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Signals Through the SCF (slimb) Ubiquitin Ligase Pathway

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13. ABSTRACT (Maximum 200 Words) NFkB is a transcription factor that functions to block the apoptotic response. Inappropriate activation of NFkB is thought to block apoptosis in breast cancer cells. NFkB activity is negatively regulated by a signaling pathway that responds to extracellular signals, including cytokines. Normally, NFkB is held in the cytoplasm by its inhibitor, IkB. In response to extracellular signals, IkB is destroyed by the process of ubiquitin mediated proteolysis. This process is activated through protein kinases that respond to cytokines such as TNFalpha. These kinases phosphorylate IkB, thereby activating it for ubiquitination. Ubiquitination involves 3 activities: an E1 activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin-protein ligase. In work supported by this grant, we have identified the molecular components involved in IkB ubiquitination. The ubiquitin ligase is composed of Skp1/Cul1/Rbx1 and the specificity factor beta-TRCP. Beta-TRCP binds IkB in a phosphorylation dependent manner and targets it for ubiquitination via the SCF pathway. These data suggest that molecules which interfere with IkB ubiquitination by the SCFbeta-TRCP complex could function as anti-apoptotic agents.				
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FOREWORD

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Introduction

The process of apoptosis is critical to the development and homeostasis of multicellular organisms. It provides a mechanism for loss of cells during organogenesis and provides a pathway for removal of cells that are undergoing inappropriate proliferative events or have received unreparable DNA damage. A theme in cancer biology is that cellular transformation requires the establishment of survival pathways that limit the process of apoptosis. Recent studies have revealed that one such survival pathway is established through the action of the transcription factor NF κ B. NF κ B plays important roles in activation of genes in response to cytokines and other stimuli, and has been well characterized with respect to its role in inflammatory diseases. Cytokines such as TNF- α activate two pathways, one that activates a cell death response, and one that activates a survival response that is dependent upon NF κ B. This finding has renewed interest in the development of inhibitors of the NF κ B pathway that can be used therapeutically to block survival pathways while simultaneously allowing for agents such as TNF or chemotherapeutics to activate apoptotic pathways. The use of such combination therapy has the potential to allow proliferative control of established tumors. Because of the widespread nature of the NF κ B survival pathway, such NF κ B inhibitors would be expected to be useful in a wide variety of proliferative diseases and mammary cancer is no exception. Recent studies have revealed that mammary tumor cells utilize NF κ B in an anti-apoptotic mechanism and display increased NF κ B activity that correlates with estrogen-independence. NF κ B function is normally controlled by I κ B, which holds NF κ B in the cytoplasmic. Signals which activate NF κ B lead to phosphorylation of I κ B, which signals it for ubiquitin-mediated proteolysis thereby allowing NF κ B to enter the nucleus. The importance of I κ B in the survival function of NF κ B is demonstrated by the fact that overexpression of non-phosphorylatable I κ B blocks NF κ B function, allowing for apoptosis.

Because of its critical position in the NFB activation pathway, the I κ B ubiquitin ligase represents an important therapeutic target. Blocking its activity would be equivalent to overexpression of non-phosphorylatable I κ B and would be expected to lead to inhibition of NF κ B. In the last progress report, we described our efforts to understand how the I κ B protein is regulated in response to its phosphorylation by I κ B kinase (IKK). It is now clear from our work and work of others that phosphorylation of I κ B by IKK allows it to interact with a ubiquitin ligase, SCF $^{\beta\text{-TRCP}}$ (Winston et al., 1999; Maniatis, 1999). Ubiquitination of I κ B by the SCF $^{\beta\text{-TRCP}}$ leads to the destruction of I κ B by the proteasome. SCF complexes function as E3 ubiquitin ligases and are composed of Skp1, the Ring-H2 finger protein Rbx1, the Cul1 protein, and an F-box protein, in this case $\beta\text{-TRCP}$. This complex recognizes I κ B in a phosphorylation-dependent manner and catalyzes I κ B ubiquitination in vitro, in conjunction with an E2 ubiquitin conjugating enzyme and an E1 activating enzyme. Analysis of the role of $\beta\text{-TRCP}$ in the destruction of I κ B was a major goal of Aim 1. In the past year, we have explored the biochemistry of the interaction of $\beta\text{-TRCP}$ with I κ B using physical and mutagenic approaches. As described below, this has revealed a consensus sequence recognized by $\beta\text{-TRCP}$, which is found in additional targets of I κ B, including $\beta\text{-catenin}$. In addition, we have identified residues on the surface of $\beta\text{-TRCP}$ required for recognition of substrates. These studies were major

goals of Aim 2.

Body

Identification of a consensus substrate recognition motif for β -TRCP.

As a first step towards understanding how β -TRCP interacts with I κ B and β -catenin destruction motifs, we wanted to search for sequences that are able to bind to β -TRCP. One approach to this problem, which we proposed, is the use of peptide libraries containing a large number of diverse sequences. Sequence analysis of peptides that bind to a particular protein provides a consensus sequence for binding which can then be followed up with more detailed experiments, depending upon the degeneracy observed. Together with Dr. Songyang who developed the peptide library approach, we performed an analysis of β -TRCP. Because large amounts of immobilized protein is required for this technique, we had to develop a system for expressing large quantities of functional β -TRCP. Preliminary experiments indicated that expression of β -TRCP in bacteria was sub-optimal and the protein that could be expressed as incapable of binding to I κ B (data not shown). Therefore, we developed an insect cell expression system wherein we co-express untagged β -TRCP with GST-Skp1. Complexes are then purified using GSH-sepharose. Using this approach we were able to generate sufficient amounts of essentially homogeneous GST-Skp1/ β -TRCP complexes for binding studies.

Because we already knew that β -TRCP interacts with phospho-serine containing destruction motifs, we used a peptide library containing two fixed phosphoserine residues with the first phosphoserine preceded by an aspartate. Peptide library was incubated with immobilized GST-Skp1/ β -TRCP and the beads washed extensively. Peptide was eluted by treating the complex with acid (pH2) and released peptides subjected to Edman degradation to determine the collection of peptide sequences. The data are shown in Fig. 1(appendix). The consensus was ϕ - ϕ -[A,N]-D-pS-[G,E,N,Y]-[Y,E]-[A,F,Y,E]-pS-[Y,F]-[Y,F] (where ϕ = a hydrophobic amino acid). Some aspects of this consensus conform to the sequences of I κ B and β -catenin while other aspects do not. For example selection of Y and F residues in the last two positions was not expected, based on the I κ B sequence. This suggests that it might be possible to generate a specific inhibitor of β -TRCP. Several other libraries were tried, including single phosphoserine libraries but these did not bind, suggesting that two phospho-serines are required for binding.

Identification of residues involved in recognition of I κ B and β -catenin by β -TRCP.

As a second step towards identifying small molecules that interact with β -TRCP and block association with I κ B and β -catenin, we sought to identify residues in β -TRCP that are required for this association. β -TRCP is a member of the WD40 repeat family of proteins and contains 7 WD40 repeats. Proteins containing 7 WD40 repeats, such as β -transducin, form a β -propeller structure in which each WD40 repeat forms a blade of the 7-blade propeller. We hypothesized that basic residues

(lysine and arginine) located on the surface of β -TRCP might function in the recognition of phosphorylated I κ B and β -catenin destruction motifs. To examine this question, we developed a model of β -TRCP based on the known structure of β -transducin. Using this model, we identified lysine and arginine residues that are conserved among β -TRCP family members but not other family WD40 containing F-box proteins. Residues were classified as either being on the surface of the face of the propeller that binds α -transducin, the face of the propeller that binds γ -transducin, or buried in the central core of the propeller. Residues identified by this exercise are shown in Fig. 2 (appendix).

Given the likelihood that ligands bind β -TRCP from the α -face, we made point mutants in all of the conserved basic residues on this surface. These residues in β -TRCP were changed to alanine by site-directed mutagenesis and the mutant cDNAs cloned into expression vectors. Proteins were expressed and tested for binding to I κ B and β -catenin destruction motif peptides in either the phosphorylated or unphosphorylated forms. As shown in Figure 3 (appendix), mutations of most of the residue had no effect on the interaction of β -TRCP with I κ B and β -catenin destruction motifs. However, mutation of two residues - Arg306 and Lys289 - led to dramatic decreases in binding affinity. These two residues are located in the first WD40 repeat, adjacent to the F-box motif. Previous deletion studies have revealed that this WD40 repeat is important for interaction with β -TRCP targets.

Specificity of ligand binding by WD40 repeat-containing F-box proteins.

As described in the previous progress report, we have cloned a family of mammalian F-box proteins, including WD40 and leucine rich repeat containing proteins. Previously, we had found 5 WD40 repeat containing proteins. In the last year, we have identified 2 additional WD40 repeat containing F-box proteins, Fbw6 and Fbw7. Given this rather large number of F-box proteins, we wondered whether there was a common theme to substrate recognition or whether different F-box proteins used different structural elements to interact with targets. To date, the only other WD40-containing F-box protein whose substrate has been identified is Fbw7. We recently found that Fbw7 is responsible for ubiquitin-mediated destruction of cyclin E. Like β -TRCP, Fbw7 interacts with a short phosphorylated destruction motif in cyclin E, Leu-Leu-phosphoThr-Pro-Pro. We used a similar strategy as described for β -TRCP to identify residues involved in binding of cyclin E to Fbw7. This analysis revealed that three arginine residues were important for the cyclin E interaction (Fig. 4, appendix). Unlike the situation with β -TRCP, these residues were located in WD40 repeats 3, 4, and 5. Mutation of these residues independently to alanine either abolished or greatly reduced the interaction of Fbw7 with cyclin E. These data suggest that different WD40 repeat elements confer substrate specificity upon different F-box proteins. This work is currently being written for publication.

WD40 elements alone are insufficient for destruction motif recognition.

Given the results with β -TRCP and I κ B/ β -catenin destruction motifs, we were interested in determining whether isolated WD40 elements could interact with phosphopeptides. We generated β -TRCP proteins that were truncated after each WD40 repeat and tested these for binding to I κ B destruction motifs. Only the full length protein containing all 7 WD40 repeats was capable of binding (data not shown). It is likely that this reflects the absence of structural stability of the WD40 β -propeller structure when one or more repeats are missing.

Discussion

Activation of NF κ B involves an extensive signal transduction pathway that culminates in the destruction of the NF κ B inhibitor I κ B. We have demonstrated that I κ B is ubiquitinated by an SCF $^{\beta$ -TRCP ubiquitin ligase complex. In principle, molecules that block I κ B destruction could act as pro-apoptotic agents. F-box proteins such as β -TRCP function by binding to destruction sequences, and in I κ B, the destruction motif is created upon phosphorylation at Ser32 and Ser34. The sequences we have identified by peptide library analysis (Fig. 1) correspond to the sequence of I κ B. These sequences also occur in β -catenin and both biochemical and genetic evidence indicate a role for β -TRCP in interacting with β -catenin or armadillo in flies (Jiang and Struhl, 1998). β -catenin is a component of the Wingless/Wnt signaling pathway and functions with Tcf/Lef transcription factors to regulate patterning and other developmental decisions. Recent work in *Xenopus* has revealed that expression a β -TRCP protein lacking the F-box leads to accumulation of β -catenin and ectopic activation of the Wnt pathway (Marikawa and Elinson, 1998). This, together with our data linking β -TRCP to direct recognition of the phosphorylated β -catenin destruction motif strongly implicates SCF $^{\beta$ -TRCP as the β -catenin ubiquitin ligase. Further studies are required to determine whether any of the many proteins containing the DSG ϕ XS motif are also substrates for SCF $^{\beta$ -TRCP.

Our results also indicate that different F-box proteins employ distinct WD40 repeats to recognize substrates. β -TRCP employs WD40 repeat number 1 primarily while Fbw7 employs WD40 repeats 3-5. This indicates that agents that interact with distinct structural components of WD40 repeat proteins may display specificity towards individual family members. The crystal structures of F-box proteins associated with their targets will help clarify how destruction motifs are recognized and will facilitate the identification of agents that can block interactions with destruction motifs.

Research Accomplishments:

Year 1

* Identification of the I κ B ubiquitin ligase

* Demonstration that the SCF $^{\beta$ -TRCP complexes recognizes I κ B in a phosphorylation dependent manner

- * Identification of the β -catenin ubiquitin ligase
 - * Identification of a second β -TRCP gene in the human genome
- Year 2
- * Identification of a consensus sequence for β -TRCP substrates
 - * Identification of residues located in WD40 repeat 1 required for interaction of β -TRCP with substrates
 - * Identification of a β -TRCP homolog and a determination of the residues in this protein required to interact with its substrate cyclin E

Reportable outcomes.

Publications:

Winston, J.T., Strack, P., Beer-Romero, P., Chu, C., Elledge, S.J., and Harper, J.W. (1999) The SCF ^{β -TRCP} ubiquitin ligase specifically associates with phosphorylated destruction motifs in I κ B and β -catenin and stimulates I κ B ubiquitination in vitro. **Genes and Development**, 13, 270-283. (recognized as a "Hot Paper" by ISI, ranked 5th among all papers for citations in 1999)

Winston, J.T., Koepp, D.M., Zhu, C., Elledge, S.J., and Harper, J.W. (1999) A family of mammalian F-box proteins. **Current Biology** 9, 1180-1182.

Koepp, D., Schaffer, L., Ye, X., Keyomarsi, K., Chu, C., Harper, J.W., and Elledge, S.J. (2001) Phosphorylation-dependent ubiquitination of cyclin E by a conserved SCF^{Fbw7} ubiquitin ligase. submitted to **Science**. (contains data in Fig. 4 related to the specificity of the interaction of related WD40 containing F-box proteins such as with their substrates)

Winston, J.T., Songyang, Z., and Harper, J.W. Recognition of of phosphopeptides by the β -TRCP and Cdc4 F-box proteins. manuscript in preparation.

Conclusion

Aim 2 of this research project seeks to understand how β -TRCP interacts with I κ B, with the hope of exploiting this interaction to block NF κ B activation. In principle, small molecules that block the association of I κ B with β -TRCP could block the anti-apoptotic activities of NF κ B by maintaining it in the inactive form bound to I κ B. One potential limitation of this approach is that β -TRCP also functions in the ubiquitination of β -catenin. In the coming year, we plan to further our understanding of the interaction of β -TRCP with other targets and to examine whether blockage of β -TRCP activity will be pro-apoptotic.

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Appendix:

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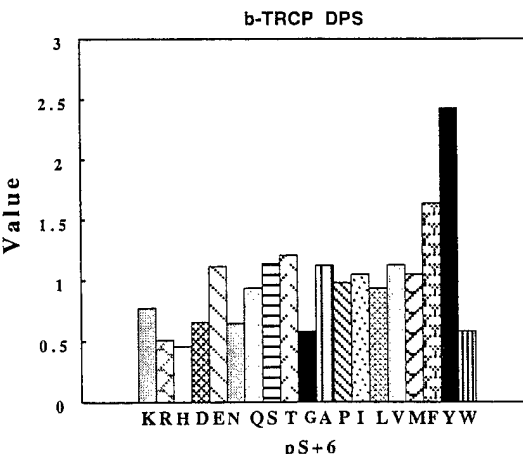
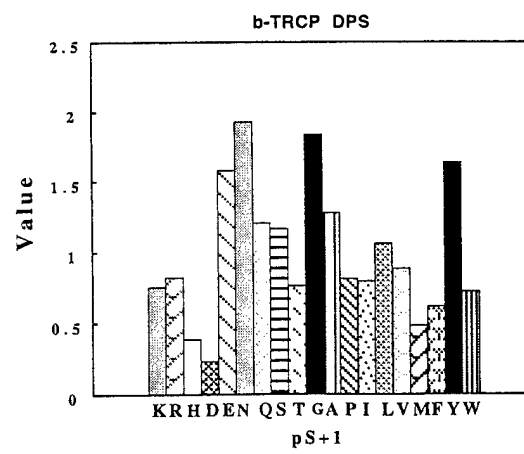
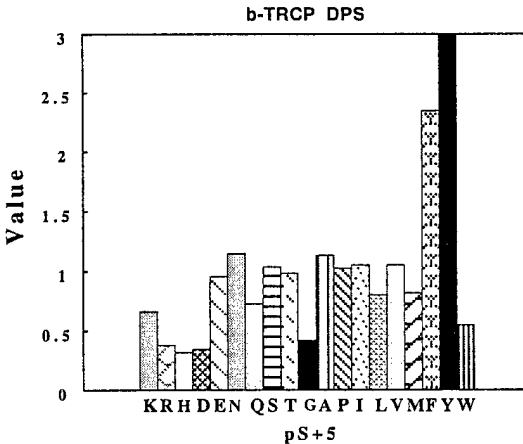
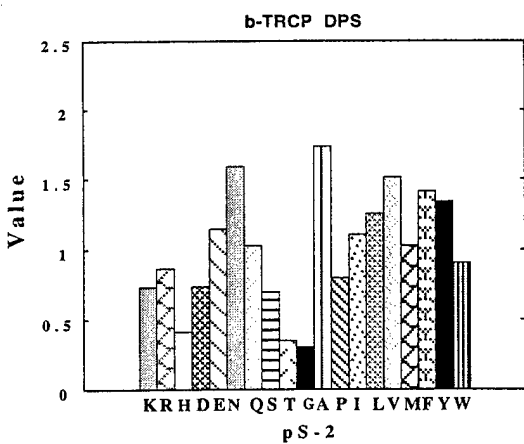
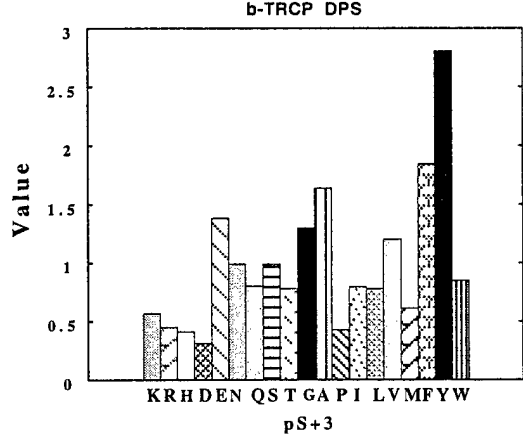
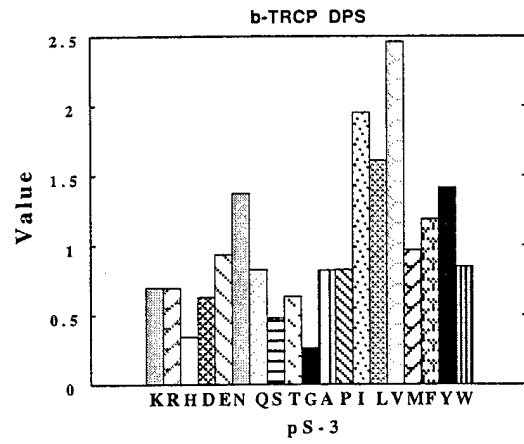
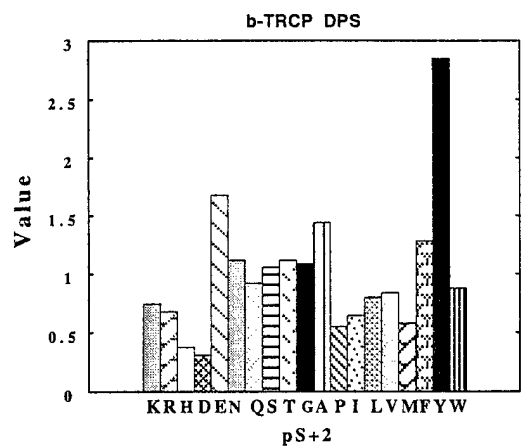
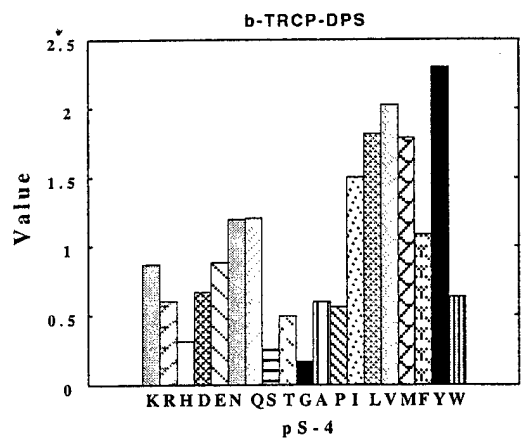
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PRINCIPAL INVESTIGATOR: Jeffrey Harper, Ph.D.

Figs 1-4

Figure 1 (legend)

Determination of consensus sequences for interaction of phospho-peptides with b-TRCP. The relative abundance of amino acids at each position in the peptide library KNXXDpSXXpSXXAK where the first pS is residue zero is indicated. These relative levels were determined by Edman degradation of peptides after selection on GST-Slk1/ β -TRCP.



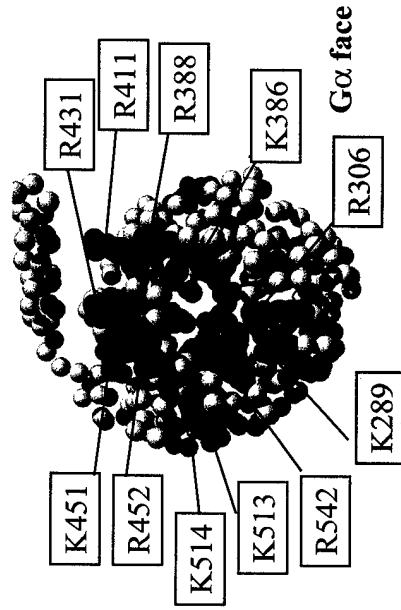


Figure 2. Model of β -TRCP based on the structure of β -transducin. Lysine and arginine residues on the surface of TRCP and corresponding to the face of β -transducin that binds α -transducin are shown in blue. The residue numbers chosen for mutagenesis are indicated in boxes. The boxes in blue represent residues that are required for interaction of TRCP with IRB and β -catenin

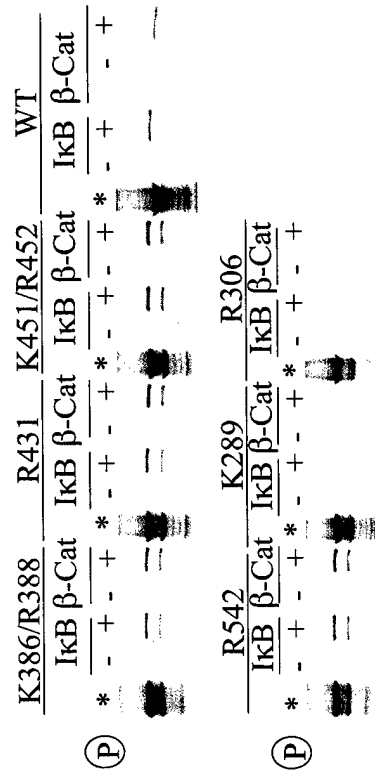


Figure 3. Binding of β -TRCP mutants to I κ B and β -catenin phosphopeptides. In vitro translated β -TRCP and mutants (*) were used for binding reactions with beads containing either unphosphorylated or phosphorylated destruction motifs from I κ B and β -catenin. After binding, proteins were separated by SDS-PAGE and detected by autoradiography.

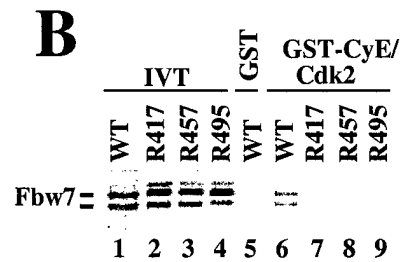
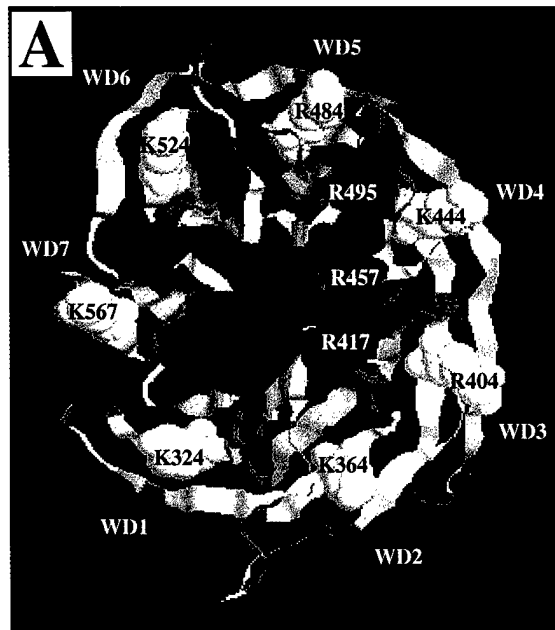


Figure 4. Analysis of the β -TRCP homolog Fbw7 and its motifs involved in interaction with cyclin E. A model of Fbw7 was generated and conserved arginine and lysine residues not present in β -TRCP found (red). These residues were mutated to alanine and in vitro translation products used for binding to GST-cyclin E/Cdk2. The R417 and R457 mutants displayed no detectable binding while the R495 mutant displayed reduced binding.