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13. ABSTRACT *(Maximum 200 words)*

The **human estrogen receptor** (hER), is the *in vivo* target of the female sex hormone estrogen. It is also the therapeutic target of various **antiestrogens** used during hormonal treatment in the battle of breast cancer. Many aspects of the hER function are regulated by phosphorylation. This includes transcriptional activity, DNA binding affinity and dimerization. This research project is investigating the role of **tyrosine phosphorylation** on the **dimerization** of the hER. It has been demonstrated *in vitro* that a 12 amino acid **phosphotyrosyl peptide** can prevent dimerization of the ER, acting as a pure antiestrogen. We have identified a region within the hER capable of binding a phosphotyrosyl peptide. The specificity of this interaction was further investigated using seven amino acid phosphotyrosyl peptides where it was found that the residues C-terminal to the Y537 (i.e., 536 to 542) are necessary and sufficient for blocking hER dimerization. Individual residues within this carboxy terminal region are more importance than others. These peptides represent a potentially new class of antiestrogens which appear to function with a completely different mechanism than the classical steroidal antiestrogens.

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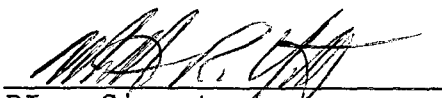

July 7, 1997

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INTRODUCTION

Background and purpose of this project:

The human estrogen receptor (hER) is a member of the steroid/thyroid hormone receptor superfamily of ligand activated transcription factors (Evans, 1989). Functionally active receptors exist as homo- or heterodimers which display high affinity ligand binding, a response to nuclear localization signals, a sequence specific DNA binding affinity, and involvement in numerous protein-protein interactions important for both signal regulation and DNA transcription.

The steroid hormone receptors are phosphoproteins (Denner et al. 1990; Denton et al. 1992). Regulation of transcription factors by protein phosphorylation has been well documented (Hunter and Karin, 1992). However, the role of phosphorylation of steroid receptors is less clear. Phosphorylation of the hER and other members of the nuclear receptor family have been implicated in the nuclear localization of the receptors, ligand binding, DNA binding, transcriptional activation, and receptor turnover (LeGoff et al. 1994; Lahooti et al. 1994). The regulation and functional importance of these phosphorylations, i.e. which sites are modified by which kinases, and why, is an area of contentious active research.

Recent studies, as outlined below, have identified phosphorylation on tyrosine 537 as a prerequisite for hER homodimerization. The scope of this research project is to identify, isolate and characterize the phosphotyrosine (pY) binding site of the hER which is believed to be the molecular basis of the coupling between monomers. In accomplishing these objectives, a greater understanding of transcription factor dimerization and of the structure-function relationship of the hER will be attained. The

phosphopeptides used in these experiments represent pure antiestrogens, and there development may lead to novel therapies for estrogen receptor dependent diseases such as breast cancer.

Overview of previous work: The dimerization of the hER was found to be dependent on phosphorylation of tyrosine 537. Dephosphorylation of the purified recombinant hER from Sf9 cells or the native hER from the MCF-7 human breast carcinoma cell line by a tyrosine phosphatase resulted in a DNA binding deficient form of the receptor as seen by gel mobility shift assay (Arnold et al 1995a). Furthermore, molecular sizing chromatography revealed that dephosphorylation of the Y537 resulted in receptor monomers, unable to dimerize and bind DNA. Two 12 residue peptides corresponding to the hER sequence from amino acids 531 to 542 were synthesized. Both tyrosine phosphorylated and non-phosphorylated peptides were prepared. Interestingly, the phosphorylated peptide, but not the non-phosphorylated peptide or an unrelated phosphotyrosyl peptide, inhibited formation of an hER-ERE complex in a concentration dependent fashion in a gel shift experiment (Arnold & Notides, 1995). The binding of the phosphotyrosyl peptide to the hER was shown by a far western assay. Furthermore, a tyrosine to phenylalanine mutation at this site results in a 'dead' receptor - unable to bind DNA, activate transcription or form homodimers (Arnold et al. 1995b, unpublished data). These data indicate the hER dimerization is phosphotyrosine directed and occurs via reciprocal phosphotyrosine binding interactions of the monomers.

BODY

Results and Discussion

1. Identification of the phosphotyrosine binding domain.

The hER binds specific for phosphopeptide Yp537---A phosphotyrosyl peptide corresponding to the sequence surrounding Y537 of the hER was synthesized as well as the unmodified tyrosyl peptide, a sulfated tyrosine derivative, and an unrelated phosphotyrosyl peptide (Table 1 and Figure 1a.). A far western affinity blot with the ^{32}P-labeled Yp537 was first used to assess peptide binding to purified recombinant hER. As previously shown, the unlabeled Yp537 peptide can compete for binding to the hER while the dephosphorylated peptide (Y537) or an unrelated phosphotyrosyl peptide (STAT) cannot (Figure 1b.). We also demonstrate that the tyrosine sulfated peptide derivative (Ys537) fails to compete with the labeled Yp537 peptide for hER binding at concentrations up to 200 μM (Fig. 1b). This suggests the Yp537 peptide-hER binding specificity for phosphate transcends electrostatic interactions.

TABLE 1. Summary of peptides discussed in the text. p = phosphate; s = sulfate; STAT and RXR are unrelated phosphotyrosyl peptide sequences.

Phosphopeptide Competitors	
Phosphopeptide	**Sequence**
Y537	NVVPLY DLLLE
Yp537	NVVPLYpDLLLE
Ys537	NVVPLYsDLLLE
STAT-Yp	GYpIKTE

The Yp537 peptide is specific for the hER.---Equivalent amounts of the hER, the thyroid hormone receptor (TR), the retinoic acid receptor β (RXRβ), and the SH2 domains from the tyrosine kinase Lck and the signaling intermediate Grb2 were probed with ^{32}P-labeled Yp537 peptide in a far western assay. Only the hER interacts with the labeled peptide (Fig. 1c). Immunopurified progesterone receptor was also unreactive to the phosphopeptide (data not shown).

GST fusion proteins with hER fragments identify a region of phosphopeptide binding---Having demonstrated a direct and sequence specific phosphotyrosyl peptide interaction with the hER, we next sought to determine the region of the receptor responsible for binding the Yp537 peptide. A variety of hER fragments or sub-domains were prepared as glutathione-S-transferase (GST) fusion proteins with boundaries based on the location of functional domains (Kumare et al. 1987) or encoded entirely by one or more exons (Ponglikitmongkol et al. 1988) (Fig 2a). Only the GST-hER(1-340) and the GST-hER(251-367) interacted with the ^{32}P-Yp537 peptide (Fig. 2b). The peptide binding region therefore lies between amino acids 251-340, which includes the hinge or D domain and the extreme amino terminal region of the ligand binding domain and is entirely coded within hER exon 4.

Phosphopeptide titration of GST-hER(251-367)---To further analyze this region for peptide binding and to compare relative peptide affinities, competition experiments were carried out by titrating unlabeled competitor peptides with equivalent amounts of GST-hER(251-367) protein, a procedure similarly used to compare relative peptide affinities for SH2 domains (Huyer et al. 1995) . Preliminary data with the phosphorylated and nonphosphorylated peptides agrees with far western

data in that only the phosphorylated peptide interacts with the hER fragment. These experiments are being confirmed and the data is not shown. The inhibitory concentration that reduces the binding of the radiolabeled peptide to the GST-hER(251-367) by 50% (IC 50) is in the range of one to four micromolar under these conditions. This assay is suitable for testing various peptide ligands as well as mutant or truncated forms of the phosphotyrosine binding region.

GST-hER(251-367) has little homology with SH2 and PTB domains--Comparison of exon 4 from the hER with several SH2 domains and the PTB domain from Shc revealed no significant sequence homology. In addition, a search of the sequence surrounding Y537 did not reveal homology with any known SH2 or PTB domain substrate (GCG, Wisconsin Package).

Immobilized peptides as an affinity support---Based on the results discussed, it was hypothesized that the phosphopeptide, when immobilized on a solid support, such as agarose, will interact with the hER and the fragment which binds the peptide. Thus far, these experiments have failed to confirm this hypothesis. A variety of phosphopeptide conjugates were prepared and used under a variety of conditions, as similarly reported for SH2 domain - Phosphopeptide interactions. The specific problem occurring in these experiments is a very high background binding of the hER to negative control affinity supports. The negative control supports include the unphosphorylated peptide conjugate as well as unconjugated agarose. The hER appears to bind the supports non-specifically and any affinity for the phosphopeptide is lower than detectable under these conditions. Currently, I am focusing on developing the assay discussed above which

involves immobilizing the protein domain and the full length hER and titrating radiolabeled and cold peptides.

2. Analysis of peptide specificity

Phosphopeptides block hER DNA binding depending on their amino acid sequence---Gel mobility shift assays were used to assess the peptide sequence specificity inherent in the hER interaction. Two truncated peptides were synthesized and used with the unphosphorylated Y537 peptide as controls. Surprisingly the truncated peptide which includes the amino acids carboxy terminal to the phosphotyrosine blocked DNA binding while the amino terminal peptide sequence did not (Figure 3a). Two phosphotyrosyl peptides with unrelated sequences (STAT-Yp and RXR-Yp) also failed to alter hER DNA binding. The tyrosine sulfated peptide used previously in far westerns was not an effective inhibitor either (Figure 3b). These data illustrate the specificity of peptide interactions for both tyrosine phosphorylation and amino acid sequence.

Inhibition of hER DNA binding with an 11-mer phosphotyrosyl peptide has an IC50 of 1.3 μM---A twelve amino acid phosphotyrosyl peptide derived from the sequence surrounding tyrosine 537 of the hER (figure 4a) was previously shown to block DNA binding by impairing receptor dimerization. Removal of the amino terminal cysteine residue from this peptide does not affect the affinity of the peptide for the hER. A scatchard analysis of the gel mobility peptide interference data estimates an affinity of this 11-mer peptide for the hER of 1.29 μM (figure 4b.)

Truncated phosphopeptides have differential binding affinity to the hER--- In order to evaluate the amino acids within the peptide sequence important for peptide binding, the 11-mer phosphopeptide, Yp537, two

seven amino acid phosphopeptides were prepared corresponding to the overlapping carboxy (-7C) or amino terminal (-7N) fragments. Far western (Figure 6) and gel mobility shift analysis of hER-peptide interaction found the Yp537- 7C to be greater than 10 times as effective than the amino terminal derivative (Figure 5). This type of peptide specificity for carboxy terminal residues is analogous to the specificity observed for SH2 domain phosphopeptide interactions.

Alanine scanning mutagenesis of phosphopeptides reveals amino acid specificity in peptide binding---The Yp7C peptide was further altered for amino acid specificity information by individually altering each residue to alanine. Additionally, the aspartate residue corresponding to amino acid 538, was altered to both asparagine and glutamate, in addition to alanine, in order to study the role of electrostatic modifications near the phosphotyrosine. The data is summarized in table 2.

Ligand binding does not interfere with peptide binding---A variety of estrogen receptor ligands were incubated without or with 50 μM phosphopeptide. In all cases phosphopeptide inhibition of DNA binding activity was unaffected. Figure 7 illustrates the differential mobility's of the various ligand-hER-ERE complexes. This effect is a result of mixed conformations of the hER depending on the ligand species bound. It clearly demonstrates that the peptide-hER interaction occurs independent of the classical hER ligands.

TABLE 2. Summary of peptide-hER interactions. Percent inhibition was determined by quantification of bands on a PhosphorImager. The band intensity of the Yp537 lane was subtracted from the band intensity of the control lane without added peptide. This value was chosen as 100% for the purposes of displaying relative effectiveness under identical conditions. All peptides were 80 μM final concentration. These data are an average of two or more experiments

Phosphopeptide Competitors		
Phosphopeptide	**Sequence**	**Percent Inhibition**
Yp537	NVVPLYpDLLLE	100 %
Ys537	NVVPLYsDLLLE	< 10 %
Y537	NVVPLY DLLLE	< 10 %
Yp537-7C	LYpDLLLE	79 %
Yp537-7N	NVVPLYpD	< 10 %
Yp537-7C(D538N)	LYpNLLLE	*(65 %)*
Yp537-7C(D538E)	LYpELLLE	82.7 %
Yp537-7C(D538A)	LYpALLLE	49.5 %
Yp537-7C(L539A)	LYpNALLE	31.8 %
Yp537-7C(L540A)	LYpDLALE	46.0 %
Yp537-7C(L541A)	LYpDLLAE	15.0 %
Yp537-7C(E542A)	LYpNLLLA	16.3 %

The importance of this data is its demonstration of peptide binding determinants. The D538E peptide appears to have slightly higher affinity for the hER than the 'wild type' sequence. The drastic effects of altering the leucine at 541 and the carboxy terminal glutamic suggests a longer peptide may bind tighter. These are the necessary first experiments with

the long term aim in developing pure peptidomimetic antiestrogens. Future goals include isolating the highest affinity peptide sequence,. a mimetic which is phosphatase or peptidase resistant, and developing assays to test them *in-vivo*.

Experimental Methods

hER expression and purification---The recombinant hER expressed in Sf9 cells was used as a whole cell extract or purified to near homogeneity using DNA-affinity chromatography in the presence of phosphatase and protease inhibitors. (Obourn ,1994)

Far Western Blot---Approximately 20 pmoles of purified hER was run on a 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked at 4°C overnight in PBS, pH 7.4, containing 2.5% PVP-40, 1% BSA and 0.1% Tween-20. . Peptides were synthesized by California Peptide Research Inc. as previously described (Huyer et. al., 1995) and analyzed by mass spectroscopy and HPLC for composition and purity. Peptides were >90% pure. The Y537 peptide was labeled with ^{32}P-γ-ATP and a tyrosine kinase (gift of Dr. W. Huckle, Merck Pharmaceutical) with a typical efficiency of 43-52% as determined by HPLC. Free ATP was removed from the labeling reaction using a Sep-Pak C18 column (Millipore). The eluted peptide was dried, resuspended in methanol, and quantified. The membranes were washed with incubation buffer (1X TEE, pH7.4, 150 mM KCl, 0.5% BSA, 0.5% PVP-40, and 0.1% Tween) and probed with 100,000 cpm/ml of labelled peptide and at least a 100 fold molar excess of unlabeled competitor peptide (when indicated) for 2 h at room temperature. The membranes were washed with incubation buffer and exposed on a phosphimager

GST-hER fragment fusion proteins--Gene fragments of the hER corresponding to the protein fragments shown in figure 2a, were generated using PCR with degenerate oligonucleotides creating unique BamHI and EcoRI restriction sites and cloned into the pGEX-2T vector (Pharmacia). The DNA sequences were confirmed by sequencing. The fusion proteins were expressed in *E.coli* strain BL21 and cell extracts solubilized in SDS buffer and analyzed by SDS-PAGE and western blotting with an anti-GST antibody (Pharmacia). The over-expressed proteins ran at the predicted molecular weights. Fusion proteins were quantified by visual comparison of Coomassie Blue stained gels using albumin standards, and equivalent amounts of each fusion protein run on a 10% SDS-PAG and transferred to a PVDF membrane (Millipore).

Peptide competition assays--- Approximately 10% of GST-hER(251-367) was expressed as soluble protein from *E. coli* by growing a 1/10 dilution of an overnight culture for 3-4 hours at 22 °C before induction with 0.1 mM IPTG, followed by an additional 4-5 hour incubation at 22 °C. Cells were harvested, lysed by sonication, and analyzed by western blotting for soluble fusion protein. Purification of the protein on GSH-Sepharose was done according to manufacturer's recommendations (Pharmacia). The soluble GST-hER(251-367) fusion protein, immobilized on glutathione agarose beads (GSH-Sepharose, Pharmacia) to approximately 1 mg/ml beads, was incubated with 0.2 μM ^{32}P-labeled Yp537 and various amounts of unlabeled competitor peptides as indicated. All points are an average of 3-4 individual experiments.

Gel mobility shift assay---Gel mobility shift assays were caried out as previously described (Koszewski and Notides 1991). Each reaction contained 6 nM of hER as measured by ^{3}H-estradiol binding, and approximately 2 ng of ^{32}P labeled ERE probe (10,000 cpm/ng), in a buffer of 25 mM HEPES, pH 7.4, with1 mM Na_3VO_4, 80 mM NaCl, 10% glycerol, 0.5 mM PMSF, 1 mM leupeptin in a final volume of 20 μl. For the competition and affinity measurements, each peptide was incubated for 1 h with the DNA binding reaction at 4°C before addition of non specific DNA and 32P labeled ERE Samples were electrophoresed through a 5%polyacrylamide gel for 2.5 h at 175 volts at 4°Cusing a 9 mM Tris-HCl, 9 mM boric acid, 2 mM EDTA, pH 8 buffer. Relative band intensities were quantified by volume integration using ImageQuant software from Molecular Dynamics.

CONCLUSIONS

Our data indicates that dimerization of the hER involves the interaction of the unique PID with phosphorylated tyrosine 537, and this interaction therefore determines the hER DNA binding and transcriptional activity. The potentially novel PID is located between the hER DNA and ligand binding domains. Future studies aimed at investigating the *in vivo* regulation of hER tyrosine phosphorylation and dimerization will determine the potential for exploiting this dimerization mechanism as a therapy in hER dependent diseases such as breast cancer and osteoporosis.

One potential strategy may rely on phosphopeptide mimetics to block hER dimerization *in vivo*. These compounds would structurally resemble the phosphopeptide inhibitors being studied here. The initial conclusions with these in-vitro approaches are:

- The apparent affinity for the peptide-hER complex is 1.29 μM. The tyrosine phosphate is absolutely required for these interactions, as substitution with tyrosine sulfate or the use of phosphoserine or tyrosine fails to mimic the results.
- The amino acids carboxy terminal to phosphotyrosine are additional determinants for specific hER interaction. The phosphopeptide Yp537-7C has a 4.5 fold higher affinity for the hER. Peptide Yp537-7N has an almost 40-fold lower affinity than Yp537.
- These effects are a consequence of direct binding to the hER and are not mediated through other protein interactions or caused by non-specific competition for the ERE.

- The mechanism of the phosphotyrosyl peptide inhibition of receptor functions occur by disrupting or preventing the formation of stable hER dimers. Thus, these peptides represent pure antagonists of hER function.

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Figure Legends

Figure 1. Phosphotyrosyl peptide interactions with the hER. a, Sequence comparison of the peptides used to determine the specificity for interaction with the hER. A partial amino acid sequence of the hER is also shown. b, Far western competition assay showing the specificity of peptide Yp537 for binding to the purified hER. The ^{32}P-labeled Yp537 (20 nM) binds the hER (lane 1) and 50 μM of unlabeled Yp537 competes for binding, while 200 μM of peptides Y537, Ys537, and STAT-Yp do not (lanes 2-5). c, Specificity of Yp537 for the hER. The thyroid hormone receptor (TR), retinoid acid receptor β (RARβ) the SH2 domains from p56^{lck} kinase and Grb2 were probed along with the purified hER for their ability to bind the Yp537 peptide. Approximately one microgram of each protein was used in this assay, as determined by BCA protein assay (Pierce) and Coomassie Blue staining of SDS-PAGE (data not shown). Positions of molecular weight markers in kilodaltons are indicated to the right of the blot.

Figure 2. Identification of a phosphotyrosine interaction domain on the hER. a, The hER fragmentation strategy. Top, schematic representation of the functional domains on the hER (A-F). Middle, location of the exon coding boundaries in the translated protein. Dashed lines indicate the exon extends beyond the translated region. Bottom, the GST-fusion proteins used to evaluate binding of ^{32}P-labeled Yp537 peptide. b, Far western blot indicating binding of labeled Yp537 peptide is localized to the hinge region (amino acids 251-340) of the hER. Positions of molecular weight markers in kilodaltons are indicated to the right of the blot.

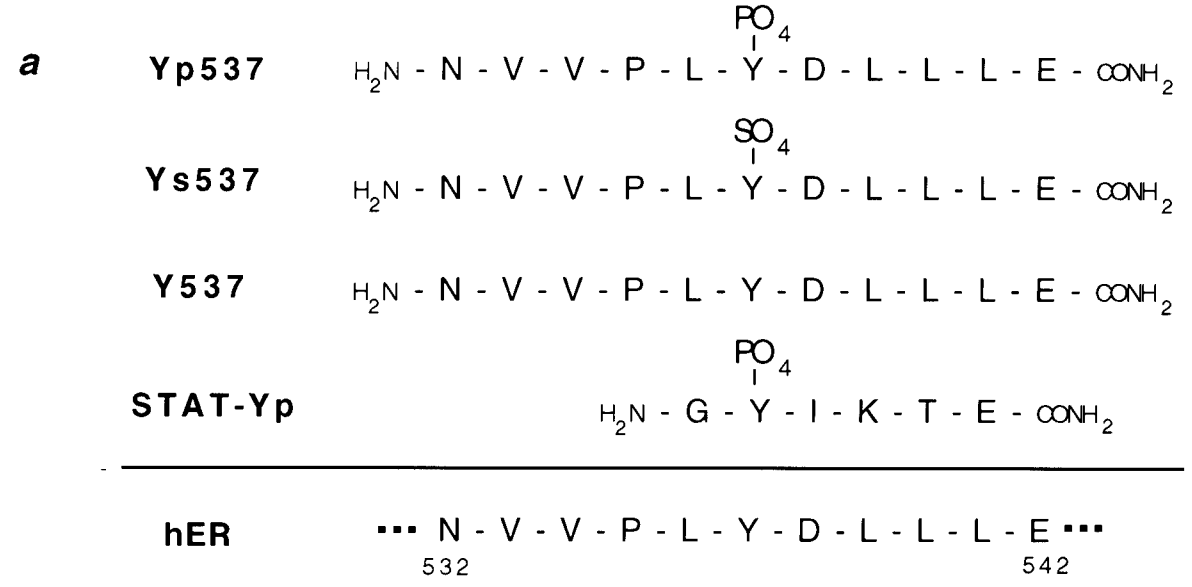
Figure 3. The phosphopeptide-hER interaction is specific for amino acids in the carboxy terminal side of phosphotyrosine. a, A gel mobility shift assay using two seven amino acids phosphopeptides corresponding to residues either amino (Yp537-7N) or carboxy terminal (Yp537-7C) to the phosphotyrosine revealed that specificity for blocking ERE-binding lies with the carboxy terminal residues. Two unrelated phosphotyrosyl peptides (RXR-Yp, and STAT-Yp) did not block hER-ERE formation. The concentrations indicated are mM. b, A twelve amino acid tyrosine sulfated peptide (Ys537) was unable to block hER DNA binding at a concentration of 500μM. The hER-ERE identity is confirmed with an hER antibody (Ab).

Figure 4. Phosphopeptide interference of human estrogen receptor (hER) ERE Binding. a, A 12 amino acid phosphotyrosyl peptide corresponding to amino acids 531 to 542 (Yp537) of the hER specifically inhibits the formation of an hER-ERE complex (lanes 1 - 8) as seen by gel mobility shift assay. The effect is dependent on the presence of the phosphate, as the dephosphorylated peptide failed to block ERE binding, even at 10-fold higher concentrations (lanes 9 - 11). The identity of the hER-ERE complex is confirmed in lane 13 with the use of an hER specific antibody (Ab). b, A Scatchard plot analysis of the hER-ERE complex found an apparent K_d for the peptide-hER interaction to be 1.29 μM. For this analysis, the concentration of peptide-hER (Bound) was determined by subtracting the intensity of each band from that of the control in lane 12.

Figure 5. The affinity of the hER for Yp537-7C lower than for Yp537, but much higher than for Yp537-7N. a, Comparison of Yp537-7N and Yp537-7C on their ability to block specific ERE-binding of the hER. The concentration range of Yp537-7C is 300mM to 3 mM while that of Yp537-7N is 1.2 mM to 100 mM. b, Scatchard analysis indicates the affinity of the Yp537-7C is 6.02 μM. Although this is 4.5 fold lower than for Yp537, the affinity of the Yp537-7N is approximately 45 μM, almost 40 fold lower (data not shown).

Figure 6. Far western competition binding analysis of 7-mer phosphopeptides to the hER. a, Far western competition assay shows that Yp537 and Yp537-7C compete with the $Y^{32}p537$ probe while the other peptides cannot. b, Immunoblot with anti-hER Ab confirming equal amounts of hER in each lane.

Figure 1.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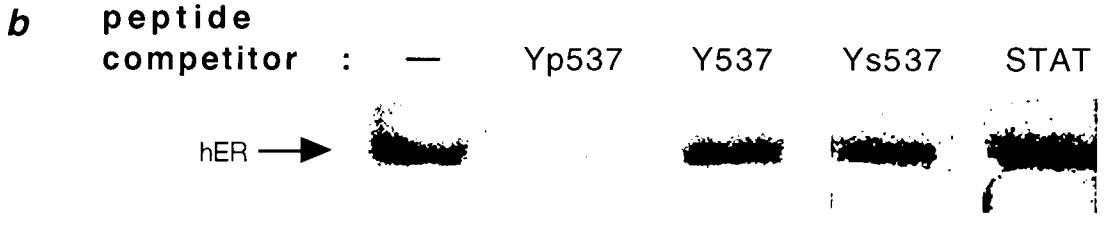
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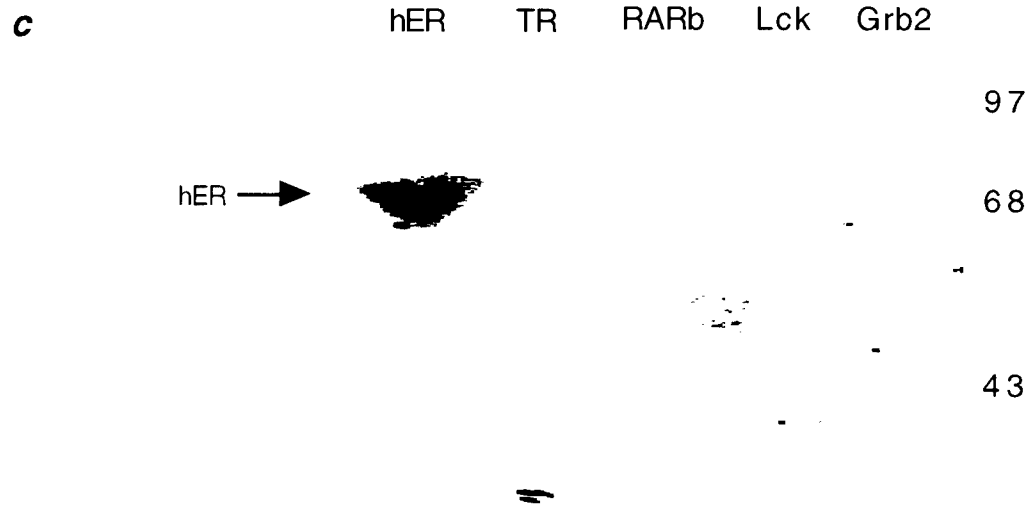
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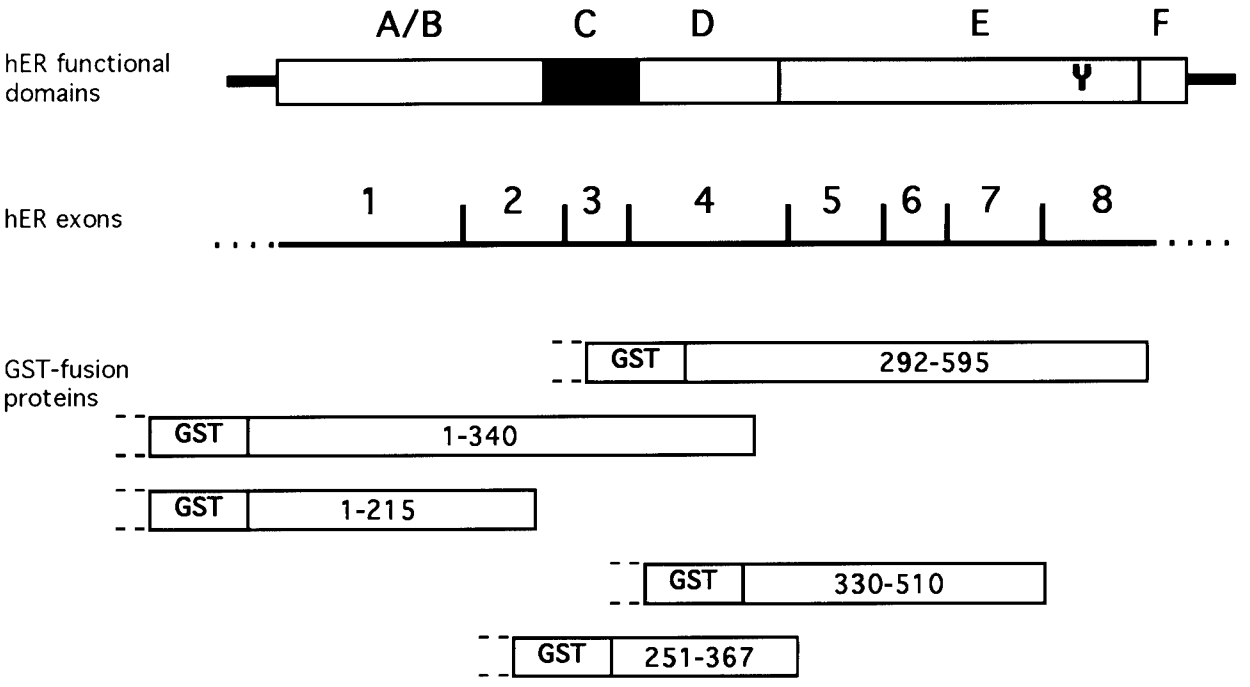
Figure 2.

a


A/B
C
D
E
F
hER functional domains
hER exons
1
2
3
4
5
6
7
8
GST-fusion proteins
GST
292-595
GST
1-340
GST
1-215
GST
330-510
GST
251-367

b

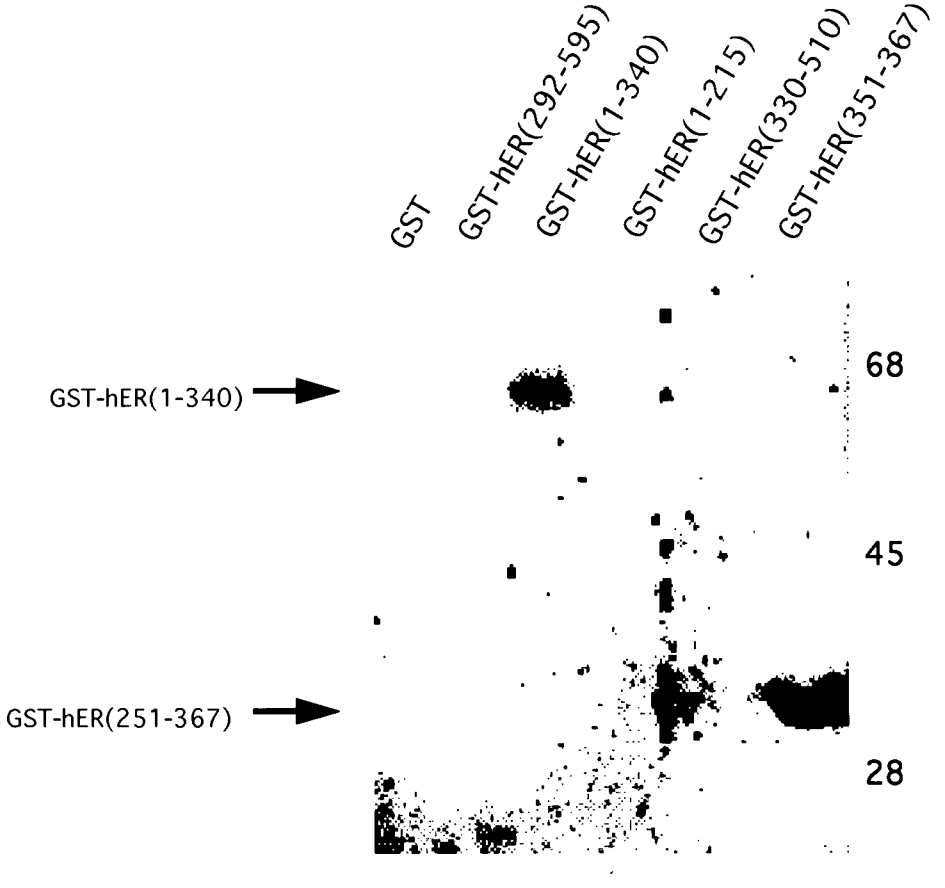
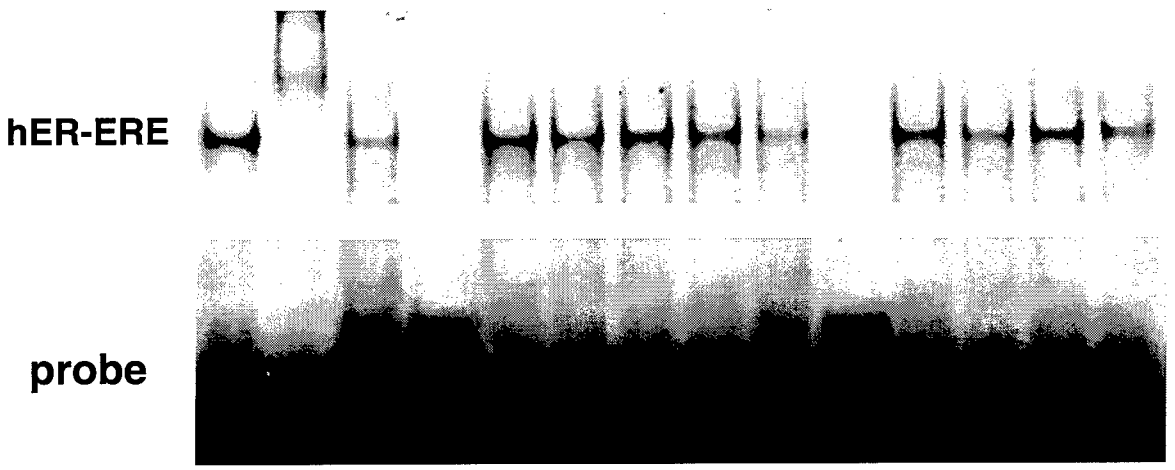

GST
GST-hER(292-595)
GST-hER(1-340)
GST-hER(1-215)
GST-hER(330-510)
GST-hER(351-367)
GST-hER(1-340)
GST-hER(251-367)
68
45
28

Figure 3.

A.

hER-Ab	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Yp537-12	-	-	10	100	-	-	-	-	-	-	-	-	-	-	-
Y537-12	-	-	-	-	10	100	-	-	-	-	-	-	-	-	-
Yp537-7N	-	-	-	-	-	-	10	100	-	-	-	-	-	-	-
Yp537-7C	-	-	-	-	-	-	-	-	10	100	-	-	-	-	-
RXR-Yp	-	-	-	-	-	-	-	-	-	-	100	1000	-	-	
STAT-Yp	-	-	-	-	-	-	-	-	-	-	-	-	100	1000	



B.

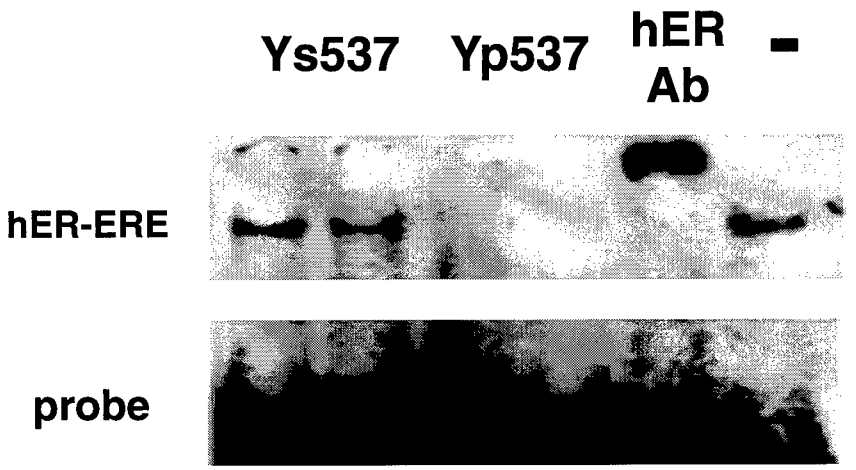
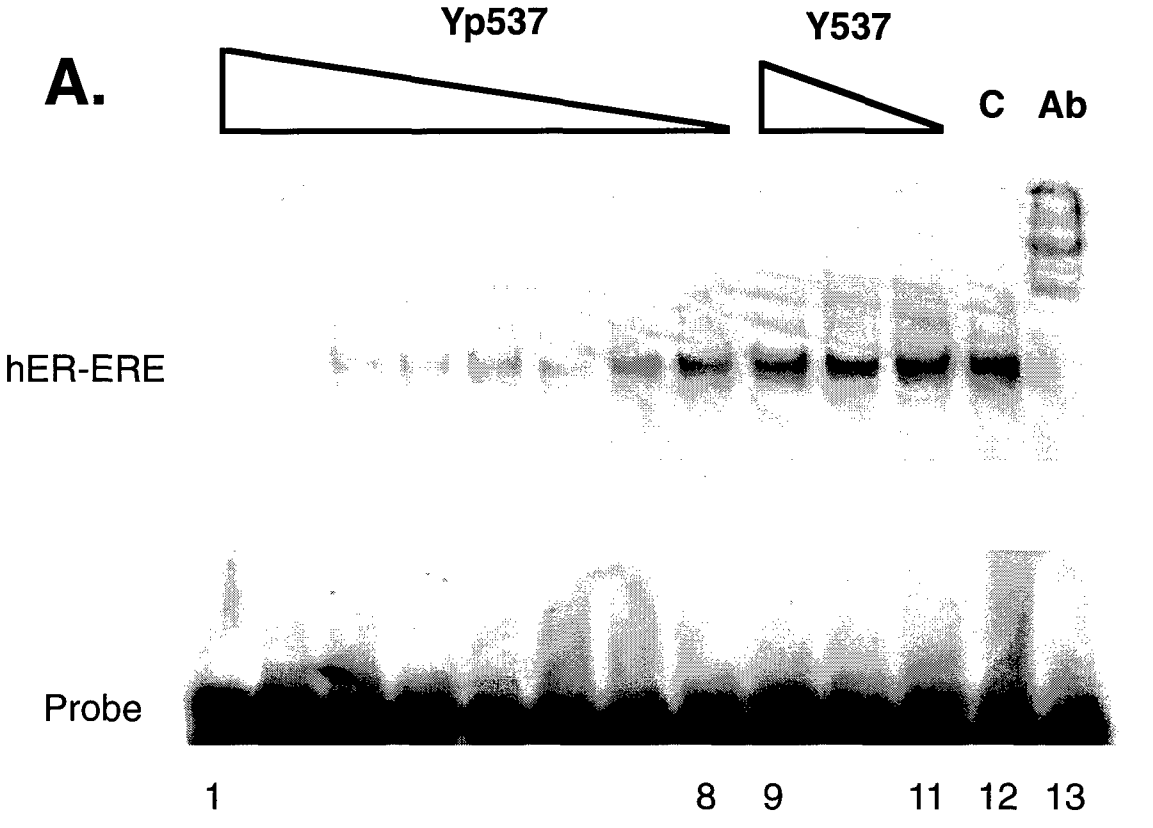


Figure 4.


A.
Yp537
Y537
C
Ab
hER-ERE
Probe
1
8
9
11
12
13

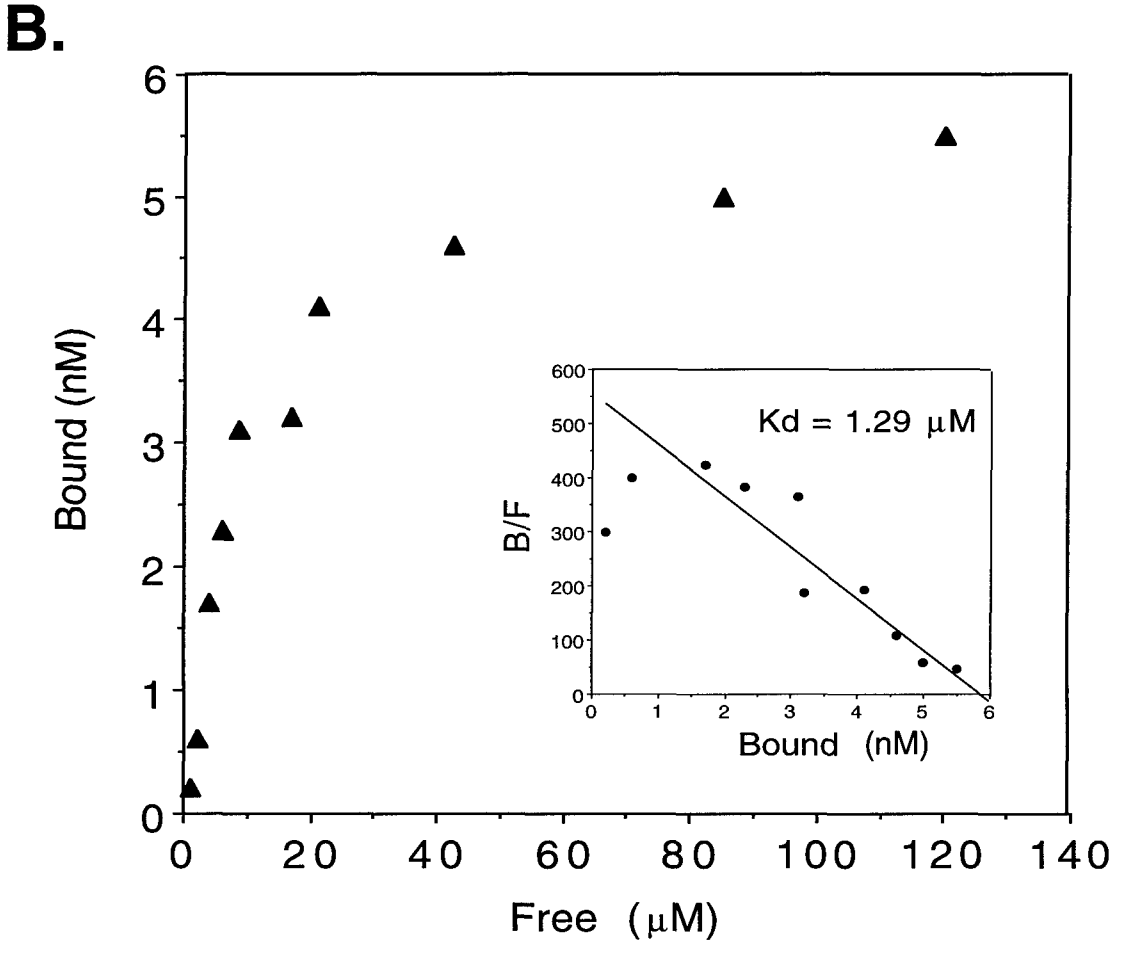
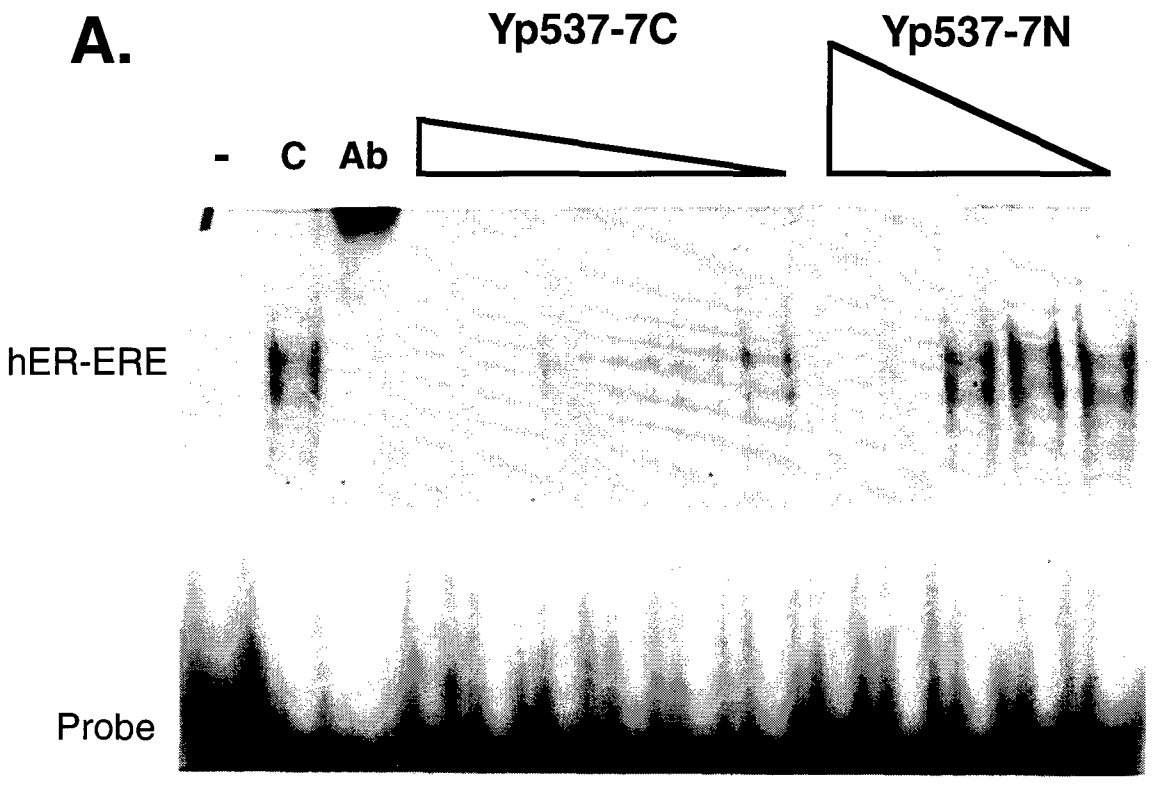

B.
Bound (nM)
Free (μM)
0
20
40
60
80
100
120
140
1
2
3
4
5
6
B/F
Kd = 1.29 μM
Bound (nM)
100
200
300
400
500
600

Figure 5.


A.
Yp537-7C
Yp537-7N
-
C
Ab
hER-ERE
Probe

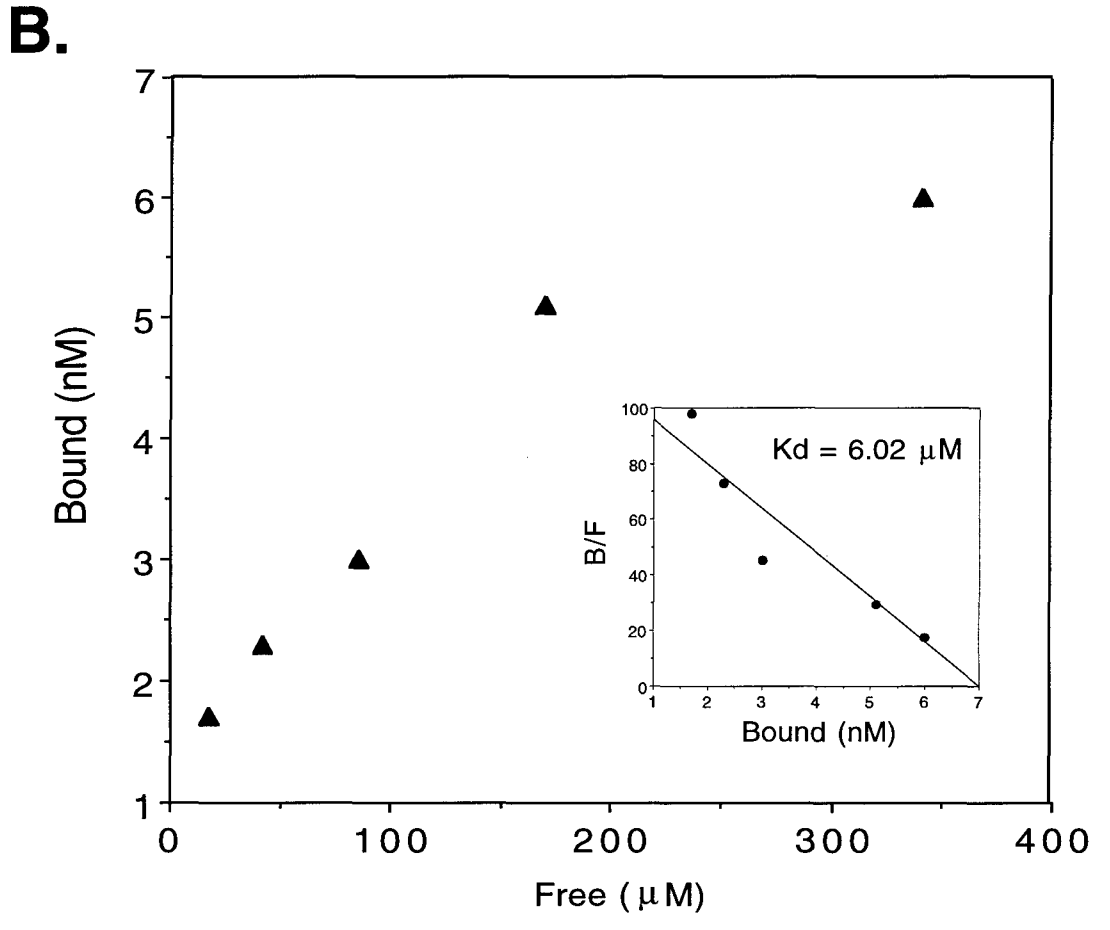
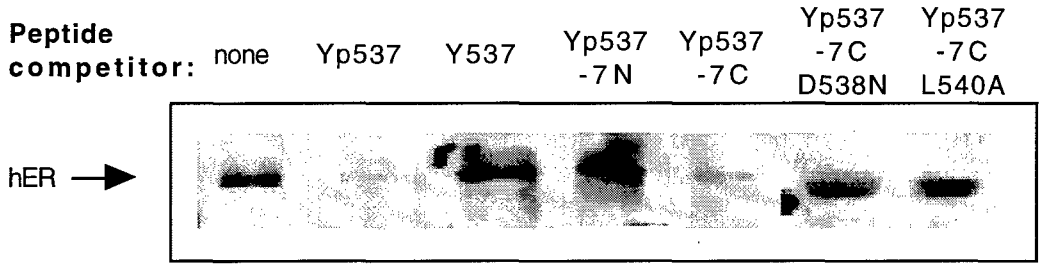

B.
Bound (nM)
Free (μM)
Kd = 6.02 μM
B/F
Bound (nM)

Figure 6.

A. Far western competition blot using a Y P537 peptide probe



B. Western blot using anti-hER Ab

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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Rec'd 10/27/2000

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 20 Oct 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grant DAMD17-96-1-6219. Request the limited distribution statement for Accession Document Numbers ADB241069, ADB257705 and ADB228950 be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by email at Judy.Pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management