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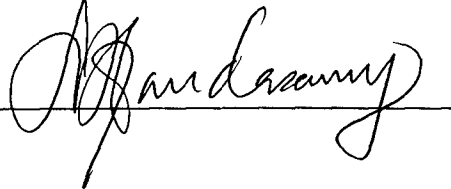
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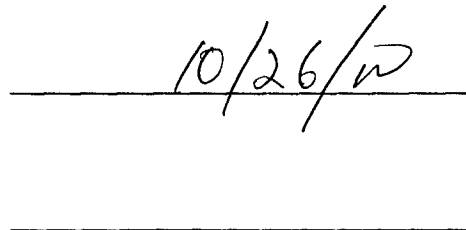
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Principal Investigator
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13. ABSTRACT (Maximum 200 words) Our interest in proteins which modulate the transcriptional activity of members of the nuclear receptor superfamily led to the identification of yeast RSP5 and its human homolog hRPF1/Nedd4, which potentiate progesterone receptor (PR)-dependent transcription. Subsequently, we have observed that hRPF1 is a potentiator of p53-dependent transcriptional activation. As hRPF1 appears to modulate two transcription factors known to play a role in breast cancer, we are interested in the identification of substrates of hRPF1's enzymatic activity which may explain its transcriptional effect. hRPF1 shares sequence homology with a known family of 'hect' E3 ubiquitin ligases. Both hRPF1 and its yeast homolog, RSP5, have been shown to bind to and ubiquitinate the large subunit of RNA polymerase II. We postulate that there may be additional hRPF1 substrates, the identification of which will help to explain our observations linking E3 ubiquitin ligase activity and the general transcriptional machinery. Using a yeast two-hybrid approach, we have identified a 68 kDa pre-mRNA cleavage factor which specifically binds to and serves as a ubiquitination substrate for hRPF1. As transcription and RNA processing are known to be integrated processes, it is likely that alterations in protein levels or function of either of these two proteins may explain the observed effects on PR- and p53-dependent transcription.					
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Maria R. Giacani 7/29/99
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Introduction:

In our search for proteins which modulate the transcriptional activity of steroid hormone receptors, we previously identified the yeast protein, RSP5, and its human homolog, hRPF1 as potentiators of progesterone receptor (PR)-dependent transcription (1). Further analyses indicated that human hRPF1/Nedd4 was also able to potentiate the transcriptional activity of the tumor suppressor, p53. hRPF1 shares significant sequence homology with the conserved family of 'hect' E3 ubiquitin-ligase proteins (2). Our hypothesis is that hRPF1 is able to ubiquitinate and/or signal the degradation of a protein substrate which is required for both PR- and p53-dependent transcription. The PR receptor signalling pathway exerts a proliferative effect in mammary carcinomas which can be blocked by anti-progestins, and p53 is known to be mutated in a significant number of human breast cancers. These two signalling molecules play key roles in the molecular pathogenesis of breast cancer; therefore the identification of protein substrates of hRPF1, a protein which potentiates both PR- and p53-dependent transcriptional responses, is crucial to our understanding of human breast cancers.

A. Identification of proteins which bind to hRPF1

Using a yeast two hybrid approach, Staub et al. identified the PY motif of the epithelial Na⁺ channels as a potential target of rat NEDD-4 activity (3). As a homolog of NEDD-4, RPF1 is an excellent candidate for a yeast two-hybrid approach to identify interacting targets.

Experimental Methods:

The yeast homolog of hRPF1, yRSP5, has previously been shown to bind to and target the large subunit of RNA polymerase II for ubiquitination and degradation. Based upon the region of RSP5 required for RNA pol II binding, we chose to use the amino terminus of hRPF1 (aa. 193-506) as bait in a yeast two-hybrid screen.

hRPF1 (aa. 193-506) was subcloned in the the Gal4DNA binding domain vector (pGBT9; Clontech) and expression was verified by western blot analysis (anti-Gal4DBD; Santa Cruz). Yeast were cotransformed with bait (pGBT9-hRPF1) and HeLa yeast two-hybrid library (Matchmaker; Clontech) plasmids and screened for a His⁺LacZ⁺ phenotype. Careful analysis of potential positives included plasmid recovery and retransformation of both bait (hRPF1) and prey (library clone) into two different yeast strains and confirmation that both plasmids were required for the His⁺LacZ⁺ phenotype. cDNA clones were subsequently sequenced, and sequence data was used to BLAST search (<http://www.ncbi.nlm>) several databases with the goal of discovering identity or homology to proteins of known function.

To independently confirm the interaction of yeast two-hybrid positives with hRPF1, we used an *in vitro* GST-pulldown approach, concurrently identifying regions in hRPF1 which were required for interaction. Briefly, library cDNAs from the yeast 'prey' vector, pGADGH, were subcloned in frame into a GST fusion vector (Pharmacia), and GST-fusion proteins were overexpressed and purified from bacteria. GST-fusion proteins were immobilized on glutathione-sepharose (Pharmacia) and incubated with *in vitro* translated, ³⁵S-methionine labelled hRPF1, or portions thereof. After thorough washing, bound proteins were resolved using SDS-PAGE and visualized by autoradiography. In

several instances, the parallel experiment was performed, with GST-hRPF1 incubated with *in vitro* translated, ³⁵S-methionine labelled two-hybrid positive proteins.

While these experiments were initially performed using partial library cDNAs, we have subsequently used 5'RACE to obtain 5' end sequence of the partial cDNA library clones. Full length cDNAs were cloned by PCR using Pfu polymerase (Stratagene), and sequenced before use in all subsequent experiments.

Results and Discussion:

Portions of six different human cDNAs were isolated from a Hela two-hybrid library (summarized in Table 1) based upon their ability to bind to the amino terminus of hRPF1. Of these six different library cDNAs, four were recovered multiple times in our screen, suggesting that we thoroughly screened a representative number of library clones.

In addition to simply providing interaction confirmation, the GST-pulldown experiments were used to identify regions of hRPF1 which were required for interaction, dividing the 6 cDNAs into two classes. The 'regulatory' class consists of two homologous human proteins which are able to bind to both the C2/calcium lipid binding domain of hRPF1 or the central WW domains. The 'substrate' class includes the four proteins identified which bind to hRPF1 solely through the WW protein-protein interaction motifs. Interestingly, these four 'substrate' proteins all contain 'PPXY' motifs within the protein fragment encoded by the library cDNA clone. As proline rich motifs such as 'PPXY' have been described as consensus sequences to which WW domains bind (4), these sequences in the proteins of the 'substrate' group likely mediate the direct binding to the WW domains of hRPF1.

This is in fact the case for one interactor, the 68 kDa RNA processing subunit, as substitution of two alanines within this 'PPXY' motif is sufficient to disrupt binding of 68 kDa protein to hRPF1 in a GST-pulldown interaction assay (Figure 1). Similar experiments testing the remaining substrates' requirement of the 'PPXY' motif for hRPF1 interaction are in progress.

While we are enthusiastic about pursuing both 'regulatory' and 'substrate' classes of hRPF1 interactors, for the purpose of this proposal, we have focused much of our efforts on characterizing the 'substrate' group of proteins, in particular the 68kDa protein subunit of the CF I_m pre-mRNA processing complex (cDNA obtained from Dr. Walter Keller, Basel, Switzerland).

The 68kDa protein is one of four subunits which comprise the CF I_m complex which is required for the 3' processing/cleavage of RNA transcripts (5). Containing several domains characteristic of RNA associated proteins, the 68 kDa subunit has two RRM's (RNA Recognition Motifs) in the amino terminus, a central proline-rich domain, and an alternating charge domain in the carboxyl terminus. As the goal for our studies has been to identify proteins which may elucidate RPF1's role in PR- and p53-dependent transcription, this 68 kDa protein is a promising candidate. RNA processing and transcription are increasingly understood to be coupled events (6-8). Therefore, it is possible that this 68kDa protein is present within the transcription complex, and alterations in stability or modification of it may be sufficient to alter a transcriptional response.

The only other 'substrate' protein with a matching cDNA in the database was the human tom-1 like protein, which has an amino terminal domain similar to that found in HRS and STAM proteins and a central coiled-coil domain. Based upon these structural homologies, it has been proposed that the human tom-1 like protein plays a role in vesicular trafficking and degradation of growth factor receptors (9).

B. *In Vitro* Ubiquitination Assays

We have identified several proteins which interact with the amino terminus of hRPF1; however, their designation as 'substrates' of hRPF1 activity is only possible after demonstration that they are capable of being ubiquitinated by hRPF1. With this goal, we have established in our laboratory, an *in vitro* ubiquitination assay using either purified yRSP5 or hRPF1 as the E3 ubiquitin-ligase enzyme.

Experimental Methods:

Similar to published descriptions (10), the ubiquitination assay consists of the incubation of *in vitro* translated, ³⁵S-methionine labelled 'substrate' protein with recombinant E1 (ubiquitin-activating), E2(ubiquitin-conjugating) and E3 (ubiquitin-ligase) enzymes, and visualization of a ubiquitin-conjugated protein ladder using SDS-PAGE followed by autoradiography. The 'substrate' candidate may be translated in either wheat germ lysate or rabbit reticulocyte lysate (TNT; Promega) and while E3 enzyme preparations are generally purified protein, the E1 and E2 are soluble extracts from E.coli which overexpress the relevant ubiquitin enzyme.

Several different techniques were utilized to produce recombinant purified E3 enzyme. While we originally proposed the purification of recombinant hRPF1 from a yeast system, we subsequently chose to use either a baculovirus/SF9 cell or bacterial overexpression system, as several other 'hect' E3 ubiquitin ligases have been purified with success using these techniques. In our first attempt to produce enzymatically active hRPF1, we first produced recombinant baculovirus using the pFastBac system (Gibco BRL). Baculovirus encoding His-RPF1 or His-RPF1-C867A was used to infect insect Sf9 cells, and His-tagged proteins were purified using Ni-NTA resin (Quiagen). Purified hRPF1 proteins were tested in standard ubiquitination assays, using bacterially produced yRSP5 and its substrate, the large subunit of RNA pol II, as a positive control.

GST-RSP5 (gift from Dr. Jon Huibregtse) was overexpressed and purified from BL21 bacterial cells and RSP5 was cleaved from the GST tag using Prescission protease (Pharmacia). hRPF1 or hRPF1 (aa. 173-900) was also expressed and purified using this bacterial system and assayed on the known substrate, the large subunit of RNA polymerase II.

Results and Discussion:

We have demonstrated that the 68 kDa subunit of the CF Im complex is an *in vitro* substrate of hRPF1 and yRSP5 activity (Figure 2). We believe that this is a specific activity, as a 'PPXY' mutant which no longer binds hRPF1 or yRSP5 was no longer a substrate of yRSP5 ubiquitination, while alanine substitutions in two distinct proline rich motifs had no deleterious effect upon binding or ubiquitination.

In fact, each member of the 'substrate' group analyzed thus far is a ubiquitination substrate of yRSP5. However, these analyses have not been trivial due to the difficulties which we have encountered in purifying enzymatically active hRPF1.

We have found all preparations of full length hRPF1 from baculovirus/SF9 cells to be inactive. A collaborator, Dr. Jon Huibregtse, has similarly observed that full length recombinant hRPF1 protein purified from various sources is enzymatically inactive. His laboratory has had success in purifying an active truncated form of hRPF1 (whect = aa. 193-900) from bacteria, and preparations of this active enzyme are able to ubiquitinate the 68 kDa pre-mRNA processing subunit, identified as a hRPF1 substrate in our laboratory. We do not yet know the physiological significance of the observation that the whect portion of hRPF1 is sufficient for ubiquitination activity *in vitro*. However, recent reports indicate that cellular hRPF1/Nedd4 is cleaved by caspases upon apoptotic stimuli into a truncated form which corresponds in size to the whect portion of the protein (11). This raises the

possibility that the amino terminal C2/Ca²⁺ lipid binding domain (absent in the enzymatically active whect enzyme preparations) is inhibitory or associates with proteins which block the ubiquitinating activity of hRPF1.

These difficulties we have encountered in producing recombinant enzymatically active hRPF1 enzyme underscore the importance in understanding the regulation of its activity. We predict that the 'regulatory' group of proteins identified in our yeast two-hybrid screen may provide future clues to the proper regulation of hRPF1 activity.

C. Ubiquitination in Cultured Cells

The goal behind the identification of substrates of hRPF1, was to elucidate the mechanism of RPF1 action on the PR-dependent and p53-dependent transcriptional responses. However, the complex transient transfection assay which is used to analyze RPF1's transcriptional effect presupposes that overexpression of hRPF1 is sufficient for the ubiquitination and regulation of a substrate protein. With this end, our preliminary *in vivo* experiments have been to overexpress hRPF1 and analyze the effect on a cotransfected tagged substrate protein.

Experimental Methods:

The 68 kDa pre-mRNA processing subunit protein and its corresponding non-ubiquitinatable mutant were subcloned into a GFP fusion vector (pEGFP-N1, Clontech) and transiently transfected into HeLa cells using Lipofectin (Gibco BRL). hRPF1 or hRPF1-C867A was cotransfected, and cell lysates were prepared 24-48 hrs post transfection. Western blot analysis was performed using a polyclonal anti-GFP antibody (Clontech) to analyze protein levels of tagged-substrate proteins.

Results and Discussion:

Coexpression of hRPF1 and GFP-68kDa or GFP-68kDa(mut) might be predicted to result in decreased protein levels of WT substrate, but not the corresponding mutant. However, we observed no significant change in substrate (exogenous or endogenous) protein levels as detected by western blot analysis. We are currently in the process of examining the stability of 68kDa or 68kDa(mut) using pulse-chase analysis. However, several possibilities exist to explain our observations.

As little is known about the *in vivo* regulation of mammalian 'hect' E3 ubiquitin-ligase proteins such as hRPF1, it is likely that simple overexpression of the full length cDNA for hRPF1 is not sufficient to mimic an activation event required for hRPF1 activity. This is consistent with our observations that full length hRPF1 has not been yet demonstrated to be enzymatically active *in vitro*. With this in mind, we also chose to overexpress the truncated hRPF1-whect (aa. 193-900) and assay for changes in cotransfected GFP-substrate protein levels. However, these analyses to date have not demonstrated a direct correlation between hRPF1 and degradation of a substrate protein *in vivo*.

We are also aware of the possibility that hRPF1 substrates identified may be ubiquitinated *in vivo*, but not subject to degradation through the proteasome pathway. There have been several examples of proteins for which ubiquitination is a reversible modification, which is removed by the action of de-ubiquitinating enzymes. This possibility is being explored using immunoprecipitation of substrates, and subsequent detection of ubiquitin-conjugates with an anti-ubiquitin antibodies.

Conclusions:

In summary, we have been successful in the identification of a 68kDa pre-mRNA processing factor as a substrate of hRPF1 ubiquitination *in vitro*. Additionally, our collaborative work with Dr. Jon Huibregtse has demonstrated the the large subunit of RNA polymerase II is also a substrate of hRPF1 ubiquitination (12). As transcription and RNA processing are known to be integrated processes, it is likely that alterations in protein levels or function of either of these two proteins may explain the observed effects on PR- and p53-dependent transcription. Experiments addressing these possibilities are underway.

However, several controlled experiments suggest that hRPF1's effect on activated transcription may not be direct. Specifically, we have observed that overexpression of hRPF1 is able to alter the levels of cotransfected progesterone receptor, suggesting an indirect mechanism for 'potentiation' of PR-dependent transcription. Nonetheless, recent reports from other laboratories have implicated 'hect' family members such as E6-AP as associating with and modulating steroid receptor dependent transcription (13). It is clear that this family of E3 ubiquitin ligase proteins is involved in transcriptional processes, though the precise mechanism remains elusive.

Key Research Accomplishments

- Completion of yeast two-hybrid screen with identification of 6 proteins which specifically bind to the amino terminal 'substrate binding domain' of hRPF1
- Confirmation of yeast two-hybrid interactions using GST-pulldown interaction assays
- Cloning of full-length cDNAs for two hRPF1-interacting proteins
- Creation of recombinant baculovirus for hRPF1 expression; purification of RPF1 and RPF1-C867A from baculovirus/SF9 cells
- Establishment of *in vitro* ubiquitination assay using yRSP5 or hRPF1 as E3 ubiquitin-ligase
- Identification and creation of amino acid substitutions in substrate proteins which abrogate ubiquitination

Name	Times Isolated	'PPXY' Motif?	<i>In vitro</i> Interactions?
#40	37 >5 cDNAs	No	RPF1 +++ C2 ++ WW ++ hect -
#74 human GEF-2 ganglioside expression factor	1	No	RPF1 +++ C2 ++ WW ++ nect -
#96 human 68 kDa pre-mRNA cleavage factor	3 2 cDNAs	'RPPPY'	RPF1 + C2 - WW +++ hect -
#113 K1AA0058	6 3 diff. cDNAs	'APPAY'	RPF1 +++ C2 - WW +++ hect n.d.
#51 ?	10 2 diff. cDNAs	'QPPPY'	RPF1 +++ C2 - WW +++ hect n.d.
#58 tom1-like protein	1	'QPPNY'	RPF1 + C2 - WW +++ hect n.d.

Table 1: Yeast Two-hybrid Clones which Interact with hRPF1
cDNAs encoding six different human proteins were isolated from a Hela two-hybrid library. These interacting protein were classified into two classes, based upon 1) the presence or absence of a 'PPXY' motif, and 2) the region of hRPF1 to which they bind. (hRPF1: aa. 25-900; C2: aa.1-190, WW: aa.180-594, hect: aa. 494-900)

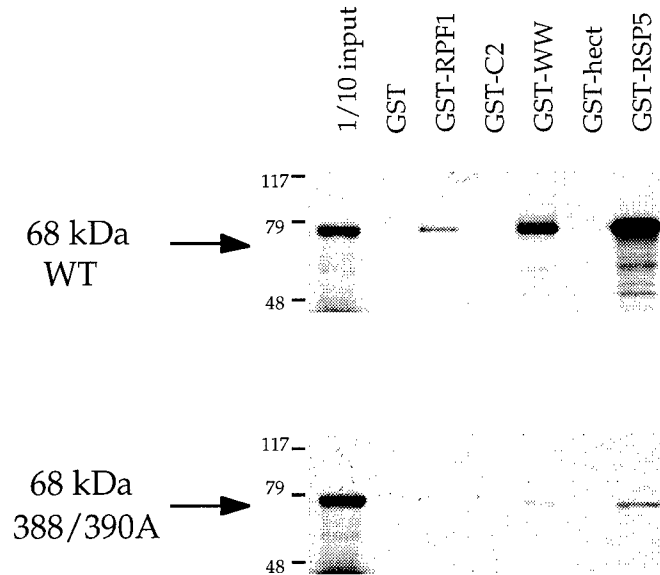


Figure 1: *In vitro* interaction of WW domains of hRPF1 and 68kDa protein.

In vitro transcribed/translated and 35 S-methionine labelled 68kDa protein (or 68 kDa 'PPXY' mutant, 388/90A) was incubated with GST alone, or with GST fused to hRPF1, RPF-N, RPF-W, RPF-hect. or yRSP5. Bound proteins were analyzed by 10% SDS-PAGE and visualized by autoradiography.

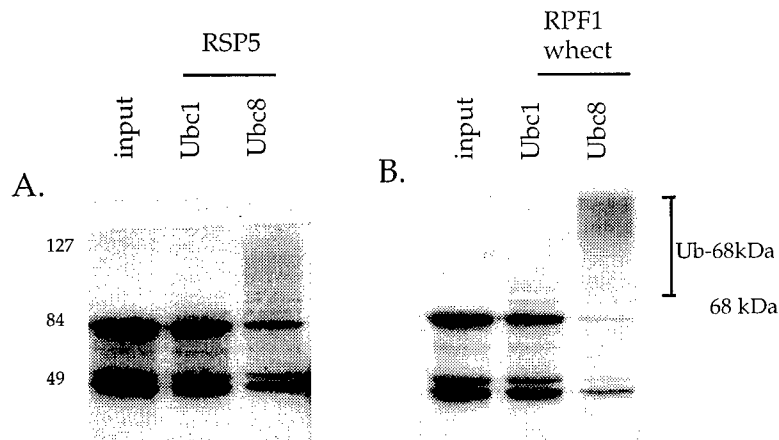


Figure 2: *In vitro* Ubiquitination Reactions. 35 S-methionine labelled 68kDa protein was *in vitro* transcribed/translated in rabbit reticulocyte lysate. Radiolabelled protein was incubated with bacterially expressed recombinant E2 protein (Ubc1 or Ubc8) and yeast RSP5 (A) or human RPF1-whect (B) for one hour at 30°C. Reactions were terminated by addition of SDS sample buffer, and slower migrating ubiquitin conjugates were resolved and detected using SDS-PAGE and autoradiography.

REFERENCES

1. Imhof, M. O. and McDonnell, D. P. Yeast RSP5 and Its Human Homolog hRPF1 Potentiate Hormone-Dependent Activation of Transcription by Human Progesterone and Glucocorticoid Receptors, *Molecular and Cellular Biology*. *16*: 2594-2605, 1996.
2. Huibregtse, J. M., Scheffner, M., Beaudenon, S., and Howley, P. M. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase, *Proc. Natl. Acad. Sci. USA*. *92*: 2563-2567 and 5249, 1995.
3. Staub, O., Dho, S., Henry, P. C., Correa, J., Ishikawa, T., McGlade, J., and Rotin, D. WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na⁺ channel deleted in Liddle's syndrome, *EMBO*. *15*: 2371-2380, 1996.
4. Sudol, M. Structure and Function of the WW Domain, *Progress in Biophysics and Molecular Biology*. *65*: 113-132, 1996.
5. Ruegsegger, U., Blank, D., and Keller, W. Human Pre-mRNA Cleavage Factor Im Is Related to Spliceosomal SR Proteins and Can Be Reconstituted In Vitro From Recombinant Subunits, *Molecular Cell*. *1*: 243-253, 1998.
6. Du, L. and Warren, S. L. A Functional Interaction between the Carboxy-Terminal Domain of RNA Polymerase II and Pre-mRNA Splicing, *Journal of Cell Biology*. *136*: 5-18, 1997.
7. McCracken, S., Fong, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S. D., Wickens, M., and Bentley, D. L. The C-terminal domain of RNA polymerase II couples mRNA processing to transcription, *Nature*. *385*: 357-361, 1997.
8. Steinmetz, E. J. Pre-mRNA Processing and the CTD of RNA Polymerase II: The Tail That Wags the Dog?, *Cell*. *89*: 491-494, 1997.
9. Seroussi, E., Kedra, D., Kost-Alimova, M., Sandberg-Nordqvist, A.-C., Fransson, I., Jacobs, J. F. M., Fu, Y., Pan, H.-Q., Roe, B. A., Imreh, S., and Dumanski, J. P. TOM1 Genes Map to Human Chromosome 22q13.1 and Mouse Chromosome 8C1 and Encode Proteins Similar to the Endosomal Proteins HGS and STAM, *Genomics*. *57*: 380-388, 1999.
10. Huibregtse, J. M., Yang, J. C., and Beaudenon, S. L. The large subunit of RNA polymerase II is a substrate of the Rsp5 ubiquitin-protein ligase, *Proc. Natl. Acad. Sci. U.S.A.* *94*: 3656-3661, 1997.
11. Harvey, K. F., Harvey, N. L., Michael, J. M., Parasivam, G., Waterhouse, N., Alnemri, E. S., Watters, D., and Kumar, S. Caspase-mediated Cleavage of the Ubiquitin-protein Ligase Nedd4 during Apoptosis, *Journal of Biological Chemistry*. *273*: 13524-13530, 1998.
12. Beaudenon, S. L., Huacani, M. R., Wang, G., McDonnell, D. P., and Huibregtse, J. M. The ubiquitin-protein ligase, RSP5 mediates the UV-induced degradation of RNA polymerase II, *Molecular Cellular Biology*. *in press*, 1999.
13. Nawaz, Z., Lonard, D. M., Smith, C. L., Lev-Lehman, E., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily, *Molecular Cellular Biology*. *19*: 1182-1189, 1999.

PUBLICATIONS:

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John D. Norris, Lisa A. Paige, Dale J. Christensen, Ching-Yi Chang, **Maria R. Huacani**, Daju Fan, Paul T. Hamilton, Dana M. Fowlkes, and Donald P. McDonnell. (1999) Peptide antagonists of the human estrogen receptor. *Science* (in press)

CONFERENCE PRESENTATIONS AND POSTERS:

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IDENTIFICATION OF SUBSTRATES OF hRPF1: A NOVEL E3 UBIQUITIN LIGASE

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The ubiquitin-proteasome pathway is responsible for the regulation of protein stability in a wide variety of cellular processes, including gene transcription, cell cycle progression and signal transduction. E3 ubiquitin ligase proteins are the components of this multi-enzyme cascade which are believed to be key players in the selection of ubiquitination substrates. Several examples of ubiquitination dysregulation and subsequent cellular transformation have been shown to occur. The defect in these systems has been shown to occur primarily at the level of E3 ubiquitin ligase-substrate recognition. Our laboratory is interested in an E3 ubiquitin ligase, hRPF1, which was originally identified as a modulator of steroid receptor transcriptional activity. The yeast homolog of hRPF1, RSP5 has been shown to bind to and ubiquitinate the large subunit of RNA polymerase II. We postulate that there may be additional hRPF1 substrates, the identification of which will help to explain these observations linking E3 ubiquitin ligase activity and the general transcriptional machinery. Using a yeast two-hybrid approach, we have identified a pre-mRNA cleavage factor which specifically binds to and serves as a substrate for hRPF1. As RNA processing is known to be coupled to transcription, we are intrigued by the possibility that components of the RNA processing machinery might be regulated by ubiquitination. *In vitro* analysis of the regions of hRPF1 required for binding, suggest that in addition to putative ubiquitination substrates, we have also identified a class of proteins which may play a regulatory role in hRPF1 activity. Present work is aimed at determining the physiological significance of these interactions.

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The Rsp5 ubiquitin-protein ligase mediates DNA damage-induced degradation of the large
subunit of RNA polymerase II in *Saccharomyces cerevisiae*

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Abstract

Rsp5 is an E3 ubiquitin-protein ligase of *S. cerevisiae*, belonging to the hect domain family of E3 proteins. We have shown previously that Rsp5 binds and ubiquitinates the largest subunit of RNA polymerase II (Rpb1) *in vitro*. We show here that Rpb1 ubiquitination and degradation is induced *in vivo* by UV irradiation and by the UV-mimetic compound 4-nitroquinoline N-oxide (4-NQO), and that a functional *RSP5* gene product is required for this effect. The 26S proteasome is also required, as mutation of *SEN3/RPN2* (*sen3-1*), which encodes an essential regulatory subunit of the 26S proteasome, partially blocks 4-NQO-induced degradation of Rpb1. These results suggest that Rsp5-mediated ubiquitination and degradation of Rpb1 is a component of the response to DNA damage. A human WW domain-containing E3 closely related to Rsp5, Rpf1/hNedd4, also binds and ubiquitinates both yeast and human Rpb1 *in vitro*, suggesting that Rpf1 and/or another WW-hect E3 protein mediates UV-induced degradation of the large subunit of pol II in human cells.

Introduction

Ubiquitin-dependent proteolysis involves the covalent ligation of ubiquitin to substrate proteins, which are then recognized and degraded by the 26S proteasome. While many of the components involved in catalyzing protein ubiquitination have been identified and characterized biochemically, we are only beginning to understand how the system specifically recognizes appropriate substrates. At least three classes of activities, known as E1 (ubiquitin-activating), E2 (ubiquitin conjugating), and E3 (ubiquitin-protein ligase) enzymes, cooperate in catalyzing protein ubiquitination (33). The enzymatic mechanisms and functions of the E1 and E2 proteins have been well characterized. In contrast, the E3 enzymes are a diverse and less well characterized group of activities, and many lines of evidence indicate that E3 activities play the major role in determining substrate specificity of the ubiquitination pathway (14, 28, 33).

The hect domain (homologous to E6-AP carboxyl terminus) defines a family of E3 proteins which were discovered through characterization of human E6-AP (17). The interaction of E6-AP with the human papillomavirus (HPV) E6 protein of the cervical cancer-associated HPV types causes E6-AP to associate with and ubiquitinate p53, suggesting that E6 functions in promoting cellular immortalization, at least in part, by stimulating the destruction of this important tumor suppressor protein (16). The hect E3s range in MW from 92 to over 500 kDa, with the hect domain comprising the approximately 350 carboxyl-terminal amino acids (17, 33). Exactly five hect E3s are encoded by the *S. cerevisiae* genome, and over 30 have been so far identified in mammalian species. An obligatory intermediate in the ubiquitination reactions catalyzed by hect E3s is a ubiquitin-thioester formed between the thiol group of an absolutely conserved cysteine within the hect domain and the terminal carboxyl group of ubiquitin (32). The E3 becomes "charged" with ubiquitin via a cascade of ubiquitin-thioester transfers, in which

ubiquitin is transferred from the active site cysteine of the E1 enzyme, to the active site cysteine of an E2, and finally to the hect E3, which catalyzes isopeptide bond formation between ubiquitin and the substrate. The E3 can apparently be re-charged with ubiquitin while bound to the substrate and can therefore catalyze ligation of multiple ubiquitin moieties to the substrate, either through conjugation to other lysines on the substrate, or to lysine residues on previously conjugated ubiquitin molecules. The resulting multi-ubiquitinated substrate is then recognized and degraded by the 26S proteasome. Structure/function analyses of human E6-AP and yeast Rsp5 have suggested a model for hect E3 function in which the large and non-conserved amino-terminal domains of these proteins contain determinants for substrate specificity, while the carboxyl-terminal hect domain catalyzes multi-ubiquitination of bound substrates (16, 37).

The *S. cerevisiae* *RSP5* gene encodes an essential hect E3 protein and has been isolated in multiple genetic screens, including as a suppressor of mutations in *SPT3* (Fred Winston and coworkers, unpublished, cited in 17, 18). Rsp5 has also been identified as being involved in down-regulation of several plasma membrane-associated permeases, including uracil permease (Fur4), general amino acid permease (Gap1), maltose permease (Mal61), and the plasma membrane H^+ -ATPase (5, 9, 13, 23). The primary structure of yeast Rsp5 reveals, in addition to its carboxyl-terminal hect domain, two types of domains within the amino-terminal region: a C2 domain (amino acids 3 to 140) and three WW domains (within amino acids 231-418). C2 domains interact with membrane phospholipids, inositol polyphosphates, and proteins, in most cases dependent on or regulated by Ca^{2+} (31). Although it has not yet been demonstrated, it is possible that the C2 domain of Rsp5 is involved in targeting its membrane-associated substrates, either by localizing Rsp5 to the plasma membrane or by directly mediating the interaction with these substrates.

WW domains are protein-protein interaction modules that recognize proline-rich sequences, with the consensus binding site containing either a PPxY (4, 21), PPLP (1, 7), or PPPGM sequence (2). WW domains, like SH3 domains, recognize polyproline ligands with high-specificity but low-affinity ($K_d=1\text{-}200\text{ }\mu\text{M}$). The basis of recognition is the N-substituted nature of the proline peptide backbone, rather than the proline side chain, itself, and it has been suggested that this explains how WW and SH3 domains can achieve specific but low-affinity recognition of ligands since proline is the only natural N-substituted amino acid (26). It has also been recently shown that WW domains can also recognize phosphoserine and phosphothreonine-containing ligands (22), which has important implications for the diversity of substrates that may be recognized by Rsp5 and other WW domain containing E3s. A structure-function analysis of Rsp5 showed that the hect domain and the region spanning WW domains 2 and 3 are necessary and sufficient to support the essential *in vivo* function of Rsp5, while the C2 domain and the first WW are dispensable, at least under standard growth conditions (37). Together, the results of our structure/function analyses imply that ubiquitination of one or more substrates of Rsp5 is essential for cell viability and that the critical substrate(s) is/are recognized by the WW domain 2 and 3 region.

We previously reported the results of a biochemical approach for identifying substrates of Rsp5, which led to the identification of Rpb1, the largest subunit of RNA polymerase II, as a substrate of Rsp5 (18). Rpb1 is very efficiently ubiquitinated by Rsp5 *in vitro* and the WW domain region mediates binding to Rpb1, with WW domain 2 being most critical. Since the requirements for Rpb1 binding and ubiquitination parallel those required for the essential function of Rsp5, Rpb1 is a candidate for being at least one of the substrates related to the essential function of Rsp5. The biological relevance of Rpb1 ubiquitination was not initially

clear, however, since Rpb1 is an abundant, long-lived protein *in vivo*. Interestingly, another study showed that the large subunit of human pol II (pol II LS) is subject to ubiquitination and degradation in response to UV irradiation (3, 30), however the enzymatic components of the ubiquitin system responsible for this phenomenon were not identified or characterized. We show here that UV irradiation or treatment with a UV-mimetic chemical induces the degradation of Rpb1 in yeast cells, and that Rsp5 and the 26S proteasome mediate this effect. Furthermore, we show that human Rpf1, a WW domain-containing hect E3, binds and ubiquitinates Rpb1 *in vitro*, suggesting that this may be the E3 that mediates UV-induced degradation of the pol II LS in human cells.

Materials and Methods

Yeast Strains and plasmids

The FY56 (*RSP5*), FW1808 (*rsp5-1*), and *Gal-RSP5* strains have been described previously (18, 37). The *sen3-1* (MHY811) and *SEN3* (MHY810) strains (6) were kindly provided by Mark Hochstrasser (University of Chicago). All plasmids for expression of Rsp5 and Rpb1 have been described previously (18, 37). Plasmids for bacterial expression of GST-Rpf1 fusion proteins were generated by PCR amplification of regions of the Rpf1 ORF from plasmid pBKC-hRPF1 (19). The GST-Rpf1 N protein contains amino acids 13–192 of Rpf1, GST-WW protein contains amino acids 193–506, GST-C contains 506–901, and GST-WW-*hect* contains 193–901. This numbering is based on the assumption that amino acid 29 of the protein sequence given in GenBank D42055 is the initiating methionine. pGEX-5x-1 (Pharmacia) was the cloning vector for expression of all GST fusion proteins except for GST-WW-*hect*, which was expressed using pGEX-6p-1.

Protein purification and biochemical assays

GST fusion proteins for ubiquitination assays and protein binding assays were expressed in *E. coli* by standard methods and affinity-purified on glutathione sepharose (Pharmacia). Ubiquitination assays utilized *hect* E3 proteins (Rsp5, the Rsp5 C-A mutant, human E6-AP, and Rpf1 WW-*hect*) that were cleaved from the GST portion of the molecule with PreScission protease (Pharmacia). These proteins were then used in ubiquitination assays using ³⁵S-labeled *in vitro* translated (TNT rabbit reticulocyte lysate system, Promega) yeast Rpb1, as described previously (18).

Rpb1 binding assays were performed by mixing 100 ng of GST-E3 fusion protein bound to 10 μ l of glutathione sepharose with 80 μ g of total HeLa cell lysate (cell lysis buffer 0.1 M Tris, pH 8.0, 0.1M NaCl, and 1% NP-40), with the remainder of the 125 μ l volume consisting of 25 mM Tris (pH 8.0), 125 mM NaCl. Reactions were rotated for 2 hours at 4°, and the beads were washed three times with 500 μ l of cell lysis buffer. SDS-PAGE loading buffer was added directly to the sepharose, heated at 95° for 5 minutes, and proteins were analyzed by SDS-PAGE and western blotting using either anti-CTD antibody (generously provided by Danny Reinberg, University of Medicine and Dentistry of New Jersey, Piscataway, NJ) or anti-pol II antibody N-20 from Santa Cruz Biotechnology (Santa Cruz, CA).

Analysis of UV and 4-NQO treated cells

HeLa cells were maintained in DMEM with 10% fetal bovine serum and UV-irradiation was performed on tissue culture dishes after removal of media. A 254 nm germicidal lamp with an incident dose rate of 1.5 J per m² per sec was used, and time of irradiation was generally 15 sec, for a total dose of 22.5 J per m². Fresh media was then added to the cells, which were then allowed to recover for various times at 37°. 4-NQO (Sigma), prepared as a 0.5 mg/ml stock in ethanol, was added directly to the media at the indicated concentrations and for the indicated times. Extracts were made by lysing cells directly in SDS-PAGE loading buffer.

higher
dose
(15 J/m²)
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Yeast were irradiated on agar plates by taking 5 O.D. units of log-phase liquid cultures, concentrating the cells by centrifugation to 0.5 ml, then spreading the cells onto 10 cm agar plates. The liquid was allowed to absorb into the plates for 30 minutes at 30°, then the plates

were irradiated as described above for HeLa cells. The cells were then collected from the plates and extracts were prepared by the method of Silver, et al. (35). Log-phase liquid yeast cultures were treated with 4-NQO by adding a 0.5 mg/ml stock in ethanol directly to the culture media at the indicated concentrations. Western analysis utilized either anti-HA rabbit polyclonal antibody (Santa Cruz Biotechnology), anti-Rsp5 mouse monoclonal antibody (37), anti-CTD rabbit polyclonal antibody, or anti-ubiquitin rabbit polyclonal antibody (StressGen, Victoria, BC, Canada). Horseradish peroxidase-linked secondary antibodies and chemiluminescent reagents were obtained from DuPont NEN.

Results

UV irradiation and 4-NQO induce the degradation of Rpb1 in both human and yeast cells

4-nitroquinoline 1-oxide (4-NQO) is considered a UV-mimetic because it is metabolized to yield a compound that reacts with purine residues of DNA, and these adducts are processed by the nucleotide excision repair (NER) system similarly to dipyrimidine photoproducts induced by 254-nm UV light (15, 29). It has been previously shown that UV irradiation of human cells induces the ubiquitination and degradation of hRpb1 (3, 30). Figure 1 demonstrates this effect in HeLa cells. Cells were irradiated with 254 nm UV light at a dose of 22.5 J per m² and cell extracts were made at times up to 4 hours after irradiation. Extracts were analyzed by SDS-PAGE followed by immunoblotting with an antibody that recognizes the amino-terminal region of hRpb1 (and therefore detects both hypo- and hyperphosphorylated forms of the protein). 4-NQO treatment also resulted in degradation of hRpb1, with similar kinetics as UV-induced degradation. With both UV and 4-NQO treatment there was a preferential disappearance of the underphosphorylated form of hRpb1. Lactacystin, a highly specific inhibitor of the proteasome, inhibited both UV- and 4-NQO-induced degradation of hRpb1 (not shown), consistent with previous reports that this effect is mediated by the 26S proteasome of the ubiquitin system (30).

Figure 2 shows that degradation of Rpb1 was also induced in *S. cerevisiae* by both UV irradiation and 4-NQO treatment. UV irradiation of intact yeast cells on agar plates led to a dose- and time-dependent decrease in the steady-state level of Rpb1 (Figure 2A). Rpb1 levels reached a minimum between one and two hours and began to return to normal after two to four hours. 4-NQO also elicited a dose-dependent decrease in Rpb1, reaching a minimum 30 to 60 minutes after addition of 4-NQO to a liquid culture (Figure 2B). Unlike human Rpb1, the hypo- and hyperphosphorylated forms of yeast Rpb1 migrate as a very closely spaced doublet and are

therefore not easily distinguishable by SDS-PAGE. Nevertheless, in none of our experiments did we observe an apparent preferential disappearance of one form over the other. Treatment of cells with cycloheximide or actinomycin D did not lead to a decrease in Rpb1 level after 60 minutes (not shown), indicating that the effect of UV irradiation or 4-NQO treatment was not simply the result of inhibition of synthesis of Rpb1 or of any other RNA or protein. The appearance of slower-migrating forms of Rpb1, suggestive of ubiquitinated intermediates, was evident at higher concentrations of 4-NQO and on longer film exposures. These slower-migrating bands were shown to be ubiquitinated forms of Rpb1 by immunoprecipitating with anti-CTD antibody, followed by immunoblotting with either anti-CTD or anti-ubiquitin antibody (Figure 3). While the accumulation of ubiquitinated forms of Rpb1 was 4-NQO dose-dependent, there was some reaction of the Rpb1 immunoprecipitate with the anti-ubiquitin antibody even in untreated cells. This may reflect a basal level of Rpb1 ubiquitination in normal cells, as suggested previously (18).

4-NQO induced degradation of Rpb1 is dependent on *RSP5* and *SEN3/RPN2*

To determine if DNA damage-induced degradation of Rpb1 was dependent on Rsp5 we first took advantage of a yeast strain that contains a single copy of a conditionally expressed wild-type *RSP5* gene. The *Gal-RSP5* yeast strain contains an epitope-tagged *RSP5* gene under control of the *GALI* promoter, integrated at the *RSP5* chromosomal locus (18). This strain was grown to early log phase in galactose-containing media, then switched to dextrose-containing media for 48 hours. Figure 4 shows that Rsp5 protein levels were dramatically reduced after 48 hours in dextrose. The cells were still fully viable at this point and resumed growth when shifted back to galactose. The dextrose-shifted cells were treated with 4-NQO and compared to log-

phase cells that had been maintained in galactose-containing media. 4-NQO induced Rpb1 degradation occurred in the cells maintained in galactose, but not in Rsp5-depleted cells. These results suggest that 4-NQO damage-induced degradation of Rpb1 is dependent on *RSP5*.

A caveat to the above experiment is that, since Rsp5 is essential for viability, the cells have drastically slowed their rate of cell division after 48 hours in dextrose and therefore indirect effects cannot be ruled out as being responsible for the block in 4-NQO-induced Rpb1 degradation. To independently confirm the importance of Rsp5 in induced degradation of Rpb1 we examined the effect of 4-NQO in the temperature-sensitive *rsp5-1* mutant. Temperature sensitivity is conferred by a single amino acid change (amino acid 733) within the hec domain which directly affects the catalytic activity of the protein (37). The *rsp5-1* strain grows with a slightly longer doubling time than an isogenic *RSP5* strain at 30° but arrests within 30 - 60 minutes after shift to 37°. Figure 5A shows that Rpb1 degradation is induced by 4-NQO in an isogenic wild-type *RSP5* strain at 37°, while little or no loss of Rpb1 is seen in the *rsp5-1* strain at 37°. Figure 5B shows the results of an experiment in which the 4-NQO-induced multi-ubiquitinated forms of Rpb1 were clearly evident. The accumulation of these forms was seen in the wild-type *RSP5* strain at both 30° and 37°, and in the *rsp5-1* strain at 30°, but not at 37°. These results confirm that 4-NQO-induced ubiquitination and degradation of Rpb1 is *RSP5*-dependent.

A strain containing a mutation in a subunit of the 26S proteasome was used to determine if 4-NQO-induced degradation of Rpb1 was proteasome-dependent. *SEN3/RPN2* encodes an essential non-ATPase regulatory subunit of the 26S proteasome (6). The *sen3-1* mutant shows a growth defect at 30° (doubling time of 4.5 h) and a more severe growth defect at higher temperature. The MAT α 2 transcription factor and certain artificial substrates of the ubiquitin

system (Ub-Pro-Bgal and Ub-Leu-Bgal) have been shown to be stabilized in this mutant at 30°. We compared the *sen3-1* mutant to an isogenic wild-type *SEN3* strain for its ability to support 4-NQO-induced degradation of Rpb1. As shown in Figure 6, the *sen3-1* mutant was defective in 4-NQO-induced Rpb1 compared to the *SEN3* strain. This result confirms that UV-induced degradation of Rpb1 occurs in the proteasome, consistent with the observation that proteasome inhibitors blocked degradation of human pol II LS in response to UV irradiation (30).

Rpf1/hNedd4, a human hect E3 related to Rsp5, binds and ubiquitinates Rpb1 *in vitro*

Rpf1, also known as human Nedd4 (hNedd4), has a C2 domain at its extreme amino-terminus, four WW domains in the central portion of the molecule, and a carboxyl-terminal hect domain (Figure 7A). Rpf1 is one of at least 7 human hect E3s that have this same general organization, with a variable number of WW domains (two to four). GST-Rpf1 proteins were expressed as indicated in Figure 7A and assayed for their ability to bind to hRpb1. The full-length Rpf1 protein was not used in this analysis because it was produced in low amounts in bacteria and, furthermore, was not catalytically active as judged by ubiquitin-thioester assays (not shown). Rpf1 WW-hect and the isolated WW domain region stably bound hRpb1 present in HeLa cell extract (Figure 7B, left panel), whereas neither the isolated C2 domain or hect domain bound to hRpb1. These results are consistent with our previous results that the WW domain region of Rsp5 is necessary and sufficient for binding to yeast Rpb1 (18, 37). In addition, a well-characterized proteolyzed form of hRpb1 (form IIb) that lacks the CTD did not bind to Rpf1, also consistent with previous results that the CTD is the binding site for Rsp5 (18, 37). There was an apparent preferential binding of Rpf1 to the hypophosphorylated (IIa) form of hRpb1 in this experiment, however the degree to which the phosphorylated (IIo) form of hRpb1 associated

with Rpf1 was dependent on the cell extraction buffer. If the cell lysis buffer conditions were harsher (RIPA buffer instead of NP-40 lysis buffer (11)) an equivalent portion of hyperphosphorylated hRpb1 bound to Rpf1 (Figure 7B, right panel). This suggests that the interaction of the hyperphosphorylated CTD with other proteins might preclude binding to Rpf1, and that Rsp5 and Rpf1 have an inherent ability to bind to both forms of the protein. This interpretation is consistent with previous results that showed that Rsp5 could bind to both the IIo and IIa forms of purified pol II holoenzyme *in vitro* (18).

To determine if Rpf1 could ubiquitinate Rpb1, the WW-hect Rpf1 protein was cleaved from the purified GST fusion protein and assayed for its ability to ubiquitinate *in vitro* translated yeast Rpb1. Rpf1 was as efficient in stimulating multi-ubiquitination of Rpb1 as yeast Rsp5 (Figure 8). Neither the active site cysteine-to-alanine mutant of Rsp5 nor human E6-AP ubiquitinated Rpb1. Together, the binding and ubiquitination results suggest that Rpf1 may mediate the DNA damage-induced degradation of the large subunit of RNA polymerase II in human cells.

Discussion

We initially identified Rpb1 as a substrate of Rsp5 based on a biochemical screen for proteins that were bound and ubiquitinated by Rsp5 *in vitro* (18). While Rsp5 was found to efficiently multi-ubiquitinate Rpb1 *in vitro*, the biological function of this was unclear since Rpb1 is an abundant and stable protein *in vivo*. The steady-state level of Rpb1 was found to increase modestly (approximately three- to five-fold) on prolonged transcriptional repression of *RSP5*, providing evidence that Rpb1 may be a *bona fide* substrate of Rsp5 *in vivo*, even if the half-life of Rpb1 under normal growth conditions is relatively long. Other studies have shown that inhibition of transcription caused by exposure of mammalian cells to DNA damaging agents, including α -amanitin, actinomycin D, cisplatin, and UV irradiation, leads to the degradation of the largest subunit of pol II (3, 27). Ratner, et al., further demonstrated that the degradation of the pol II LS induced by UV irradiation was ubiquitin- and proteasome-dependent. Together, these results suggested that recognition of Rpb1 by Rsp5 might be enhanced in response to DNA damage. The experiments described here show that, as in human cells, DNA damage induces the ubiquitination and degradation of Rpb1 in *S. cerevisiae*, and that this is dependent on the Rsp5 ubiquitin-protein ligase. In addition, a human hect E3 closely related to Rsp5, Rpf1/hNedd4, is shown to bind and ubiquitinate Rpb1 *in vitro*, suggesting that this hect E3 might mediate UV-induced degradation of Rpb1 in human cells.

It has long been recognized that RNA synthesis is down-regulated in response to DNA damage and that stalled RNA polymerase at sites of DNA damage might serve as a signal for recruitment of the nucleotide excision repair (NER) machinery (10, 24). This is thought to be the basis of a specialized form of NER, called transcription-coupled repair (TCR), in which lesions within the transcribed strand of genes are repaired more rapidly than lesions on the

nontranscribed strand or outside of transcription units. TCR also occurs in *E. coli*, where the transcription repair coupling factor, TRCF, binds to and releases RNA polymerase stalled at a lesion and then stimulates the recruitment of the repair machinery (34). Several lines of evidence suggest that the mechanism of TCR is more complex in eukaryotes, and it is generally thought that a stalled RNA polymerase can resume transcript synthesis following repair. This is based in part on the stability of stalled RNA polymerase/template/RNA complexes *in vitro* and the idea that it would be energetically wasteful to abort transcript synthesis entirely. The finding that a fraction of the large subunit of pol II is ubiquitinated and degraded in response to DNA damage suggests an alternative mechanism for down-regulation of transcription in response to DNA damage: irreversible disassembly of transcription complexes by degradation of the major catalytic subunit of pol II.

It is not yet clear which form of pol II is targeted for ubiquitin-mediated degradation following DNA damage. The CTD, which is necessary and sufficient for Rsp5 binding, is subject to phosphorylation and dephosphorylation events during the transcription cycle and is also the site of interaction of many components of the transcription machinery (25). The CTD is hypophosphorylated (IIa) in pol II transcription initiation complexes, and undergoes phosphorylation upon promoter clearance to yield a hyperphosphorylated (IIo) form that persists throughout transcription elongation. Ratner et al. (30) reported that ubiquitinated forms of human Rpb1 detected after UV irradiation reacted with an antibody that is specific for the hyperphosphorylated form of hRpb1, suggesting that pol II complexes arrested at intragenic damage sites might be the preferential substrate for ubiquitination. This is not consistent, however, with the observation that the hypophosphorylated form of hRpb1 preferentially disappears in response to either UV irradiation or 4-NQO treatment (30, and this study). In order

to explain this discrepancy, Ratner et al. suggested that the apparent loss of hypophosphorylated hRpb1 upon UV irradiation might reflect a rapid conversion of hypo- to hyperphosphorylated Rpb1 in order to compensate for the loss of hyperphosphorylated Rpb1. While we cannot exclude this possibility, the data are also consistent with a model in which the hypophosphorylated form of pol II is actually the preferential substrate for ubiquitination, but that the kinetics of its ubiquitination and degradation are too rapid to allow detection of ubiquitinated intermediates. That is, the fact that ubiquitinated intermediates can be detected may indicate that the hyperphosphorylated form is actually less efficiently targeted.

While further studies are clearly necessary to determine which form of pol II is targeted for ubiquitin-mediated degradation in response to DNA damage *in vivo*, our *in vitro* results suggest that there is not a specific requirement for recognition of Rpb1 by Rsp5 in terms of the phosphorylation state of the CTD. Phosphorylation of the CTD is not a prerequisite for Rsp5 recognition, since *in vitro* translated Rpb1 and GST-CTD produced in bacteria are both efficiently recognized by Rsp5. We also showed previously that the hypo- and hyperphosphorylated forms of purified human pol II holoenzyme bind equally well to GST-Rsp5 (18). In addition, both Rsp5 and Rpf1 bind to the hypophosphorylated form of hRpb1 present in human cell extracts, however the degree to which Rsp5/Rpf1 can bind to hyperphosphorylated hRpb1 is a function of the cell extraction buffer, with more stringent extraction buffers resulting in more binding of the hyperphosphorylated forms. Together, these results suggest that the association of other transcription factors with pol II, and specifically with the CTD, might block recognition by Rsp5 *in vivo*. Changes in pol II transcription complexes in response to DNA damage, such as dissociation of specific CTD-associated proteins or dissociation of the

elongating polymerase complex from the template, might then allow Rsp5 to bind and ubiquitinate Rpb1.

Rsp5 is the only hect E3 protein in yeast that has a C2 domain and WW domains, while at least seven human C2/WW-hect E3s have been identified. The WW domains, as well characterized protein-protein interaction modules, are likely to mediate the interaction with at least some of the substrates of Rsp5, including Rpb1 (37). WW domains bind proline-rich ligands, with the best-characterized ligand being the "PY" motif (containing a PPxY sequence). In addition, it has recently been shown that WW domains can also recognize phosphoserine- and phosphothreonine-containing ligands (22), suggesting that there are two disparate types of WW domain ligands. The CTD heptapeptide consensus (YSPTSPS) may be a non-consensus PY motif in the context of the repeating heptapeptide (YxPxxPxYxPxxPx). Alternatively, if the phosphorylated form of Rpb1 is the *in vivo* substrate of Rsp5, phosphorylation at the serine and/or threonine residues may contribute to recognition, although as mentioned above, phosphorylation is not required for binding of Rsp5 to the CTD *in vitro*. Our finding that Rpf1/hNedd4 can bind and ubiquitinate hRpb1 *in vitro* suggests that this may be the E3 enzyme responsible for this effect in human cells. Preliminary results, however, indicate that other WW domain hect E3s can also bind to Rpb1 *in vitro* (S. L. B. and J. M. H., unpublished). It is possible that while several of the WW domain hect E3s can bind and ubiquitinate Rpb1 *in vitro*, intracellular localization is the key determinant of which E3 can target Rpb1 *in vivo*. Mouse Nedd4 and yeast Rsp5 are primarily cytoplasmic (12, and G. Wang and J. M. H., unpublished), however there is now precedent for ubiquitin-mediated degradation of nuclear proteins being linked to their export from the nucleus to the cytoplasm (8, 36).

While it is now established that DNA damage induces the degradation of Rpb1 in both yeast and human cells, the relevance of this to DNA repair is not yet clear. Rsp5 mutants do not show any apparent UV sensitivity, although we cannot yet rule out more subtle effects of Rsp5 on efficiency of DNA repair. The fact that both CSA and CSB Cockayne syndrome cells were found to be defective in UV-induced Rpb1 degradation in human cells suggested that this is related to the process of transcription-coupled DNA repair (TCR). However, a null mutant of *rad26*, the yeast CSB homolog and the only yeast protein known to be required for TCR but not for NER, exhibited no defect in 4-NQO-induced Rpb1 degradation (data not shown). This suggests that TCR may not be directly linked to DNA damage-induced degradation of Rpb1, at least in yeast, and again raises the question of which form of pol II is the *in vivo* substrate of Rsp5. Expression of Rpf1/hNedd4 in yeast cannot functionally substitute for *RSP5*, in terms of either cell viability or the UV-induced effect on Rpb1 (not shown). The basis of this non-complementation is not known, but could be related to an inability of Rpf1 to productively interact with other components of the ubiquitin system in yeast.

Several examples of regulated substrate ubiquitination have now been characterized. In many cases, modification of the substrate, often by phosphorylation, can serve as a signal for recognition by specific E3 ubiquitin-protein ligases, as in the recognition of phosphorylated Sic1 by SCF^{Cdc4} (28). In other cases unmasking of ubiquitination signals can occur when a substrate dissociates from an interacting protein, as in the case of the mutual destruction of the MAT α 2 and MAT α 1 transcription factors upon dissociation of the heterodimer (20). An unmasking of recognition signals on Rpb1 in response to DNA damage may account for the observations that Rpb1 is freely and efficiently recognized by Rsp5 under several different experimental conditions *in vitro*, yet Rpb1 is normally a stable and long lived protein *in vivo*. It seems likely

that the nature of the Rpb1 CTD, as an organizational center for many components of the basal transcription machinery, might preclude Rsp5 from interacting with Rpb1 during the normal transcription cycle. DNA damage may signal alterations in pol II complexes in a manner that allows Rsp5 to recognize and ubiquitinate Rpb1. Further studies on the effects of DNA damage of pol II holoenzyme complexes will aid in addressing this hypothesis.

Acknowledgements

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References

1. **Bedford, M. T., D. C. Chan, and P. Leder.** 1997. FBP WW domains and the Abl SH3 domain bind to a specific class of proline-rich ligands. *EMBO J.* **16**:2376-83.
2. **Bedford, M. T., R. Reed, and P. Leder.** 1998. WW domain-mediated interactions reveal a spliceosome-associated protein that binds a third class of proline-rich motif: the proline glycine and methionine-rich motif. *Proc. Natl. Acad. Sci. USA* **95**:10602-10607.
3. **Bregman, D. B., R. Halaban, A. J. van Gool, K. A. Henning, E. C. Friedberg, and S. L. Warren.** 1996. UV-induced ubiquitination of RNA polymerase II: a novel modification deficient in Cockayne syndrome cells. *Proc. Natl. Acad. Sci. USA* **93**:11,586-11,590.
4. **Chen, H. I., and M. Sudol.** 1995. The WW domain of YES-associated protein binds a proline-rich ligand that differs from the consensus setablished for Src homology 3-binding modules. *Proc. Natl. Acad. Sci. USA* **92**:7819-7823.
5. **de la Fuente, N., A. M. Maldonado, and F. Portillo.** 1997. Glucose activation of the yeast plasma membrane H⁺-ATPase requires the ubiquitin-proteasome proteolytic pathway. *FEBS Lett* **411**:308-12.
6. **DeMarini, D. J., F. R. Papa, S. Swaminathan, D. Ursic, T. P. Rasmussen, M. R. Culbertson, and M. Hochstrasser.** 1995. The yeast SEN3 gene encodes a regulatory subunit of the 26S proteasome (
7. **Ermekova, K. S., N. Zambrano, H. Linn, G. Minopoli, F. Gertler, T. Russo, and M. Sudol.** 1997. The WW domain of neural protein FE65 interacts with proline-rich motifs in Mena, the mammalian homolog of *Drosophila* enabled. *J. Biol. Chem.* **272**:32869-32877.
8. **Freedman, D. A., and A. J. Levine.** 1998. Nuclear export is required for degrdation of endogenous p53 by MDM2 and human papillomavirus E6. *Mol. Cell. Biol.* **18**:7288-7293.

9. **Galan, J. M., V. Moreau, B. Andre, C. Volland, and R. Haguenauer-Tsapis.** 1996. Ubiquitination mediated by the Npi1p/Rsp5p ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease. *J. Biol. Chem.* **271**:10946-52.
10. **Hanawalt, P. C.** 1994. Transcription-coupled repair and human disease. *Science* **266**:1957-1958.
11. **Harlow, E., and D. Lane.** 1988. *Antibodies: a laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
12. **Hatakeyama, S., J. P. Jensen, and A. M. Weissman.** 1997. Subcellular localization and ubiquitin-conjugating enzyme (E2) interactions of mammalian HECT family ubiquitin protein ligases. *J. Biol. Chem.* **272**:15085-15092.
13. **Hein, C., J.-Y. Springael, C. Volland, R. Haguenauer-Tsapis, and B. André.** 1995. *NP11*, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. *Mol. Micro.* **18**:77-87.
14. **Hochstrasser, M.** 1996. Ubiquitin-dependent protein degradation. *Annu Rev Genet* **30**:405-39.
15. **Hoeijmakers, J. H.** 1993. Nucleotide excision repair. II: From yeast to mammals. *Trends Genet.* **9**:211-217.
16. **Huibregtse, J. M., C. G. Maki, and P. M. Howley.** 1997. Ubiquitination of the p53 tumor suppressor. in *Ubiquitin and the Biology of the Cell*, Plenum, N. Y., Peters, J.-M., Harris, J. R., and Finley, D., eds.
17. **Huibregtse, J. M., M. Scheffner, S. Beaudenon, and P. M. Howley.** 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc. Natl. Acad. Sci. USA* **92**:2563-2567.

18. **Huibregtse, J. M., J. C. Yang, and S. L. Beaudenon.** 1997. The large subunit of RNA polymerase II is a substrate of the Rsp5 ubiquitin-protein ligase. *Proc. Natl. Acad. Sci., USA* **94**:3656-3661.
19. **Imhof, M. O., and D. P. McDonnell.** 1996. Yeast RSP5 and its human homolog hRPF1 potentiate hormone-dependent activation of transcription by human progesterone and glucocorticoid receptors. *Mol. Cell. Biol.* **16**:2594-2605.
20. **Johnson, P. R., R. Swanson, L. Rakhilina, and M. Hochstrasser.** 1998. Degradation signal masking by heterodimerization of MATA α 2 and MATA1 blocks their mutual destruction by the ubiquitin-proteasome pathway. *Cell* **94**:217-227.
21. **Linn, H., K. S. Ermekova, S. Rentschler, A. B. Sparks, B. K. Kay, and M. Sudol.** 1997. Using molecular repertoires to identify high-affinity peptide ligands of the WW domain of human and mouse YAP. *J. Biol. Chem.* **378**:531-537.
22. **Lu, P.-J., X. Z. Zhou, M. Shen, and K. P. Lu.** 1999. Function of WW domains as phosphoserine- or phosphothreonine-binding modules. *Science* **283**:1325-1328.
23. **Medintz, I., H. Jiang, and C. A. Michels.** 1998. The role of ubiquitin conjugation in glucose-induced proteolysis of *Saccharomyces* maltose permease. *J. Biol. Chem.* **273**:34454-34462.
24. **Mellon, I., G. Spivak, and P. C. Hanawalt.** 1987. Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. *Cell* **51**:241-249.
25. **Myers, L. C., C. M. Gustafsson, D. A. Bushnell, M. Lui, H. Erdjument-Bromage, P. Tempst, and R. D. Kornberg.** 1998. The Med proteins of yeast and their function through the RNA polymerase II carboxy-terminal domain. *Genes Dev.* **12**:45-54.

26. **Nguyen, J. T., C. W. Turck, F. E. Cohen, R. N. Zuckermann, and W. A. Lim.** 1998. Exploiting the basis of proline recognition by SH3 and WW domains: design of N-substituted inhibitors. *Science* **282**:2088-2092.
27. **Nguyen, V. T., F. Giannoni, M.-F. Dubois, S.-J. Seo, M. Vigneron, C. Keding, and O. Bensaude.** 1996. In vivo degradation of RNA polymerase II largest subunit triggered by α -amanitin. *Nucleic Acids Res.* **24**:2924-2929.
28. **Peters, J.-M.** 1998. SCF and APC: the Yin and Yang of cell cycle regulated proteolysis. *Curr. Opin. Cell Biol.* **10**:759-768.
29. **Prakash, S., P. Sung, and L. Prakash.** 1993. DNA repair genes and proteins of *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **27**:33-70.
30. **Ratner, J. N., B. Balasubramanian, J. Corden, S. L. Warren, and D. B. Bregman.** 1998. Ultraviolet radiation-induced ubiquitination and proteasomal degradation of the large subunit of RNA polymerase II. Implications for transcription-coupled DNA repair. *J. Biol. Chem.* **273**:5184-9.
31. **Rizo, J., and T. C. Sudhof.** 1998. C2-domains, structure and function of a universal Ca^{2+} -binding domain. *J. Biol. Chem.* **273**:15879-15882.
32. **Scheffner, M., U. Nuber, and J. M. Huibregtse.** 1995. Protein ubiquitination involving an E1- E2- E3 enzyme thioester cascade. *Nature* **373**:81-83.
33. **Scheffner, M., S. Smith, and S. Jentsch.** 1997. The ubiquitin-conjugation system. in *Ubiquitin and the Biology of the Cell*, Plenum, NY, Peters, J.-M., Harris, J. R., and Finley, D.
34. **Selby, C. P., and A. Sancar.** 1995. Structure and function of transcription-repair coupling factor. I. Structural domains and binding properties. *J. Biol. Chem.* **270**:4882-4889.

35. Silver, P. A., A. Chiang, and I. Sadler. 1988. Mutations that alter both localization and production of a yeast nuclear protein. *Genes Dev.* **2**:707-717.
36. Tomoda, K., Y. Kubota, and J. Kato. 1999. Degradation of the cyclin-dependent-kinase inhibitor p27^{kip1} is instigated by Jab1. *Nature* **398**:160-165.
37. Wang, G., J. Yang, and J. M. Huibregtse. 1999. Functional Domains of the Rsp5 ubiquitin-protein ligase. *Mol. Cell. Biol.* **19**:342-352.

Figure Legends

Figure 1. hRpb1 levels following UV irradiation and 4-NQO treatment of HeLa cells. HeLa were irradiated with 254 nm UV light at 22.5 J per m² as described in Materials and Methods and cell extracts were prepared immediately or 1 or 4 hours post-irradiation. For 4-NQO treatment, the chemical was added directly to the culture media at a final concentration of 0.5 µg/ml and cell extracts were prepared immediately or 1 or 4 hours later. Relative hRpb1 levels were determined by SDS-PAGE and immunoblotting. The migration positions of form IIo (hyperphosphorylated) and IIa (hypophosphorylated) are indicated.

Figure 2. A . Rpb1 levels following UV irradiation of yeast. Yeast (strain FY56) were irradiated at 22.5 J per m² as described in Materials and Methods and whole cell extracts were made at the indicated times post-irradiation. Rpb1 was detected by SDS-PAGE followed by immunoblotting. **B.** Rpb1 levels following 4-NQO treatment. 4-NQO was added to liquid cultures of log-phase yeast at the indicated concentrations and cells were collected at the indicated times following addition. Whole cell extracts were prepared and Rpb1 was detected by SDS-PAGE and immunoblotting.

Figure 3. Anti-ubiquitin antibody recognizes high molecular weight forms of Rpb1 from 4-NQO-treated cells. Yeast were treated with 4-NQO at the indicated concentrations for 30 minutes and whole cell extracts were prepared. Rpb1 was immunoprecipitated in duplicate from each sample with anti-CTD antibody. The immunoprecipitates were then analyzed by SDS-PAGE followed by immunoblotting using either anti-CTD antibody (left) or anti-ubiquitin antibody (right).

Figure 4. 4-NQO treatment of the *Gal-RSP5* strain maintained in galactose or shifted to dextrose. The *Gal-RSP5* strain was grown to early log phase in galactose containing media, then the cells were either shifted to dextrose-containing media for 48 hours or maintained in galactose-containing media. The cultures were then treated with 4-NQO at the indicated concentrations for 30 minutes and whole cell extracts were prepared and analyzed by SDS-PAGE and immunoblotting using either an anti-Rsp5 monoclonal antibody (bottom) or anti-CTD antibody (top).

Figure 5. 4-NQO treatment of the *rsp5-1* temperature-sensitive mutant. **A.** Strains FY56 (*RSP5*) and FW1808 (*rsp5-1*) were grown to mid-log phase at 30° and then shifted to 37° for 1 hour. 4-NQO was then added at the indicated concentrations for 30 minutes. Whole cell extracts were prepared and Rpb1 was detected by SDS-PAGE and immunoblotting. **B.** Similar experiment as in A., except cells were treated with 4-NQO at both 30° and 37°.

Figure 6. 4-NQO treatment of *SEN3* and *sen3-1* strains at 37°. 4-NQO was added at the indicated concentrations for 30 minutes. Whole cell extracts were prepared and Rpb1 was detected by SDS-PAGE and immunoblotting.

Figure 7. **A.** Schematic representation of yeast Rsp5 and human Rpf1/Nedd4. GST-Rpf1 fusion proteins were made to the regions of Rpf1 indicated by the solid bars. **B.** Left panel. HeLa cell

extract was prepared in NP-40 lysis buffer (see Materials and Methods). Binding of hRpb1 to GST-Rpf1 fusion proteins immobilized on glutathione sepharose was analyzed by SDS-PAGE and immunoblotting. Lane 1 represents hRpb1 present in the extract with form IIo, IIa, and IIb indicated. The right panel shows a similar experiment using HeLa cell extract prepared in RIPA buffer. The input and binding to GST-WW are shown.

Figure 8. Ubiquitination of Rpb1 by Rpf1 *in vitro*. Rpb1 was translated *in vitro* in rabbit reticulocyte lysate in the presence of ^{35}S -methionine. Purified E3 proteins (human E6-AP, ^{aa(193-901)} human Rpf1, yeast Rsp5, and the active-site cys-to-ala (C-A) mutant of Rsp5) were incubated as indicated with Rpb1 in the presence of ATP, ubiquitin, E1 enzyme, and E2 enzyme (*A. thaliana* Ubc8) as described (18).

Fig. 1

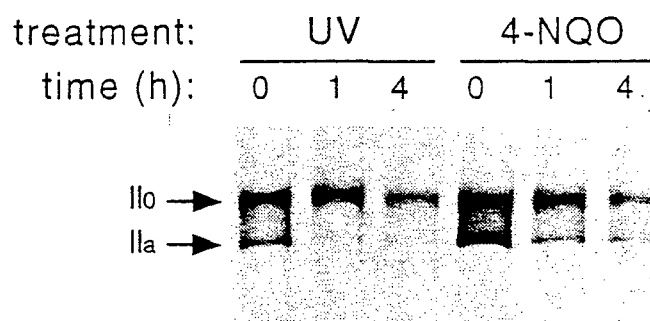
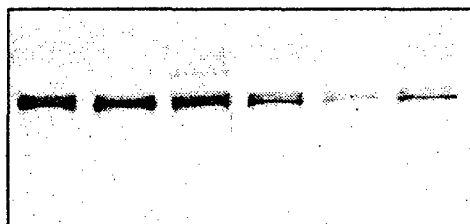


Fig. 2

A

UV irradiation:	-	+	+	+	+	+
recovery time (h):	-	-	.5	1	2	4



B

	30 min.				60 min.			
4-NQO ($\mu\text{g/ml}$):	0	.5	1	2	0	.5	1	2

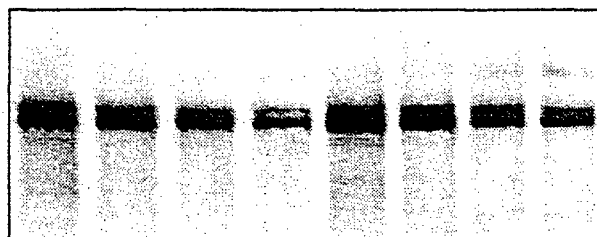


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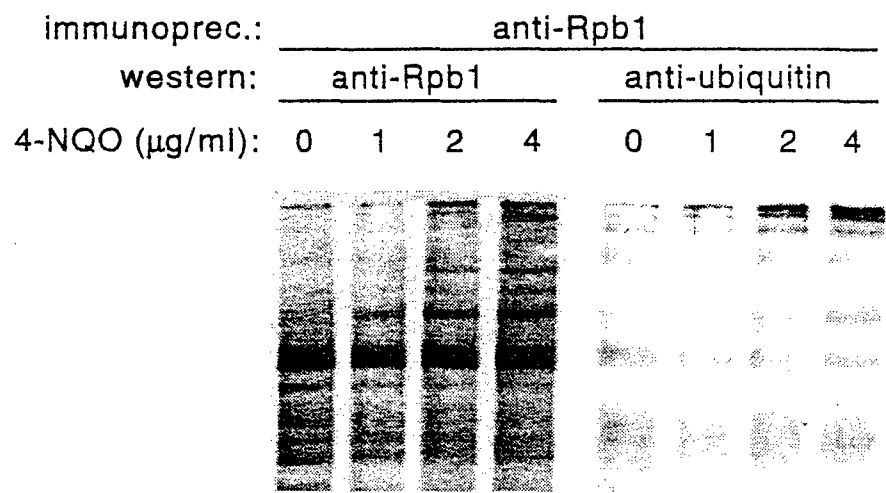


Fig. 4

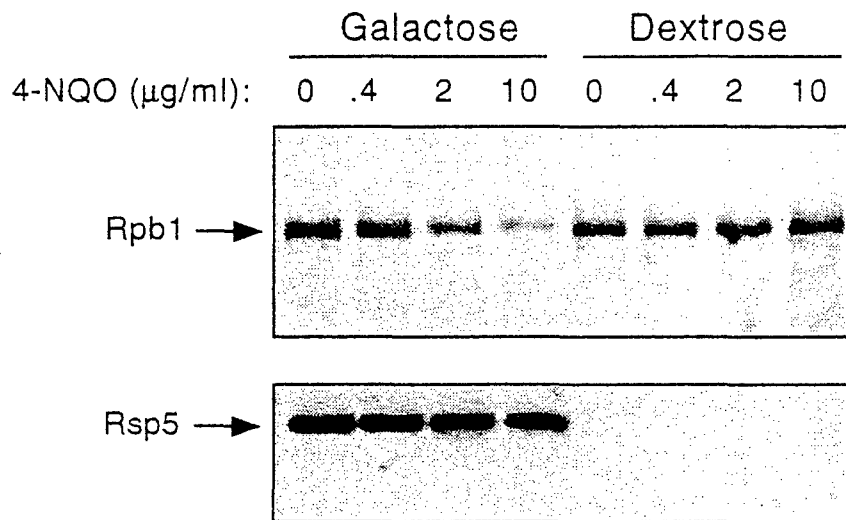
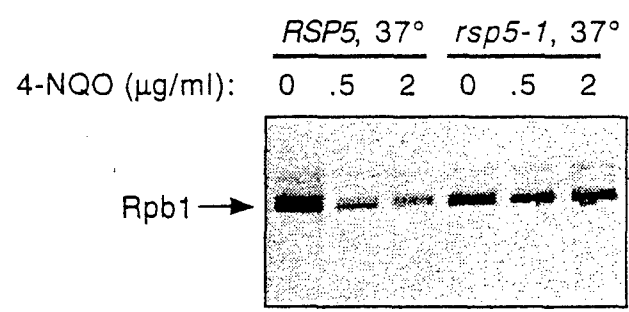


Fig. 5

A



B

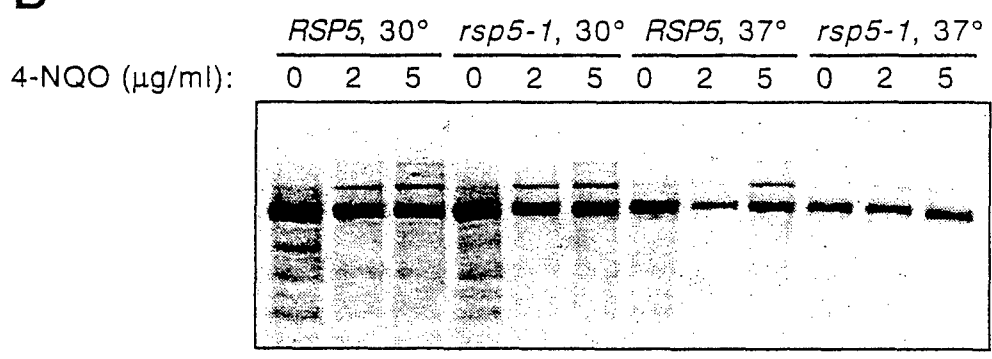


Fig. 6

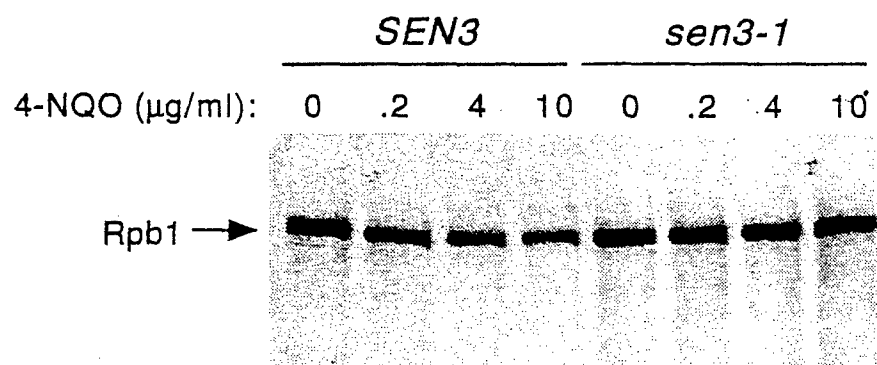
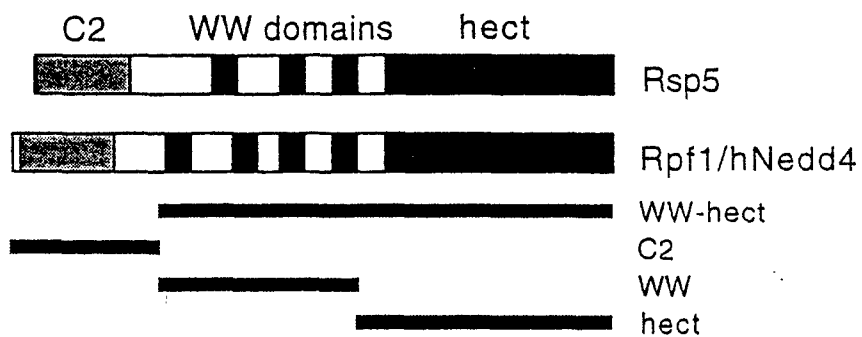


Fig. 7

A



B

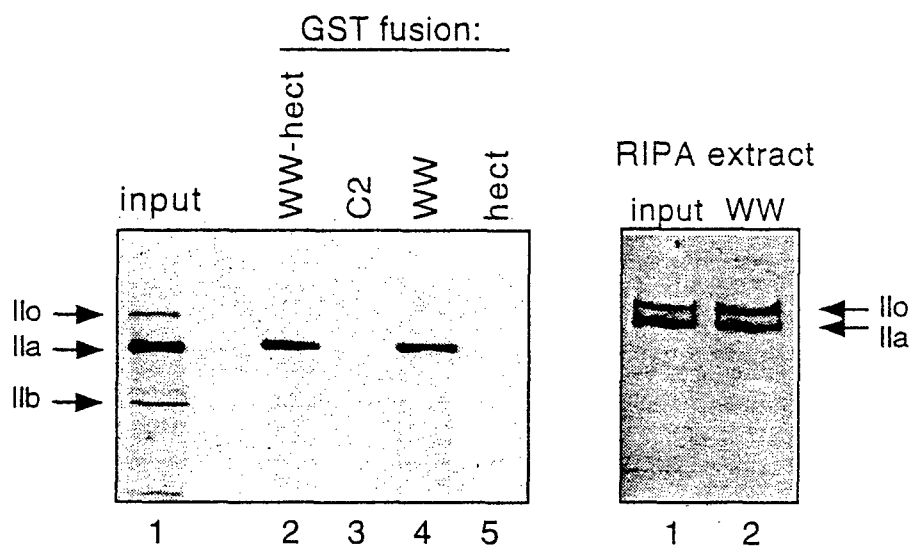
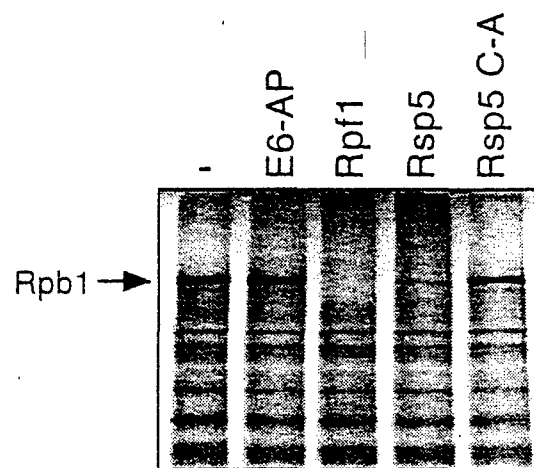


Fig. 8



Peptide antagonists of the human estrogen receptor

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Abstract

Estrogen receptor α transcriptional activity is regulated by distinct conformational states that are the result of ligand binding. Phage display was used to identify peptides that interact specifically with either estradiol or tamoxifen activated estrogen receptor α . When these peptides were coexpressed with estrogen receptor α in cells, they functioned as ligand specific antagonists indicating that estradiol-agonist and tamoxifen-partial agonist activities do not occur by the same mechanism. The ability to regulate estrogen receptor α transcriptional activity by targeting sites outside of the ligand binding pocket has implications for the development of estrogen receptor α antagonists for the treatment of tamoxifen refractory breast cancers.

Approximately 50% of all breast cancers express the ER α protein and recognize estrogen as a mitogen (1). In a subpopulation of these tumors, antiestrogens, compounds that bind ER and block estrogen action, effectively inhibit cell growth. In this regard, the antiestrogen tamoxifen has been used widely to treat ER positive breast cancers (2). Although antiestrogen therapy is initially successful, most tumors become refractory to the antiproliferative effects of tamoxifen within 2 to 5 years. The mechanism by which resistance occurs is controversial; however, it does not appear to result as a consequence of ER mutations or altered drug metabolism (3). It may relate instead to the observation that tamoxifen is a selective estrogen receptor modulator (SERM), functioning as an ER-agonist in some cells and as an antagonist in others (4). Consequently, the ability of tumors to switch from recognizing tamoxifen as an antagonist to an agonist has emerged as the most likely cause of resistance. Upon binding ER, both estradiol and tamoxifen induce distinct conformational changes within the ligand binding domain (5). The tamoxifen induced conformational change may expose surfaces on the receptor that allow it to engage the general transcription machinery. We used phage display to identify specific peptides that interacted with the estradiol- and tamoxifen-ER complexes and used these peptides to show that estradiol and tamoxifen manifest agonist activity by different mechanisms.

Affinity selection of phage displayed peptide libraries was performed to identify peptides, that could interact specifically with the agonist. 17 β -estradiol (estradiol), or 4-OH tamoxifen (tamoxifen), activated ER α or ER β (6). Representative peptides from each of four classes presented in this study are shown in Fig. 1A. Several peptides that were isolated using estradiol activated ER α (represented by α/β I) contained the LXXLL motif found in nuclear receptor coactivators (7). α II was isolated using either estradiol or tamoxifen activated ER α . Two classes of peptides, α/β III and α/β V, were identified that

interact specifically with tamoxifen activated ER α and ER β , respectively. The α/β V peptide was subsequently shown to interact with tamoxifen activated ER α (6). Several additional peptides homologous to α/β V were also identified. A BLAST search of the National Center for Biotechnology Information (NCBI) database using the derived consensus of the α/β V peptide class revealed that the yeast protein, RSP5, and its human homologue, receptor potentiating factor (RPF1), both contain sequences homologous to α/β V. These proteins were previously shown to be coactivators of progesterone receptor B (PRB) transcriptional activity (8).

Peptide-peptide competition studies were performed using time-resolved fluorescence (TRF) to determine if the α II, α/β III, and α/β V peptides were binding the same or distinct "pockets" on the tamoxifen-ER α complex (9). The α/β III and α/β V peptides cross-compete and at equimolar peptide concentrations, 50% inhibition is observed (Fig. 1B). This indicates that these two peptides bind to the same or overlapping sites on ER α . We believe that the α II peptide binds to a unique site as its binding was not competed by α/β V and only 50% inhibited by a 10-fold excess of the α/β III peptide.

We next assessed whether the peptides interacted with ER α in vivo using the mammalian two-hybrid system (10). The α/β I peptide interacted with ER α in the presence of the agonist estradiol but not the SERMs tamoxifen, raloxifene, GW7604, idoxifene, and nafoxidine or the pure antagonist ICI 182,780 (Fig. 2). The failure of antiestrogen activated ER α to interact with the α/β I peptide is consistent with previous studies which predict the molecular mechanism of antagonism results from a structural change in the receptor ligand binding domain that prevents coactivators from binding (5).

α II interacts with the receptor in the presence of all modulators tested, with the unliganded (vehicle) and ICI 182,780 bound receptors showing the least binding activity. α/β III and α/β V interact almost exclusively with the tamoxifen bound ER α . ER α does not interact with the Gal4 DBD (control) alone in the presence of any modulators tested. Further studies indicate that binding of α II, α/β III and α/β V occurs within the hormone binding domain between amino acids 282 and 535 (11) and unlike α/β I, do not require a functional activation function 2 (AF-2) (WWW.sciencemag.org/feature/data/1039590.shl). These data indicate that SERMs induce different conformational changes in ER α within the cell and firmly establish a relationship between the structure of an ER α -ligand complex and function.

When we examined the specificity of interaction between the peptides and heterologous nuclear receptors we found, as expected, that the α/β I peptide interacted with ER β , PRB, and the glucocorticoid receptor (GR) when bound by the agonists estradiol, progesterone, and dexamethasone, respectively (Fig. 3A, B and C). The α/β V peptide interacted with tamoxifen bound ER β and unexpectedly with PRB in the presence of the antagonists RU 486 or ZK 98299 (Fig. 3A and B). The α/β V peptide, however, did not interact with the glucocorticoid receptor (GR) when bound by RU 486 or ZK 98299. α II and α/β III peptides failed to interact with ER β , PRB, or GR.

We next tested the ability of the peptide-Gal4 fusion proteins to inhibit ER α transcriptional activity. Tamoxifen displays partial agonist activity when analyzed using the ER responsive complement 3 (C3) promoter in HepG2 cells (Fig. 4A). This activity can

reach 35% of that exhibited by estrogen and is mediated by three non-consensus EREs located in the C3 promoter (12). When expressed in this system, the α/β I and α II peptides inhibited the ability of estradiol to activate transcription up to 50% and 30%, respectively (Fig. 4B). Two copies of the LXXLL sequence found in α/β I enhance the inhibitory effect of this peptide and block estradiol mediated transcription by approximately 90% (13). The inability of α/β III and α/β V to block estradiol mediated transcription correlates well with their inability to bind the receptor when bound by agonist. Expression of α II, α/β III, and α/β V peptides blocked the partial agonist activity of tamoxifen (Fig. 4C). α II and α/β V were the most efficient disrupters of tamoxifen mediated transcription inhibiting this activity by approximately 90%. All peptide-Gal4 fusion proteins were expressed at similar levels indicating that the relative differences in inhibition are not due to peptide stability (14). We also demonstrated that receptor stability and DNA binding are not affected by peptide expression (15). As expected, α/β I is unable to inhibit tamoxifen mediated transcription. These findings are in agreement with the binding characteristics of these peptides and suggest that the pocket(s) recognized by α II, α/β III, and α/β V are required for tamoxifen partial agonist activity. Although α/β V was shown to interact with PRB when bound by RU 486 (Fig. 3B), it was unable to block the partial agonist activity mediated by PRB/RU 486 (16). This suggests that ER α /tamoxifen and PRB/RU 486 partial agonists activities are manifest differently. However, since α/β V was selected against ER α , this peptide may not bind PRB with high enough affinity to permit it to be useful as a PRB peptide antagonist.

Finally, we examined the ability of these peptides to inhibit ER transcriptional activity mediated through AP-1 responsive genes. This pathway has been proposed to account for some of the cell specific agonist activity of tamoxifen (17). Both estradiol and tamoxifen activated transcription from the AP-1 responsive collagenase reporter gene, pCOL-Luc (Fig. 4D). This activity is manifest in the absence of an ERE and is believed to occur through a mechanism involving an interaction between ER α and the promoter bound AP-1 complex (17). Regardless of the mechanism, each peptide was able to inhibit ER α mediated transcriptional activity in a manner that reflected its ability to interact with the receptor in a ligand dependent manner (Fig. 4E).

The mechanism by which tamoxifen manifests SERM activity is not yet known. Evidence presented in this study suggests that the tamoxifen bound receptor exposes a binding site that is occupied by a coactivating protein, not primarily utilized by the estradiol activated receptor. The α II peptide, which interacts with both estradiol and tamoxifen bound receptors, inhibits the partial agonist activity of tamoxifen efficiently, while minimally affecting estradiol mediated transcription. This suggests that this site, although crucial for tamoxifen mediated transcription, is dispensable for estrogen action. In addition, the ability of α/β III and α/β V to bind tamoxifen specific surfaces and inhibit tamoxifen mediated partial agonist activity suggests that these peptides may potentially recognize a protein contact site on ER that is critical for this activity. In this regard, we can demonstrate that similar to α/β V, overexpression of RPF1 specifically represses tamoxifen mediated partial agonist activity (Fig. 4F). However, the physiological significance of this activity remains to be determined. In summary, we have identified a series of peptide antagonists of ER α and hence validated additional target sites other than the ligand binding pocket for new drug discovery.

Figure 1. Isolation of ER α interacting peptides. **(A)** ER α interacting peptides were isolated by phage display (6). Eighteen libraries were screened each containing a complexity of approximately 1.5×10^9 phage. Several LXXLL (boxed) containing peptides were isolated of which α/β I is shown. One peptide each was isolated for the α II and α/β III peptide classes. Six peptides were isolated, including α/β V, that contained a conserved motif (boxed). Two proteins, RSP5 and RPF1, containing sequence homology to α/β V are shown. **(B)** Time resolved fluorescence (TRF) was utilized in competition mode to determine if ER α /tamoxifen interacting peptides recognize a common site on ER α (9). The peptide conjugate used for detection is indicated in each graph with the competing peptides as follows ($\blacktriangle$) no competitor, ($\circ$) α II, ($\bullet$) α/β III, ($\blacksquare$) α/β V.

Figure 2. ER α -peptide interactions in mammalian cells. The coding sequence of a peptide representative from each class identified was fused to the DNA binding domain (DBD) of the yeast transcription factor Gal4. HepG2 cells were transiently transfected with expression vectors for ER α -VP16 and the peptide-Gal4 fusion proteins. In addition, a luciferase reporter construct under the control of five copies of a Gal4 upstream enhancer element was also transfected along with a pCMV- β galactosidase vector to normalize for transfection efficiency. Transfection of the Gal4 DBD alone is included as control. Cells were then treated with various ligands (100nM) as indicated and assayed for luciferase and β galactosidase activity. Normalized response was obtained by dividing the luciferase activity by the β galactosidase activity. Transfections were performed in triplicate and error bars represent standard error of the mean (SEM). Triplicate transfections contained 1000ng ER α -VP16, 1000ng 5x Gal4-tata-Luc, 1000ng peptide-Gal4 fusion construct and 100ng pCMV- β -Gal (10).

Figure 3. Specificity of nuclear receptor-peptide interactions. Two-hybrid experiments were performed as in Fig. 2 between peptide-Gal4 fusion proteins and either (A) ER β -VP16 (B) PRB-VP16 or (C) GR-VP16 (18). RU 486 and ZK 98299 are pan-antagonists of PRB and GR.

Figure 4. Disruption of ER α mediated transcriptional activity. (A) HepG2 cells were transfected with the estrogen responsive C3-Luc reporter gene (12) along with expression vectors for ER α (19) and β galactosidase and normalized as in Fig. 2. Cells were induced with either estradiol or tamoxifen as indicated and analyzed for luciferase and β galactosidase activity. NH = no hormone. (B) HepG2 cells were transfected as above except that expression vectors for peptide-Gal4 fusions were included as indicated. Control represents the transcriptional activity of estradiol (10 nM) activated ER α in the presence of the Gal-4 DBD alone and is set at 100% activity. Increasing amounts of input plasmid for each Gal4-peptide fusion is also shown (triangle) with the resulting transcriptional activity presented as % activation of control. Data is averaged from three independent experiments (each performed in triplicate) with error bars representing SEM. Triplicate transfections contained 1000ng C3-Luc, 1000ng ER α expression vector, 100ng pCMV- β -Gal, and either 100, 500, or 1000ng peptide-Gal4 fusion construct. (C) Same as in (B) except that 4-OH tamoxifen (10 nM) was used to activate the receptor. (D) HepG2 cells were transfected with the AP-1 responsive collagenase reporter gene construct (pCOL-Luc) (12) and expression vectors for ER α and β -galactosidase. Cells were then induced with either estradiol or tamoxifen as indicated. (E) Same as (D) except that peptide-Gal4 fusion constructs were also transfected as indicated. Control represents the transcriptional activity of either estradiol or tamoxifen (100 nM) activated ER in the presence of the Gal4 DBD alone and is set at 100% activity. The transcriptional activity of estradiol and tamoxifen is shown in the presence of each Gal4-peptide fusion with the resulting transcriptional activity presented as % activation of control. Triplicate transfections contained 1000ng pCOL-Luc, 1000ng ER α expression vector, 1000ng peptide-Gal4 fusion construct, and 100ng pCMV- β -Gal. Data is presented as in (B) and (C). (F) HeLa cells were transfected with the 1X-

ERE-tata-Luc reporter gene along with expression vectors for ER α , β galactosidase, and either RPF1 (pCDNA3-RPF1) or control vector (pcDNA3 (Invitrogen, Carlsbad, CA)). Cells were induced with ligand (10nM) as indicated. Data is presented as fold induction which represents the ratio of ligand induced vs. vehicle for each transfection.

References and Notes

1. J. A. Scott and W. L. McGuire, in *Endocrine-Dependent Tumors*, K. D. Voight and C. Knabbe, eds. (Raven Press, New York, 1991), pp. 179-196.
2. C. K. Osborne, R. M. Elledge, S. A. W. Fuqua, *Sci. Am. Sci. Med.* **3**, 32 (1996).
3. B. S. Katzenellenbogen, M. M. Montano, M. E. Ekena, E. M. McInerney, *Breast Cancer Res. Treat.* **44**, 23 (1997).
4. M. Sato, M. K. Rippey, H. U. Bryant, *FASEB J.* **10**, 905 (1996).
5. A. M. Brzozowski *et al.*, *Nature* **389**, 753 (1997); A. K. Shiau *et al.*, *Cell* **95**, 927 (1998).
6. Phage display was performed as described (L. A. Paige *et al.*, *Proc. Natl. Acad. Sci. USA* **96**, 3999 (1999)). Immulon 4 96 well plates (Dynex Technologies, Inc.) were coated with streptavidin in NaHCO₃ buffer (pH 8.5) at 4°C for approximately 18 hrs. Wells were blocked with bovine serum albumin (BSA) and then washed with TBST (10mM Tris-HCl, pH 8.0/ 150mM NaCl/ 0.05% Tween 20) and 2 pmol of biotinylated vitellogenin ERE was then added per well. Plates were washed with TBST and 3 pmol of baculovirus purified ER α or ER β (Pan Vera Corp., Madison, WI) was then added and plates were incubated at room temperature for 1 hr. Hormone was then added (1 μ M) along with phage library (containing approximately 1.5 X 10⁹ phage) in TBST and incubated at room temperature for 1 hr. Non-binding phage were removed by washing with TBST. Bound phage were eluted in pre-warmed (50°C) 50 mM glycine-HCL. Eluent was neutralized by the addition of 200mM Na₂HPO₄ (pH 8.5) and phage were amplified in *E. coli* (DH5 α F'). Affinity selection was repeated 3 times and individual phage were isolated from either the second or third round of amplification. Peptide sequences were then deduced by DNA sequencing.
7. D. M. Heery, E. Kalkhoven, S. Hoare, M. G. Parker, *Nature* **387**, 733 (1997).

8. M. O. Imhof and D. P. McDonnell, *Mol. Cell. Bio.* **16**, 2594 (1996).
9. Time resolved fluorescence (TRF) assays were performed at room temperature as follows: Costar high-binding 384 well plates were coated with streptavidin in 0.1 M sodium bicarbonate and blocked with BSA. Twenty μL of biotinylated ERE (100nM in TBST) was added to each well. Following a 1 hr incubation, biotin (50 μM in TBST) was added to block any remaining binding sites. The plates were washed, and 20 μL of ER α (100nM in TBST) was added to each well. Following a 1 hr incubation, the plates were washed and 5 μL of 5 μM 4-OH tamoxifen was added to each well followed by 15 μL of solution containing the peptides conjugated to unlabeled streptavidin (prepared as described below) at a range of concentrations (from 1.67 μM in two fold dilutions). Following a 30 min incubation with the 4-OH tamoxifen and conjugate, 5 μL of 400nM europium labeled streptavidin (Wallac)-biotinylated peptide conjugate (prepared as described below) was added and incubated for 1 hr. The plates were then washed and the europium enhancement solution was added. Fluorescent readings were obtained with a POLARstar fluorimeter (BMG Lab Technologies) using a < 400 nm excitation filter and a 620 nm emission filter. The streptavidin-biotinylated peptide conjugates were prepared by adding 4 pmol biotinylated peptide per pmol of streptavidin. After incubation on ice for 30 min, the remaining biotin binding sites were blocked with biotin prior to addition to the ER coated plate.
10. HepG2 cells were maintained in modified eagles medium (MEM) (Life Technologies, Grand Island NY) supplemented with 10% fetal calf serum (FCS) (Life Technologies). Transfections were performed as described (J. D. Norris *et al.*, *J. Biol. Chem.* **270**, 22777 (1995)). pCMV- β -Gal and 5x GAL4-tata-Luc were described previously (B. L. Wagner, J. D. Norris, T. A. Knotts, N. L. Weigel, D. P. McDonnell,

Mol. Cell. Bio. **18**, 1369 (1998)). Gal4 DBD-peptide fusions were created as follows: Peptide coding sequences were excised from mBAX vector with XhoI/XbaI and subcloned into pM vector (Clontech) with a linker sequence to generate SalI and XbaI sites for cloning. ER α -VP16 was generated by PCR of human ER α -cDNA containing EcoRI sites flanking both 5' and 3' termini. The resultant PCR product was then subcloned into pVP16 (Clontech). All PCR products were sequenced to ensure the fidelity of the resultant construct. 17 β -estradiol, 4-hydroxy-tamoxifen and nafoxidine were purchased from Sigma Chemical Co. (St. Louis, MO.). ICI 182,780 was a gift from Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Raloxifene was a gift from Dr. Eric Larson (Pfizer Pharmaceuticals, Groton, CT.). Idoxifene was a gift from Dr. Maxine Gowan (SmithKline Beecham Pharmaceuticals, King of Prussia, PA.). GW 7604 was a gift from Dr. Tim Willson (Glaxo Wellcome Research and Development, Research Triangle Park, NC).

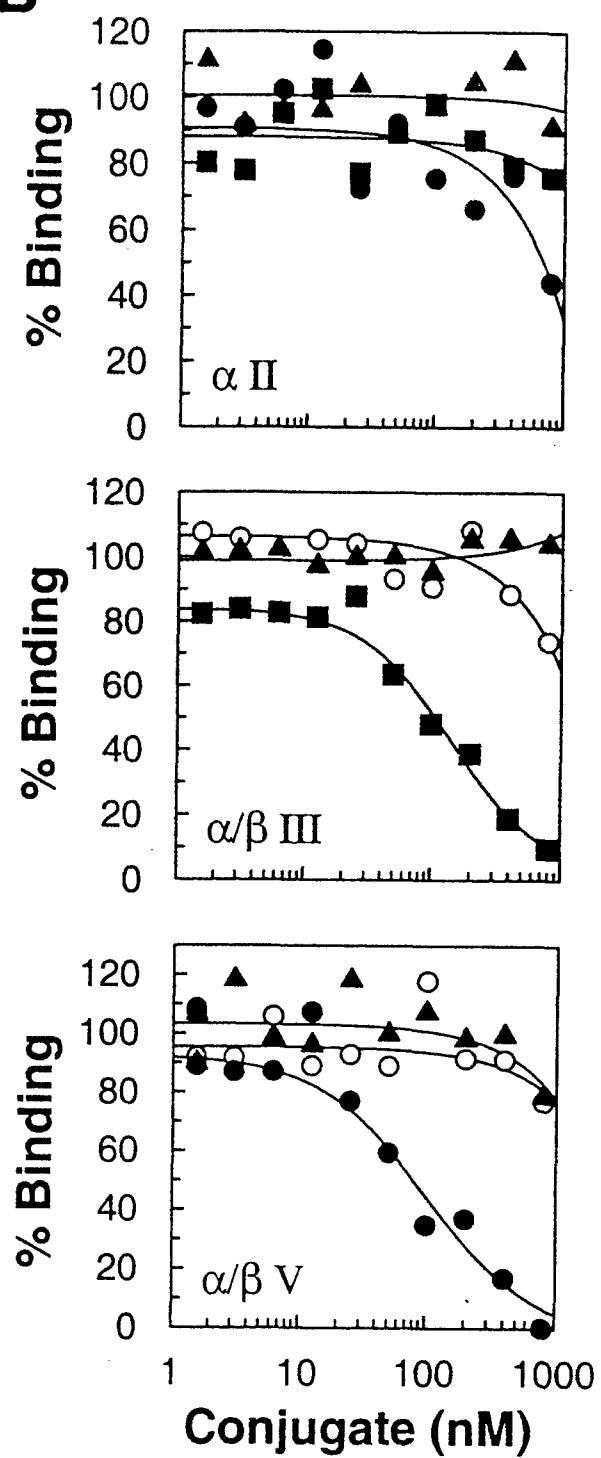
11. J. D. Norris and D. P. McDonnell, unpublished data.
12. J. D. Norris, D. Fan, B. L. Wagner, D. P. McDonnell, *Mol. Endocrinol.* **10**, 1605 (1996).
13. C. Chang and D. P. McDonnell, unpublished data.
14. J. D. Norris and D. P. McDonnell, unpublished data.
15. J. D. Norris and D. P. McDonnell, unpublished data.
16. J. D. Norris and D. P. McDonnell, unpublished data.
17. P. Webb, G. N. Lopez, R. M. Uht, P. J. Kushner, *Mol. Endocrinol.* **9**, 443 (1995).
18. ER β -VP16 was generated by PCR of ER β cDNA and resultant product was cloned into pVP16. PRB-VP16 and GR-VP16 were gifts from D. X. Wen and J. Miner (Ligand Pharmaceuticals, San Diego, CA.). Dexamethasone and progesterone were purchased

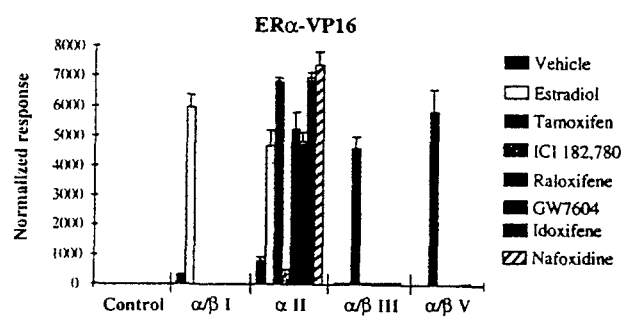
• from Sigma Chemical Co. RU 486 and ZK 98299 were gifts from Ligand Pharmaceuticals Co. and Schering-AG Pharmaceuticals (Berlin, Germany), respectively.

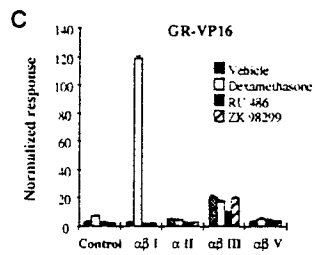
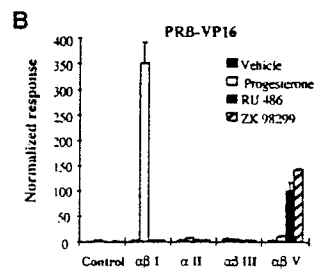
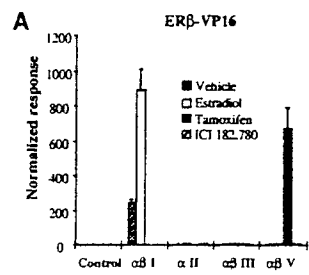
19. ER α expression vector pRST7-hER is reported elsewhere (S. L. Dana, P. A. Hoener, D. L. Wheeler, C. L. Lawrence, D. P. McDonnell, *Mol. Endocrinol.* **8**, 1193 (1994).

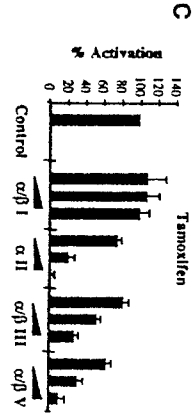
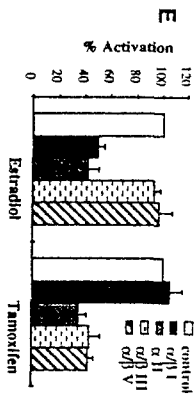
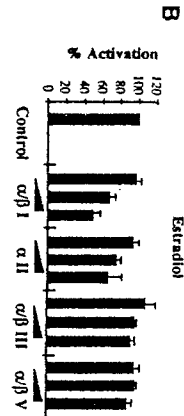
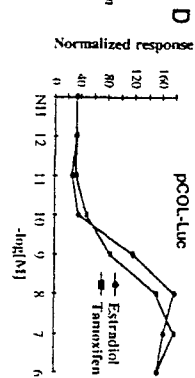
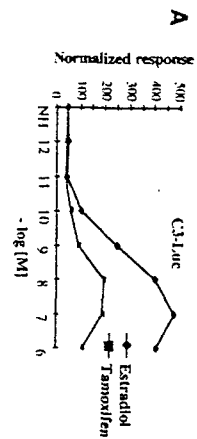
20. Supported by a grant to DPM from the NIH (DK48807).

A Peptide	Sequence	Isolation Condition
$\alpha\beta$ I	SSNHQSSR LIELL SR	Estradiol
α II	SSLTSRDEFGSWYASR	Estradiol or Tamoxifen
$\alpha\beta$ III	SSWDMHQFFWEGVSR	Tamoxifen
$\alpha\beta$ V	SSPG SREWF KDM L SR SSTT MFDF FYERLSSR SSISTYH MGEWF FYAMLSSR SSIDLYSQ MREFF QINLSSR SSWN SREFF LSQLSSR SSVA SREWF VRELSSR	Tamoxifen
<u>Consensus</u>	(S/M)X(D/E)(W/F)(W/F)XXXL	
RSP5	496-YGGV SREFF FLLSH M-510	
RPF1	727-YGGV AREWF FLISKE-741	

B









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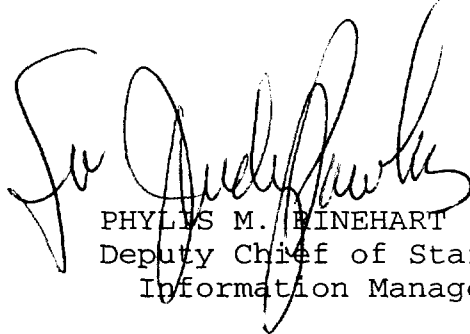
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