteasome was measured using the fluorescent substrate Suc-LLVY-AMC.. Treatment with 100nM MG132 reduced peptidolysis to background levels. B. The ATPase activity of proteasome was measured by coupling the production of ADP to the oxidation of NADH. The background level of the assay is shown in column 4. C. Repeat of panel B, but using 19S proteasome in place of 26S proteasome

Another mechanism by which Ub could evince its protective effect on the activator-DNA complex is by modifying the interaction between the proteasomal ATPases and the AD of the activator. We demonstrated previously that a complex of the Gal4 DBD alone (lacking an AD) with DNA is insensitive to 19S-

mediated destabilization, arguing that the AD-ATPase interactions are essential for this effect (107). This is consistent with previously reported direct interactions between the Gal4 and VP16 ADs and Rpt4 and Rpt6. (55,98,99,121,145). Thus, if Ub binding to the 19S weakens the AD-ATPase contacts, the strong prediction is that the destabilization process would be attenuated.

A variant of the destabilization assay was used to address the role of the Ub moiety in modulating AD-ATPase interactions in the context of the mono-ubiquitylated activator. GST-Gal4-VP16 bound to biotinylated DNA was mono-ubiquitylated, or not, by exposure to HeLa NE and a 3-fold excess amount of Ub in the presence or absence of ATP. The ability of these two forms of the activator to retain the proteasomal ATPases was then assessed by a pull-down protocol followed by SDS-PAGE and Western blotting with the appropriate antibodies. As shown in Figure 4-9, significant ubiquitylation of the activator was observed in the presence of ATP (lane 4, second panel) but not in its absence (lanes 3 and 5, second panel), as expected. The ubiquitylated and non-ubiquitylated forms of the activator-DNA complex were then exposed to immunopurified 26S proteasome in the absence of ATP (to prevent stripping of the non-ubiquitylated activator from the DNA). The third and fourth panels, respectively, show the amounts of Rpt6 and the 20S proteins retained by the activator in each case. An activator-DNA complex that had not been exposed to the HeLa NE retained slightly more than 10% of the input Rpt6 protein (and the other ATPases) but no detectable 20S

proteins (lane 2). This is in agreement with previously published results that acidic ADs bind to the ATPases in a fashion that is incompatible with simultaneous 20S association (99). A similar result was obtained for the activator-DNA complex that had been exposed to HNE in the absence of ATP, conditions under which ubiquitylation did not occur (lanes 3 and 5). In this case slightly less than 10% of the input Rpt6 protein was retained and again, little or no 20S. In stark contrast, the ubiquitylated activator retained much less Rpt6 than the non-ubiquitylated protein (compare lane 4 with lanes 3 and 5). We conclude that mono-Ub fused covalently to the Gal4 DBD antagonizes interaction of the activator with the proteasomal ATPases.

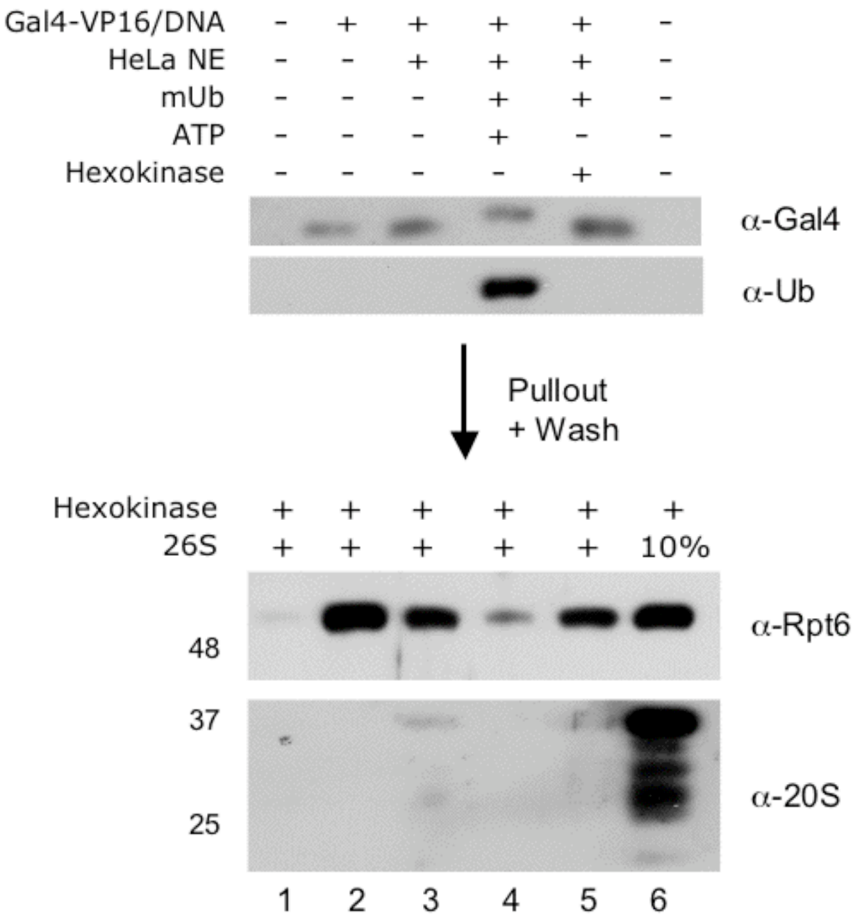

Figure 4- 9 Effect of activator mono-ubiquitylation on the interaction of the GST-Gal4-VP16 activator with the proteasome. The ability of mono-ubiquitylation to affect GST-Gal4-VP16/proteasome interaction was probed by a pull-down. 300 nM total GST-Gal4-VP16 was bound to DNA and placed in HeLa NE with or without ATP and a ~ 3-fold excess of mono-Ub. The upper two panels demonstrate the ubiquitylation of the activator (lane 4). The mono-ubiquitylated or non mono-ubiquitylated activator-DNA complex was isolated and incubated with 110 nM 26S proteasome in the absence of ATP. After incubation for 30 minutes the activator-DNA complexes and associated proteins were isolated and washed. The blots with antibodies raised against Rpt6 and 20S show the amount of the proteasome complexes retained by the activator-DNA complex. 10% of the 26S proteasome used in the pull down (lane 6) is shown for comparison.

The ADs of Gal4 and VP16 compete for proteasome interaction

The experiment in Figure 4-9 clearly shows that that mono-ubiquitylation reduces the AD/proteasome interaction. However, this experiment was done using the VP16 AD and not the native Gal4 AD. Both the Gal4 and VP16 ADs bind to the Rpt4 and Rpt6 subunits of the 19S so it seems plausible that these two ADs could be recognized equivalently by the UPS, but we wanted to add support for that idea with further experiments. We used a competition between the Gal4 AD and the GST-Gal4-VP16 protein. To do this, we again turned to oxidative chemical cross-linking (116), but in this case employed synthetic AD peptides that contained biotin and DOPA (98). Specifically, a peptide was synthesized that contains the 34 amino acid Gal4 AD (residues 841-875), an N-terminal DOPA and a C-terminal biotin tag (DOPA-Gal4 AD). We have shown previously that when incubated with the proteasome, the DOPA-Gal4 AD peptide undergoes periodate-triggered cross-linking to the Rpt4/Sug2 protein (98). As shown in Figure 4-10A addition of an excess of GST-Gal4-VP16 decreased the DOPA-Gal4 AD/Rpt4 cross-link yield (compare lanes 1 and 4), but addition of the construct lacking the VP16 AD had did not decrease the cross-link yield (lane 5). This experiment shows that the VP16 and Gal4 ADs bind to the proteasomal ATPases in a similar fashion.

As a further demonstration of the interchangeable nature of the Gal4 and VP16 ADs, the synthetic DOPA-Gal4 AD was added to a destabilization assay containing the immobilized DNA-GST-Gal4-VP16 complex and 26S proteasome. The DOPA-Gal4 AD peptide partially blocked disruption of the DNA-protein complex but an unrelated DOPA and biotin-containing peptide did not (Figure 4-10B). This demonstrates that the VP16 and Gal4 ADs will functionally compete in an assay based on 19S RP function, consistent with the above experiment that they will compete physically for binding to the ATPases.

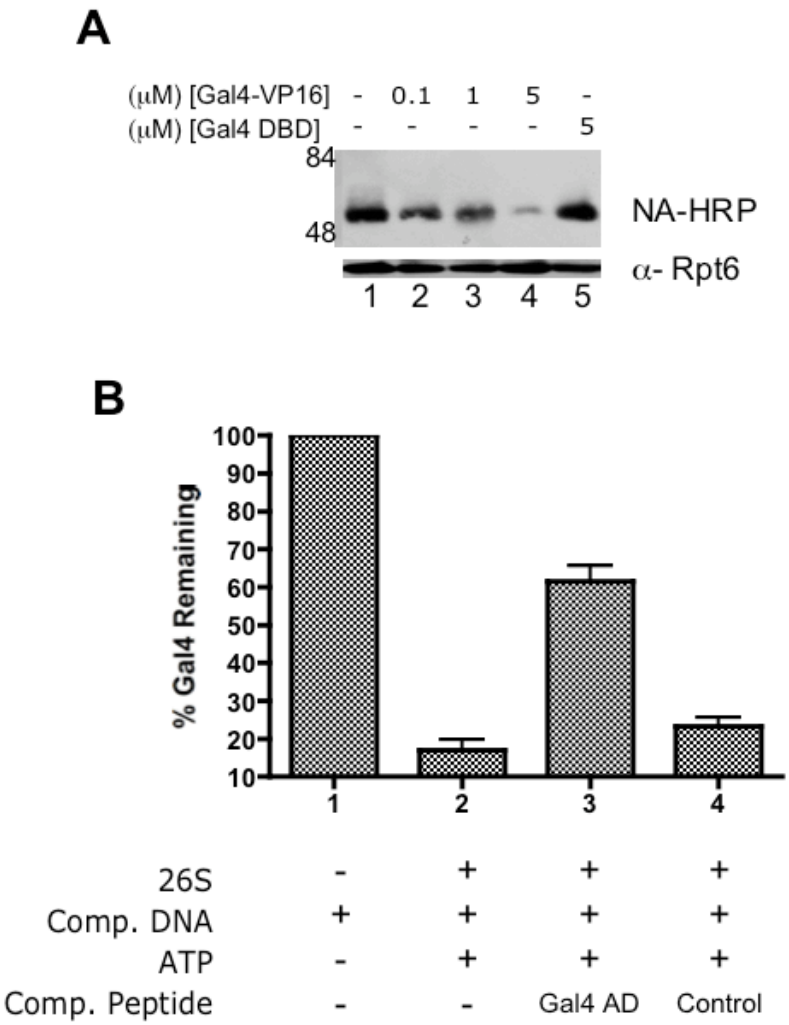

Figure 4- 10 The ADs of VP16 and Gal4 compete for proteasome binding

A. DOPA-Gal4 AD/proteasome interaction was probed by oxidative chemical cross-linking in the presence or absence of the VP16 AD. The blot for Rpt6/Sug1 shows equal amounts of proteasome in the reaction. B. The graph shows the results of a destabilization reaction performed with or without the addition of 10 μM of the Gal4 AD peptide (Ln 3) or a control peptide (Ln 4).

The ability of the Gal4 and VP16 ADs to compete for proteasomal subunits suggests that addition of ubiquitin should also inhibit interaction between the Gal4 AD and Rpt4 and Rpt6. To test that directly, cross-linking with the DOPA-Gal4 AD peptide was used to determine if Ub alters the interaction between the Gal4 AD and the proteasomal ATPases (98). As shown in Chapter Two, this peptide cross-links to the Rpt4 subunit. The NeutrAvidin-HRP blot in Figure 4-11A shows the cross-linked product produced when the DOPA-containing Gal4 AD is mixed with the 26S proteasome and $NaIO_4$ (lane 2). The addition of increasing amounts of Ub decreased the amount of AD-Rpt4 cross-linked product by about 80% (lanes 3-5). Note that the dose dependence of this effect is similar to that observed for Ub-mediated inhibition of the destabilization process (Figure 4-2B). Addition of 10 μM SUMO (lane 6) or 10 μM K48 linked tetra-Ub (lane 7) did not decrease the amount of cross-linked product. The latter result again highlights the stark differences in the properties of Ub monomers and chains in these assays (also see Figure 4-1 and 4-3). To determine if Ub would disrupt other Gal4 AD interactions, the interaction between the Gal4 AD and the known binding partner Gal80 was monitored (65). The cross-linked product formed between DOPA-Gal4 AD and His6-Gal80 (Figure 4-11B lane 2) was not decreased by addition of Ub (lane 3-5), SUMO (lane 6), or K48 linked tetra-Ub (lane 7). This experiment also demonstrated that Ub does not interfere with the chemistry of the periodate-mediated reaction. We conclude that Ub inhibited

interaction of the Gal4 AD with the proteasomal ATPases but not with other Gal4 AD targets.

A pull-down assay was used to further confirm this result. The AD of Gal4 is known to bind the 26S proteasome and extract the base of the RP. 1 μM immobilized GST-Gal4 AD fusion protein (Figure 4-11C, lane 2) retained the Rpt4 protein in the absence of Ub, but addition of mono-Ub to 10 μM almost completely abolished the interaction. Poly-Ub chains and SUMO had little effect on the interaction at the same concentration, confirming the results of the cross-linking assay. As a control, we also tested if Ub would inhibit interactions between the 26S proteasome and the Ub-like domain (Ubl) of Rad23, a known proteasome binding protein (21) that contacts Rpn1 directly (23). GST-Ubl pulled down the 26S proteasome (Figure 4-11D lane 2) and this interaction was unaffected by addition of Ub, K48 linked tetra-Ub or SUMO (lane 3-5). These results show that the Ub effect is specific for the AD-ATPase interaction.

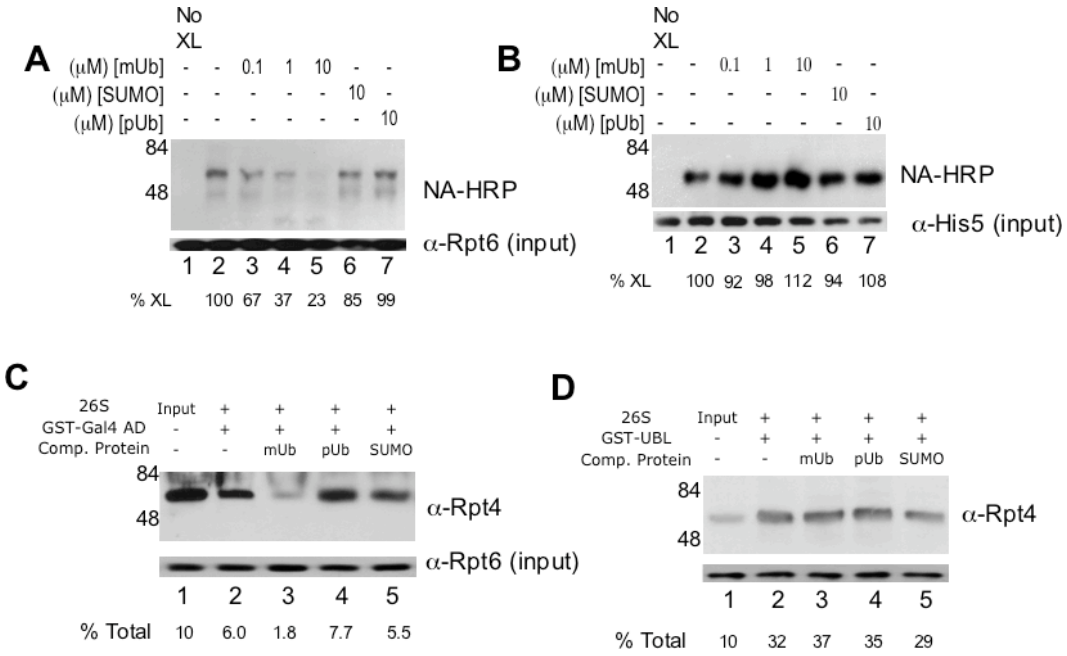

Figure 4- 11 Effect of mono-Ub on the interaction between the Gal4 AD and the proteasome A. Cross-linking was used to monitor the interaction between the DOPA-Gal4 AD peptide and its binding partners in the proteasome, Rpt4 and Rpt6. The NA-HRP blot shows the cross-linked product between the DOPA-Gal4 AD and the proteasome in the presence of the indicated protein. The Rpt6 blot of 5% of the input shows equal amounts of proteasome. The % XL under the graph is the amount of NA-HRP reactive signal in the top panel quantitated by Image J from 3 reactions with lane 2 being set as 100%. B. Same as panel A but 26S proteasome was replace by His6-Gal80. C. 1 μM GST-Gal4 AD and 50 nM 26S proteasome were incubated with or without 10 μM SUMO or Ub forms. GST-Gal4 AD and associated proteins were isolated and washed before analysis. The blot against Rpt4 shows retention of a proteasomal ATPase by the Gal4 AD. Lane 1 is 10% input of the 26S proteasome used in the experiment. % Total was quantitated as in A with lane 1 set as 10% input. D. Repeat of panel C, but using the UBL domain of Rad23 instead of the Gal4 AD.

Importance of the hydrophobic patch of ubiquitin *in vivo*

The ability of mono-ubiquitin or K63-linked tetra-ubiquitin to inhibit destabilization, but the inability of SUMO or K48-linked tetra-ubiquitin to do so, suggests that the hydrophobic patch of ubiquitin plays an important role. If the

ability to inhibit destabilization is lost upon mutation of the patch, this would strengthen the claim of the importance of the hydrophobic patch. To test this idea, point mutations were made in ubiquitin in the context of the ubiquitin-Gal4D fusion proteins. As seen in Chapter Three, fusion of ubiquitin to Gal4D partially restored activity and DNA occupancy of the protein. Mutation of I44 to alanine abrogated the activity of ubiquitin to promote the ability of the of Gal4D fusion protein to occupy the *GAL7* promoter as measured by ChIP, but the D58A mutation did not (Figure 4-12A). Mutation of the hydrophobic patch abolished the protective effect of mono-ubiquitin.

The expression level of Gal4D is known to alter the activity of the construct. Massive over expression of the protein will result in a partial recovery of the activity of Gal4D (47). To be certain that the changes seen in DNA occupancy were not due to expression level, the steady-state level of the ubiqutin-Gal4D fusions were compared to Gal4D (Figure 4-12B). There was no detectable difference in a cellular lystate.

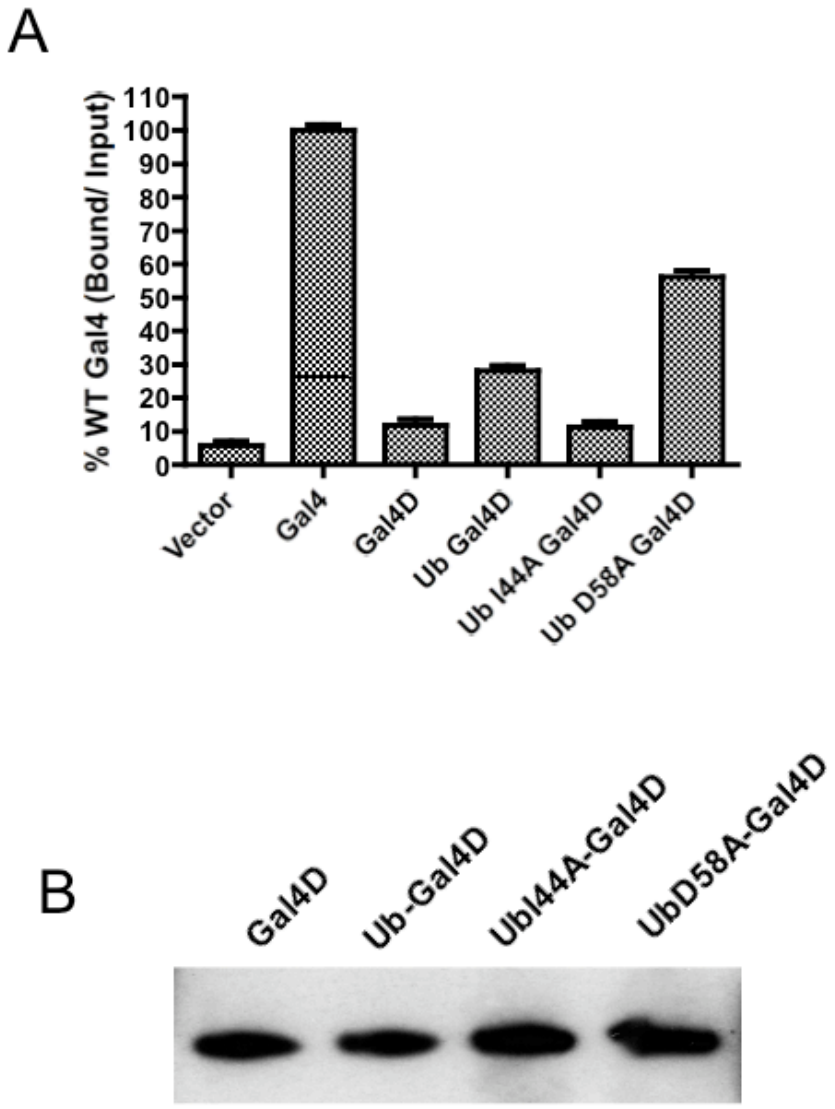

Figure 4- 12 Mutation of the hydrophobic patch of ubiquitin prevents rescue of Gal4D. A. A ChIP assay was used to monitor the DNA occupancy of the different Gal4D constructs. The data were quantitated as in Figure 3-4. B. Expression levels of Gal4D constructs are similar. The steady-state level of the indicated protein was measured by western blot with an antibody raised against Gal4.

The activities of the ubiquitin mutant Gal4D constructs were also measured using an enzyme assay to monitor production of the *MEL1* gene product and by measuring the transcript levels produced from the *GAL1* gene (Figure 4-13). Both assays demonstrated that mutation of the hydrophobic patch (I44A) decreased activity of the fusion protein to levels similar to Gal4D lacking the ubiquitin fusion. Mutation of other residues (D58A) did not decrease the activity of the protein. We conclude that the hydrophobic patch of ubiquitin is required for protection of activators from proteasomal-mediated destabilization. The differences seen between different lysine linkage of the chains can be accounted for by the availability of the hydrophobic patch for binding to the proteasomal subunits.

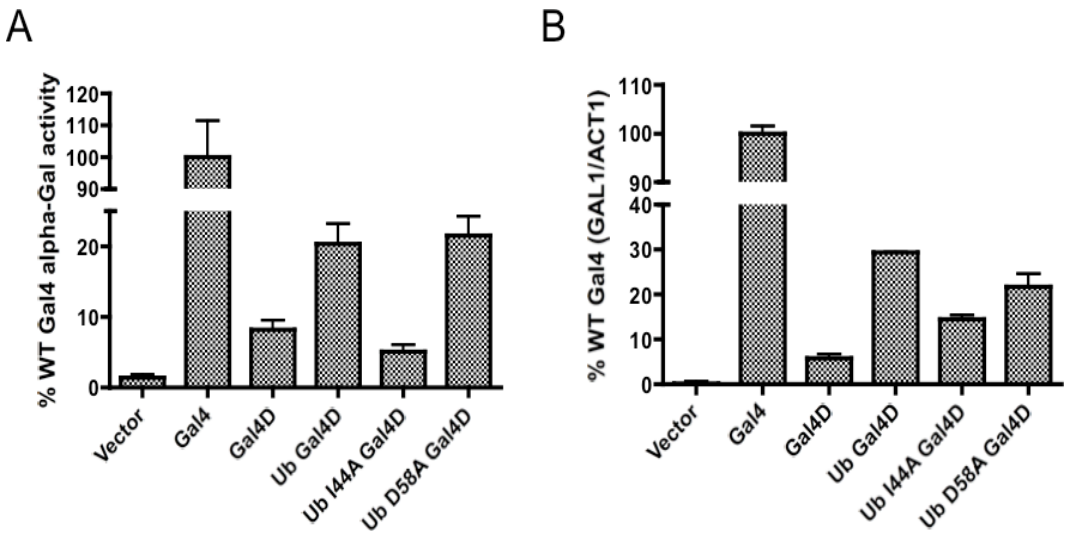

Figure 4- 13 The I44A mutation abolishes the ability of ubiquitin to promote Gal4D activity. A. An alpha-galatosidase assay measuring the protein produced from the indicated constructs. The average and SEM of three experiments are shown. B. The ratio of *GAL1* and *ACT1* mRNA is graphed based on qPCR quantitation. The average and SEM of three experiments are shown.

DISCUSSION

Previous studies from our laboratories had demonstrated that activators such as Gal4 must recruit the proteasomal ATPases in order to achieve efficient gene transcription (99,102,103). ATPase recruitment to the *GAL* genes requires direct interaction of the Gal4 AD with the Sug1/Rpt6 and Sug2/Rpt4 proteins, two of the six proteasomal ATPases (98,99). However, we also showed recently that when the ATPases engage activators, they treat them as substrates for their

protein unfolding activity (107). In the context of an activator-promoter complex, this results in potent, reversible disruption of the activator-DNA complex; a reaction that inhibits activated transcription *in vitro* and *in vivo*. This raised the question of how activators resist this activity in order to function efficiently. Using various Gal4 derivatives, we correlated the ability to resist proteasomal ATPase-mediated destabilization with ubiquitylation of an as yet uncharacterized lysine residue (107) and demonstrated that forced mono-ubiquitylation will restore activity to a truncation of Gal4 that lacks the part of the AD necessary for ubiquitylation (Archer *et al. JBC* In Press)

In this chapter we have determined the biochemical mechanism of protection against the destabilization activity of the proteasomal ATPases. A model incorporating the data presented in this study is shown schematically in Figure 4-14. Based on previous biochemical studies (99), the model supposes that the first event that occurs upon interaction of the mono-ubiquitylated activator and the proteasome is that the AD extracts the proteasomal ATPases, Rpn1 and Rpn2 (the base of the 19S RP (146), also known as the APIS complex (99)) from the proteasome, leaving the 20S core complex and the lid sub-assembly behind. Note that even if the activator is mono-ubiquitylated at this point, the Ub moiety will not bind to the 26S proteasome (Figure 4-5). The activator-APIS complex can then proceed down two pathways. The first is to dissociate, which we imagine promotes transcription by allowing the ATPases to associate with the

proximal RNA polymerase II holoenzyme and subsequently stimulate its escape from the promoter and the elongation phase of the transcription cycle. The second is to begin to unfold the activator, resulting in the destabilization of the activator-promoter complex. We propose that in the absence of activator ubiquitylation, the latter process dominates, resulting in potent inhibition of activated transcription (107). However, when the activator is mono-ubiquitylated, the Ub moiety then contacts the APIS complex directly via Rpn1 and/or Rpt1 (Figure 4-6 and 4-7) and this association results in a weakening of the AD-Rpt4/Rpt6 interactions (Figure 4-9 and 4-11), thus promoting APIS dissociation from the activator.

Put another way, we propose that the Ub-APIS contact limits the half-life of the AD-Rpt4/Rpt6 complex and reduces greatly the degree of activator unfolding that can be achieved by the ATPases before dissociating. Note that our data argue, as shown in figure 4-14, for an ordered series of events since Ub cannot interact with the intact 26S proteasome but can bind to 19S proteins after the AD has extracted the 19S base/APIS complex from the 26S proteasome (see Figure 4-5C, lane 2). As mentioned above, once the ATPases dissociate from the activator, it is appealing to imagine that they then associate with the pre-initiation complex (PIC) and help to remodel the PIC into an elongation complex and thus stimulate promoter escape and elongation (102). Our data provide no conclusive evidence for or against the continued association of the mono-Ub residues with

the APIS complex after the latter dissociates from the activator, although the poor ability of the ubiquitylated, DNA-bound activator to pull-down the ATPases (Figure 4-9) is more consistent with rapid dissociation.

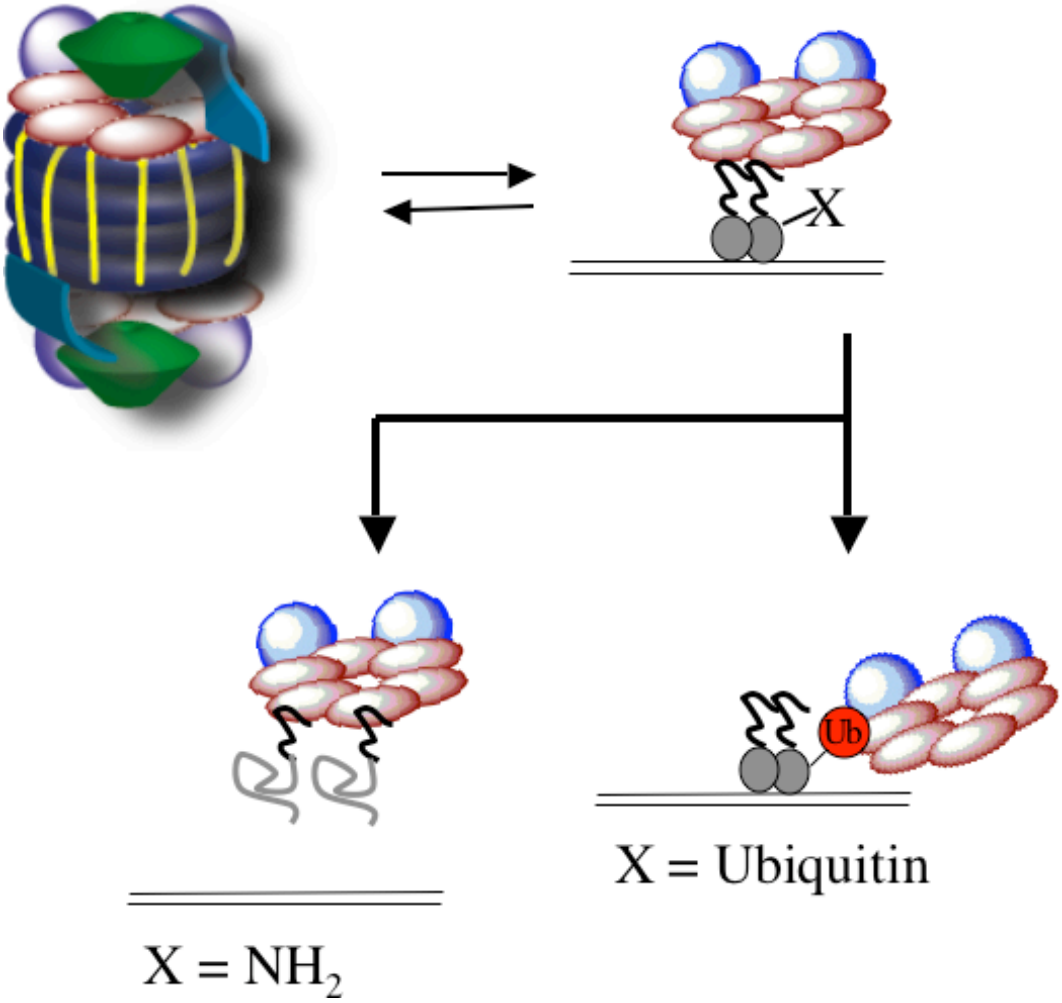

Figure 4- 14 A "hand-off" model for how activator mono-ubiquitylation facilitates activator function. DNA-bound Gal4 associates with APIS (the base of the 19RP) through direct interaction with the AD (black line). If the DBD is ubiquitylated, the Ub moiety binds to Rpn1 and/or Rpt1, resulting in the dissociation of the APIS complex from the AD. This may facilitate subsequent transfer of APIS to the pre-initiation complex (not shown) where it can stimulate promoter escape and elongation. If the activator DBD is not ubiquitylated, APIS proceeds to unfold it, resulting in dissociation of the activator-promoter complex. See text for details. X = NH_2 signifies a lysine side chain lacking the mono-ubiquitin modification. X is shown on only one of the two units of the Gal4 dimer for simplicity.

It is worthwhile reemphasizing the striking differences between the functional and physical interactions of the proteasome with different forms of ubiquitin seen in this study. Our data highlight the importance of the hydrophobic patch, centered around isoleucine 44 of ubiquitin, for inhibition of proteasomal-mediated destabilization. Proteins that lack this hydrophobic patch or have the patch hidden by structural arrangements are unable to inhibit destabilization (Figure 4-1 and 4-3). Mutation of the hydrophobic patch will abolish the ability of mono-ubiquitin to promote activity Gal4D *in vivo* (Figure 4-12 and 4-13). The availability of relatively small structural features can result in very large differences in function.

Finally, the development of the novel cross-linking/label transfer scheme (shown in Figure 4-4) has made possible the evaluation of the interaction of mono-Ub with intact proteasome complexes or sub-complexes in a relatively straightforward fashion. The results, buttressed by more classical pull-down studies, argue that mono-Ub is not a 26S proteasome-binding protein whereas previous workers have demonstrated that poly-Ub chains bind tightly to the 26S complex (18,147). On the other hand, mono-Ub binding to Rpn1 and/or Rpt1 is readily detectable by label transfer only in the absence of the 20S, revealing mono-Ub as a 19S RP/APIS-binding protein. Note that Rpn1 and Rpt1 have not, to the best of our knowledge, been implicated as poly-Ub receptors (147). At a

functional level, free Ub blocks APIS-mediated dissociation of activator-DNA complexes whereas K48 linked tetra-Ub chains at the same concentration do not exhibit this activity. These experiments highlight the fact that these different forms of Ub represent completely different modifications of a protein that will cause it to interact with the proteasome in very different ways.

CHAPTER FIVE
Conclusions and Recommendations

The data presented in this dissertation provide a biochemical mechanism for how mono-ubiquitylation of activators could promote transcription activation. This mechanism explains the Gal4D phenotype and suppression of the defective protein with the *sug1-1* and *sug2-1* mutations. Additionally, we have found another functional difference in specificity between mono-ubiquitin and tetra-ubiquitin chains and have been able to explain the difference due to the solvent exposure of the hydrophobic patch centered around isoleucine 44 of ubiquitin. Finally, we have been able to demonstrate the utility of a novel type of cross-linking and label transfer protocol.

Cross-linking

Much of the detection of protein-protein interactions in this work was done by different cross-linking technologies. The work in Chapter Two was directed towards developing this technology. This work (and the previous work by Dr. Burdine and others) demonstrated that cross-linking based on the oxidation of the DOPA group could be useful as a tool to study protein-protein interaction. We directly applied this technology in Chapter Four to study ubiquitin interacting proteins. Here the cross-linking and label transfer protocol demonstrated its

usefulness. Identification of Rpt1 and Rpn1 as the major mono-ubiquitin binding proteins in the context of the RP would have been difficult to impossible with other methodologies.

How can the cross-linking and label transfer protocol be useful in future applications? One of the easiest ways to increase the utility of the methodology is to apply it to different systems. The synthesis of the DBF compound has been standardized, the only modification required to apply this technology to other systems is to include the CCPGCC tag on the protein of interest. This ease of portability will make the DBF reagent useful for many investigators.

The one concern with portability to a new system is that optimal conditions must be determined for each system under study. In the ubiquitin/proteasome system, addition of the tag to the N-terminus was sufficient for detection of the interaction. But in many cases, the location of the tag will influence the results. In the case of the GST-Gal80 fusions (Figure 2-8), addition of the FRP tag on the C-terminal side of the Gal80 interacting peptide resulted in no label transfer, even though the GST-Gal80-FRP protein bound to Gal80 as well as the construct that contained the FRP on the N-terminal side of the Gal80 binding peptide. From our experiences, it appears that for each case the optimal conditions must be determined empirically.. Additionally, this limitation inhibits the development of a high-throughput cross-linking and label transfer system to catalogue binding proteins of all types of targets. Simply inserting that DNA

encoding your protein of interest into a stock vector containing an N-terminal FRP sequence followed by a multi-cloning site may not be very useful as it would most likely generate a large number of false negatives.

The next major advance in the cross-linking technology presented here would be to perform the reactions inside living cells. The native protein-protein interactions in their normal context could be probed in this manner. However, the cross-linking experiments attempted in this dissertation were all performed with multi-protein complexes or in the context of a cellular lysate but not performed inside cells. While the FlAsH-FRP system has been used to label proteins inside cells, the DBF reagent would not be cell permeable due to the biotin tag used for visualation. One could overcome this by exchanging the tag used. A small cell permeable epitope tag that could be recognized by an antibody would facilitate used of the reagent inside cells.

The final major concern would be the actual cross-linking chemistry. The DOPA based chemistry was shown to be amenable to the conditions present inside cells. The reducing conditions present inside the cytosol would actually help prevent activation of the compound prior to triggering the reaction. However, it is not clear how one could activate the DOPA for cross-linking, as this is done by treating the compound with an oxidizing agent. Triggering the reaction with periodate would required flooding the cell with periodate. This may or may not activate the DOPA group and could present problems within the cell

by oxidization of a large number of other proteins. It seems like a better triggering mechanism would be useful to use this reagent inside cells.

Mono-Ubiquitylated Activators

The major effort of my work was in understanding how mono-ubiquitylation of Gal4 would prevent the activator from being 'stripped' off of DNA by the proteasomal ATPases. Besides providing a detailed mechanism of this novel activity, this work explains the defect of the Gal4D protein and why it is suppressed by certain mutations in the ATPases. Finally, we were able demonstrate another functional difference between mono-ubiquitin and K48-linked tetra-ubiquitin chains and explain this difference in activity is due to availability of the hydrophobic patch of ubiquitin.

As mentioned in the introduction, many activators are known to be mono-ubiquitylated. While in some cases this mono-ubiquitylation is tied to eventual degradation, presumably through extension of the mono-ubiquitin in to a poly-ubiquitin chain, some activators do not seem to go down this route. In the case of CTIIA or SRC-3, mono-ubiquitylation leads to an increase in transcription due to recruitment of co-activators. From the studies of these proteins it was unclear if mono-ubiquitin directly recruited the co-activators or if the recruitment occurred through an indirect mechanism. We were able to show that Gal4 transcriptional activity was not increase by mono-ubiquitylation due to an enhanced recruitment of co-activators. Instead, Gal4 activity was increased because of decreased

interaction between the activation domain of Gal4 and two subunits of the proteasome, Rpt4 and Rpt6. This is a direct consequence of the interaction of mono-ubiquitin with different proteasome subunits, Rpn1 and Rpn10. Interaction with mono-ubiquitin prevents a negative activity of the proteasomal ATPases (destabilization) and allows the proteasomal ATPases to perform other activities that promote transcription.

The insights provided by the data reported here might also be important in understanding other nuclear processes that involve protein mono-ubiquitylation and non-proteolytic functions of the proteasomal ATPases. For instance, histone H2B mono-ubiquitylation functions cooperatively with the FACT complex to help the elongating polymerase move through chromatin templates (148). The proteasomal ATPases are also important for elongation (102,103) and, furthermore, FACT associates with the APIS complex (149). Another interesting case is the reported linkage of histone H2B mono-ubiquitylation and histone H3 methylation, a coupling that is somehow dependent on non-proteolytic activities of the proteasomal ATPases (101). It is possible that mono-ubiquitin-Rpn1/Rpt1 interactions are important in these events as well, either in attracting the unfoldases to histones (and perhaps thus promoting structural rearrangements that are required for subsequent covalent modifications) and/or in limiting the lifetime of a different chromatin-proximal ATPase-protein complex.

Finally, it is worthwhile reemphasizing the striking differences between the functional and physical interactions of the proteasome with mono-ubiquitin and K48-linked tetra-ubiquitin chains seen in this study. The development of the novel cross-linking/label transfer scheme first described in Chapter Two has made possible the evaluation of the interaction of mono-ubiquitin with intact proteasome complexes or sub-complexes. The results, buttressed by more classical pull-down studies, argue that mono-ubiquitin is not a 26S proteasome-binding protein whereas previous workers have demonstrated that poly-ubiquitin chains bind tightly to the 26S complex (18,147). On the other hand, mono-ubiquitin binding to Rpn1 and/or Rpt1 is readily detectable by label transfer only in the absence of the 20S, revealing mono-ubiquitin as a 19S RP/APIS-binding protein. Note that Rpn1 and Rpt1 have not, to the best of our knowledge, been implicated as poly-ubiquitin receptors (147). At a functional level, free ubiquitin blocks APIS-mediated dissociation of activator-DNA complexes whereas K48 linked tetra-ubiquitin chains at the same concentration do not exhibit this activity. These experiments highlight the fact that these different forms of ubiquitin represent completely different modifications of a protein that will cause it to interact with the proteasome in very different ways.

One of the major pieces missing from this system is the identification of the E3 ligase responsible for the mono-ubiquitylation. Other ligases have been reported to modify Gal4 and control degradation rates, but experiments suggested

that these ligases do not alter destabilization activity of the proteasomal ATPases or Gal4-mediated transcription (data not shown). Identification of this ligase would be useful so one could understand what are the possible signals that control modification of the activator. It is unknown if mono-ubiquitylation is a regulated step, a checkpoint, that is controlled by a signaling pathway. Alternatively, mono-ubiquitylation might be an indirect activity of a ligase that is recruited to the promoter by Gal4 that just happens to prevent destabilization by the proteasome. Various over-expression screens or deletion of the non-essential ligases in yeast have not uncovered the ligase in question (Melissa O'Neal and TK, unpublished data). It is unknown if these strategies failed because the ligase is essential and cannot be knocked out, the activity is redundant and deletion is compensated by another ligase, or the assumptions made in the over-expression strategy were incorrect.

An alternative strategy would be to use a biochemical approach. A standard biochemical fractionation of lysate should result in the identification of the ligase. This would allow an unbiased approach, and there is a simple *in vitro* assay available to test fractions. However, fractionation would be complicated. Mono-ubiquitylation requires prior phosphorylation of a serine in the DBD. Additionally, an E1 and E2 are also required. The identification of all of these components is still in progress. Thus, to find fractions with E3 ligase activity would either require that all of the components co-fractionate, correct

assumptions about the identity of the other required proteins so they can be added into each fraction, or rather complicated combining of fractions. Nevertheless, this strategy could be done.

A second biochemical strategy would take advantage of some of the information gleaned from this work. The C-terminal region of the Gal4 AD (AD CTR) is required for mono-ubiquitylation and will even inhibit mono-ubiquitylation if added in excess, presumably because of interaction with the ligase. This result suggests that this AD CTR could be used as an 'affinity tag' for purification of the ligase. Indeed, an initial attempt of this strategy was tried by performing a pull-down of yeast lysate with the AD CTR followed by mass spectroscopy analysis of the isolated proteins. There were only a handful of proteins specific for the AD CTR, but none of these proteins were E3 ligase components or any member of the ubiquitylation machinery (CTA, LB, Alan Tackett, and TK, unpublished data). Many of the proteins isolated were highly abundant proteins (histone components) or RNA modification enzymes. This approach is still reasonable, but the pull-down step and subsequent washes must be modified to remove the highly abundant proteins that are currently interfering with the analysis.

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