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14. ABSTRACT
We began to test the hypothesis that ERRα may play a major role in the etiology or progression of tamoxifen-resistant breast cancers, doing so by heterodimerizing with ERα and, thus, enabling transcription, despite the presence of tamoxifen, of some key estrogen responsive element-regulated genes involved in growth control of breast cells. Supporting this hypothesis, we identified a breast cancer cell line, MCF-7/HER2-18, that regains sensitivity to killing by tamoxifen when treated with XCT790, a drug that specifically knocks down ERRα protein levels. We also identified an ERRα-specific monoclonal antibody that can be used in ChIP-seq experiments. We used it to note putative sites in the human genome where ERα-ERRα heterodimers may bind in the presence of estrogens and, presumably, tamoxifen to regulate expression of genes that could play key roles in the development of tamoxifen-resistant breast cancer. Our findings provide additional evidence that drugs that specifically disrupt formation of ERα-ERRα heterodimers may serve as a novel, specific therapeutics for treating patients with tamoxifen-resistant breast cancer. More generally, these studies suggest that patients with tamoxifen-resistant breast cancers may derive more therapeutic benefit from treatment with new or existing drugs (e.g., Herceptin) that affect the activities of ERRα than from ERα-targeted therapeutics.

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INTRODUCTION

Estrogen receptor α (ERα) has long been a focus of the etiology and therapy of breast cancers. Estrogen-related receptor α (ERRα) is a hormone receptor superfamily member structurally related to ERα and ERβ (reviewed in 1-3). Approximately 55 percent of primary breast carcinomas score as ERRα –positive (+) by immunohistochemistry, with many, but not all, of these tumors scoring as ERα as well (4,5). Strikingly, ERRα status strongly correlates as an independent biomarker of breast cancer recurrence, adverse clinical outcome, and resistance to tamoxifen therapy (4). Furthermore, treatment with an ERRα-specific antagonist inhibits growth of ERα(+), ERRα(+) breast cancer cells in a mouse xenograph model (6).

ERRα's DNA binding specificity partially overlaps with that of the ERs (e.g., 7,8). However, ERRα is bound by neither estrogen, tamoxifen, nor any other known natural ligand. Rather, its activities are determined by post-translational modifications of the protein (e.g., 9,10). Importantly, ERRα can homodimerize, bind DNA, and interact with co-activators in the absence of ligands (e.g., 11,12). Our preliminary unpublished data from bioluminescence resonance energy transfer (BRET) (13) and other assays indicated that ERRα can also heterodimerize with ERα, doing so in a ligand-dependent manner. Among the ligands that enable formation of ERα-ERRα heterodimers are estrogen and tamoxifen (e.g., Fig. 1). Taking these findings together, we hypothesized that ERRα may play a major role in the etiology or progression of many tamoxifen-resistant breast cancers, doing so by heterodimerizing with ERα and, thus, enabling transcription, despite the presence of tamoxifen, of some key estrogen responsive element (ERE)-regulated genes involved in growth control of breast cells. If so, drugs that specifically disrupt this heterodimerization may serve as a novel therapeutic for treating patients with tamoxifen-resistant breast cancer. This DOD BCRP CONCEPT grant was designed to enable us to obtain some additional preliminary data to further test the validity of this hypothesis.

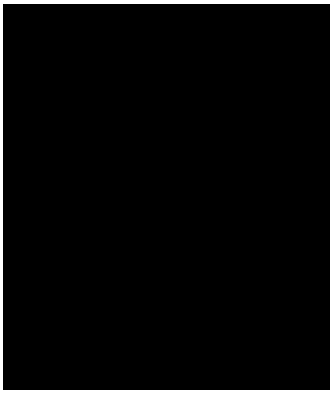

Fig. 1. BRET analysis of ERα-ERα homodimerization (- - -) and ERα/ERRα heterodimersization (—) at various concentrations of 4-OHT. Kraus *et al*., in prep.

BODY

Specific Aim #1 - Determine whether tamoxifen resistance in some breast cancer cell lines involves ERRα.

Consistent with our hypothesis, we have successfully achieved the first, key part of Aim 1, namely, showing that there exists an ERα(+), tamoxifen-resistant breast cancer cell line that reacquires sensitivity to killing by tamoxifen when ERRα is knocked down. The breast cancer cell line we identified with this property is called MCF-7/HER2-18 (14). This tamoxifen-resistant cell line had been generated by stable addition of a HER2 expression plasmid into MCF-7 cells, an ERα(+) cell line that is usually quite sensitive to killing by tamoxifen. In our experiment, we knocked down ERRα by incubation of the MCF-7/HER2-18 cells with XCT790, a drug that has been identified to specifically lead to proteasomal degradation of ERRα (15,16). As shown in Figure 2, we observed almost complete cell death when the cells were incubated for 2 days with 10^{-7} M 4-hydroxy-tamoxifen (4-OHT) in the presence of 2.5 μM XCT790, but not in the absence of XCT790.

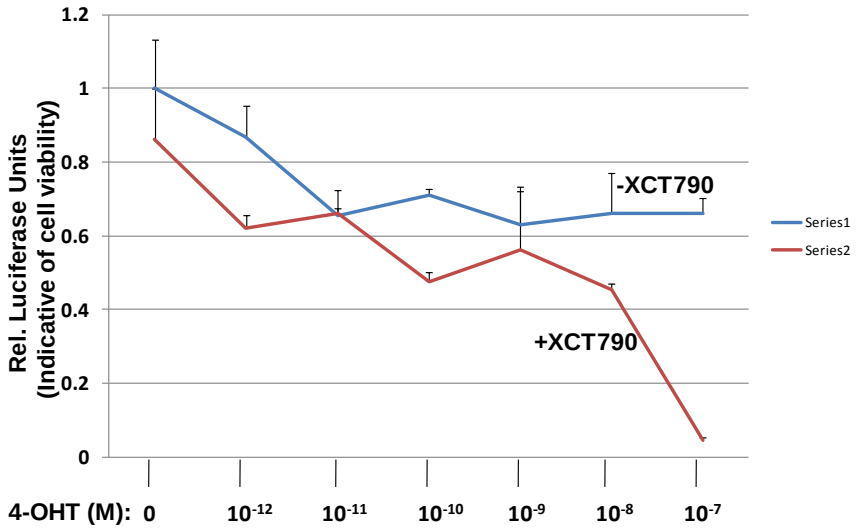

Fig. 2. ERRα Knockdown Resensitizes Tamoxifen-Resistant MCF-7 Cells to Tamoxifen. MCF-7/HER2-18 cells were incubated for 48 h with the indicated concentrations of 4-OHT in the presence or absence of 2.5 μM XCT790. Cells were then assayed for death by the Cell-titer Glo method (Promega). Shown here are the results from one typical experiment performed in triplicate.

This finding also provides strong additional support for our hypothesis that ERRα's transcriptional activity is regulated in part via HER2 signaling (9). In other words, over-expression of HER2 makes MCF-7 cells resistant to killing by tamoxifen in part by inducing phosphorylation of ERRα, switching the latter from its repressor to activator state; incubation with XCT790 reverses this phenotype by knocking down expression of ERRα in the cells. Now that we have successfully identified a cell line with these desired properties, we should be able in the near future to achieve Task 2 by infecting MCF-7/HER2-18 cells with a lentivirus that expresses an shRNA against ERRα and isolating clonal derivatives of these cells that have reduced levels of ERRα and are, once again, sensitive to killing by tamoxifen.

Specific Aim #2 - Identify some key cellular genes involved in growth control whose promoter regions are bound by ERα-ERRα heterodimers.

A necessary prerequisite for achieving Aim 2 involved identifying an ERRα-specific antibody that meets the gold standard for use in ChIP-seq experiments, *i.e.,* chromatin immunoprecipitation followed by sequencing of the specific DNA sites bound by ERRα. Thus, we first set out to finalize our characterization, publication, and licensing to biotechnology companies of our previously isolated panel of 9 mouse monoclonal antibodies (mAbs) specific to ERRα. This work has now been completed. We recently published an article describing the isolation and extensive characterization of these nine antibodies (17; Appendix 1). We have also successfully licensed our panel of ERRα-specific mAbs to Santa Cruz Biotechnology so they are now readily available to anyone for non-commercial uses. In addition, we optimized reaction conditions for performing ChIP assays using a combination of our ERRα-specific mAbs. With this knowledge in hand, we then collaborated with the laboratory of Dr. Peggy Farnham, a leader in the ENCODE Project. They thoroughly tested some of our antibodies using their ChIP-seq protocols with chromatin obtained from MCF-7 cells grown in standard medium and serum. They found that our ERRα-specific mAb 2ERR7 met their "gold standard" for use in ChIP-seq experiments, including confirming that ERRα bound strongly to its own promoter. They then used these data to tentatively identify 5,119 peaks on the human genome that appear to be bound by both ERRα and ERα. Thus, we are now very well positioned to achieve Task 3, *i.e.,* identify by ChIP-seq specific sites that are bound by both ERα and ERRα in a tamoxifen-dependent manner in the genomes of the MCF-7/HER2-18 cell lines identified in Aim 1 as dependent upon ERRα for tamoxifen resistance; we hope to do so in the near future.

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Specific Aim #3 - Test whether expression of these above-identified genes is, indeed, regulated, in part, by the combination of these two hormone receptors together with tamoxifen.

We had proposed to achieve Aim 3 by performing Task 4, *i.e.,* testing by ChIP and RT-qPCR assays of RNA isolated from the tamoxifen-sensitive clonal derivatives isolated in Task 2 and cells pre-treated with shRNAs specific to ERα or ERRα or drugs such as ICI 182,780 or XCT790 that specifically lead to degradation of these receptors, respectively, whether expression of the genes identified in Task 3 is truly activated, in part, by binding to these genes of the combination of these two receptors in the presence of tamoxifen. Clearly, this task cannot be initiated until after we have completed Tasks 2 and 3. Given we are somewhat beyond schedule in completing Tasks 2 and 3, we have yet to begin Task 4.

Other Experiment Directly Related to This Project - We have been attempting to complete the last set of experiments we need to be able to publish our key background BRET data showing tamoxifen-dependent formation of ERα-ERRα heterodimers. All of our data are now publishable quality (e.g., Fig. 1 above) except for documenting co-immunoprecipitation of endogenous ERα with ERRα. We have achieved clear, albeit low-level co-IPing of these two proteins, possibly because they may be dissociating during the extensive washings. We have been attempting to overcome this problem using a new technique, iFAST, that enables one to perform the equivalent of 2 or 3 washes within 1 minute (18).

KEY RESEARCH ACCOMPLISHMENTS

- Identification of a tamoxifen-resistant breast cancer cell line whose resistance to this drug is dependent upon the presence of ERRα.
- Publication and commercial licensing of a panel of monoclonal antibodies specific to ERRα, including one that meets the gold standard for use in ChIP-seq experiments.
- Tentative identification of sites in the human genome of MCF-7 cells that may be bound by both ERα and ERRα under at least some growth conditions.

REPORTABLE OUTCOMES

Publications:

Esch, A.M., Thompson, N.E., Lamberski, J.A., Mertz, J.E., and Burgess, R.R. Production and characterization of monoclonal antibodies to estrogen-related receptor alpha (ERRα) and use in immunoaffinity chromatography. Protein Expression & Purification *84*: 47-58, 2012.

Abstracts and Presentations:

Mertz, J. E., Esch, A. M., and Burgess, R. R.. Estrogen-related Receptor Alpha Target Genes: Identification of Novel Biomarkers of Aggressive Breast Cancer. DOD-BCRP Era of Hope Conference, Orlando, FL, August, 2011.

Mertz, J. E. Kraus, R. J., and Lillios, N. P. Regulation of Estrogen-related Receptor Alpha Activity by its Tyrosine at Residue 400. DOD-BCRP Era of Hope Conference, Orlando, FL, August, 2011.

Mertz, J. E., Kraus, R. J., Golden, S. K., and Esch, A. M. Estrogen-related Receptor Alpha and Estrogen Receptor alpha form Heterodimers in a Ligand-dependent Manner. DOD-BCRP Era of Hope Conference, Orlando, FL, August, 2011.

Licenses Issued:

Burgess, RR, Thompson, NE, Lamberski, JA, Mertz, JE, Vu, EH, Lesi, AL, and Kraus, RJ. UW-Madison Invention Disclosure Report: Mouse monoclonal antibodies (1ERR21, 1ERR87, and 2ERR10, and others) that react with the human nuclear receptor estrogen-related receptor alpha (ERRα).

The Wisconsin Alumni Research Foundation (WARF) has nonexclusively licensed our nine anti-ERRα monoclonal antibodies to Santa Cruz Biotechnology. These antibodies are now readily available to researchers for non-commercial use.

Degrees Obtained / Employment Based upon Experience:

Amanda Esch received her Ph.D. in Cancer Biology from the University of Wisconsin, Madison in August, 2011 based upon a thesis entitled, "Identification of surrogate biomarkers of estrogen-related receptor alpha (ERRα) activity in breast cancer." She is now employed as a postdoctoral fellow at the Oregon Health Sciences Center in Portland, OR.

Personnel Paid in Part By This Grant: Dr. Janet E. Mertz; Richard J. Kraus.

Funding Applied For: Several grants applications have been submitted; none have been funded to date.

CONCLUSIONS

Here, we set out to test our hypothesis that ERRα may play a major role in the etiology or progression of many tamoxifen-resistant breast cancers, doing so by heterodimerizing with ERα and, thus, enabling transcription, despite the presence of tamoxifen, of some key estrogen responsive element (ERE)-regulated genes involved in growth control of breast cells. In strong support of this hypothesis, we identified a breast cancer cell line, MCF-7/HER2-18, that regains sensitivity to killing by tamoxifen when treated with XCT790, a drug that specifically leads to knock down of ERRα protein levels in cells. We also identified an ERRα-specific monoclonal antibody that meets the gold standard for use in ChIP-seq experiments. We began to use it to note putative locations in the human genome that may be sites where ERα-ERRα heterodimers bind in the presence of estrogens and, presumably, tamoxifen to regulate expression of genes that could play key roles in the development of tamoxifen-resistant breast cancer. Thus, our findings well supported our hypothesis. The experiments have proceeding well, but we ran out of time and money to complete all of the ones proposed for this small, 1-year CONCEPT grant. The data obtain should help us to succeed in obtaining a larger grant that requires more preliminary data.

Regarding applications, our findings provide additional evidence that drugs that specifically disrupt formation of ERα-ERRα heterodimers may serve as novel, specific therapeutics for treating patients with tamoxifen-resistant breast cancer. More generally, these studies suggest that patients with aggressive breast cancers, especially tamoxifen-resistant ones for which there are currently no good therapies, may derive more therapeutic benefit from treatment with new or existing drugs (e.g., Herceptin) affecting the activities of ERRα and its target genes rather than from ERα-targeted therapeutics such as tamoxifen. Hopefully, our studies may also lead to the rational development of novel breast cancer prognosticators and predictors of responses to current therapies.

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APPENDIX - Esch *et al.* Protein Expression & Purification *84*: 47-58, 2012.

Protein Expression and Purification 84 (2012) 47–58

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Production and characterization of monoclonal antibodies to estrogen-related receptor alpha (ERRα) and use in immunoaffinity chromatography

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ABSTRACT

Estrogen-related receptor alpha (ERRα) is an orphan nuclear receptor whose elevated expression is thought to contribute to breast, colon, and ovarian cancers. In order to investigate the role of ERRα in human disease, there is a need for immunological reagents suitable for detection and purification of ERRα. We expressed recombinant human ERRα in *Escherichia coli,* purified the protein, and used it to generate monoclonal antibodies (mAbs) to ERRα. Nine high-affinity mAbs were chosen for their abilities to detect overexpressed ERRα in enzyme-linked immunosorbent assays (ELISAs) and Western blots, after which isotyping and preliminary epitope mapping was performed. The mAbs were all IgG subtypes and reacted with several different regions of full-length ERRα. A majority of the mAbs were found to be useful for immunoprecipitation of ERRα, and several could detect DNA-bound ERRα in electrophoretic mobility supershift assays (EMSAs) and chromatin immunoprecipitation (ChIP). The suitability of mAbs to detect ERRα in immunofluorescence assays was assessed. One mAb in particular, 2ERR10, could specifically detect endogenous ERRα in mammary carcinoma cells. Finally, we performed assays to screen for mAbs that gently release ERRα in the presence of a low-molecular-weight polyhydroxylated compound (polyol) and nonchaotropic salt. Using gentle immunoaffinity chromatography, we were able to isolate ERRα from mammalian cells by eluting with a polyol-salt solution. Our characterization studies show that these monoclonal antibodies perform well in a variety of biochemical assays. We anticipate that these novel reagents will prove useful for the detection and purification of ERRα in research and clinical applications.

Introduction

Estrogen-related receptor alpha (ERRα)[1] is a nuclear receptor transcription factor which was discovered in low-stringency screens of cDNA libraries which used the estrogen receptor DNA-binding domain as a probe [1]. Despite its structural similarities with estrogen receptor and other nuclear receptor family members, ERRα is considered to be an orphan receptor because it has no known natural ligand. Its transcriptional activities are thought to be modulated by post-translational modifications and interactions with cofactors [2,3].

Full-length ERRα consists of 423 amino acids which encode a 46-kDa protein. It comprises four major canonical nuclear receptor domains, indicated by amino acid (aa) position (see Fig. 3B). The N-terminal A/B domain (aa 1–78) is involved in ligand-independent functions of nuclear receptors. The C region (aa 79–144) contains the DNA-binding domain (DBD), which shares nearly 70% sequence identity with the DBD of estrogen receptor alpha. Like estrogen receptor, ERRα can bind estrogen response elements (EREs) containing the inverse palindromic sequence 5′-AGGTCANNNTGACCT-3′; ERRα can also bind to extended half-site sequences known as estrogen-related response elements (ERREs), which have the general sequence 5′-TNAAGGTCA-3′ [4–8]. Adjacent to the DBD is the D domain (aa 145–198), also known as the hinge region. The C-terminal E/F domain (aa 199–423) typically contains a ligand-binding region for most nuclear receptors; however, phenylalanine residues fill the binding pocket of ERRα, holding it in an active conformation similar to that of estrogen-bound estrogen receptor [9]. It is thought that this structural configura-

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[1] *Abbreviations used:* ERRα, estrogen-related receptor alpha; aa, amino acid; DBA, DNA-binding domain; EREs, estrogen response elements; mAbs, monoclonal antibodies; ELISAs, enzyme-linked immunosorbent assays; EMSAs, electrophoretic mobility supershift assays; IP, immunoprecipitation; IF, immunofluorescence; LB, Luria–Bertani; IPTG, isopropyl-β-D-1-thiogalactopyranoside; Ni–NTA, nickel–nitrilotriacetic acid; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; HEK 293T, human embryonic kidney 293T cells; PEI, polyethyleneimine; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; MES, 2-(N-morpholino)ethanesulfonic acid; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; NBT, nitroblue tetrazolium; HRP, horseradish peroxidase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; ChIP, chromatin immunoprecipitation; DAPI, 4′,6-diamidino-2-phenylindole; DBD, DNA-binding domain; ERRE, estrogen-related response element.

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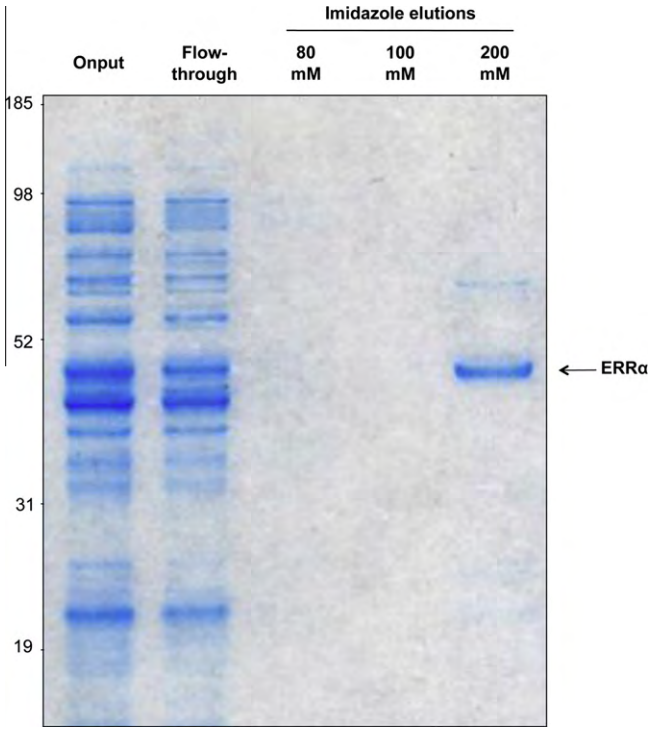

Fig. 1. Purification of His_6-ERRα. His_6-tagged full-length ERRα was overexpressed in *E. coli* and purified on a Ni–NTA column. Purification fractions were separated by SDS–PAGE and stained with Coomassie. Full-length ERRα has a molecular weight of 46 kDa; His_6-tagged ERRα has an apparent molecular weight of about 49 kDa.

tion is responsible for the hormone-independent activity of ERRα. The E/F domain also contains LXXLL motifs which allow ERRα to interact with cofactors [10].

ERRα is localized predominantly in cell nuclei and is most highly expressed in skeletal muscle, kidney, heart, brain, and intestine [1,7,11]. Recent studies have demonstrated that ERRα regulates transcription of genes involved in metabolism [12–16]. Elevated ERRα protein levels have been associated with certain breast, endometrial, ovarian, prostate, and colorectal cancers [17–26], but its exact role in cancer progression is not clear. In order to investigate the contributions of ERRα in disease development, it would be useful to have high-affinity immunological reagents able to detect and purify ERRα in a variety of applications.

To address this need, we generated murine monoclonal antibodies (mAbs) to human recombinant ERRα. Nine high-affinity mAbs were chosen for their abilities to react with ERRα in enzyme-linked immunosorbent assays (ELISAs) and Western blot analyses. We evaluated the capacity of each mAb to react with ERRα in electrophoretic mobility supershift assays (EMSAs), immunoprecipitation (IP), chromatin immunoprecipitation (ChIP), and immunofluorescence (IF) applications. Using gentle immunoaffinity chromatography, we were able to purify overexpressed and endogenous ERRα from mammalian cell lysates in a single chromatographic step. Our characterization studies demonstrate that these mAbs retain high affinity for ERRα and are suitable for use in a wide range of biochemical assays. We anticipate that these immunological reagents will prove useful in research and clinical applications for the detection and purification of ERRα.

Materials and methods

Reagents and buffers

General-purpose molecular biology-grade reagents were obtained from established vendors. Reagents specific to particular applications are indicated in the text.

Expression of His_6-ERRα in Escherichia coli

The coding region of human full-length ERRα (contained in pcDNA3.1/V5-His, described in [8]) was amplified using the forward primer 5′-GCCACCATGGCATCCAGCCAGGTGGTGGGCATTG-3′, which contained an *Nco*I site (underlined). The reverse primer, 5′-CGCTGCGGCCGCGTCCATCATGGCCTCGAGCATC-3′, contained a *Not*I site (underlined). The amplified product was gel-purified and cloned into the *Nco*I/*Not*I sites of the pET28b(+) vector (EMD Biosciences, Gibbstown, NJ). This construction resulted in full-length ERRα containing an in-frame hexahistidine (His_6) tag at the C-terminus. The sequence of the insert was confirmed and the plasmid was transformed into *E. coli* BL21(DE3)pLysS. Trans-

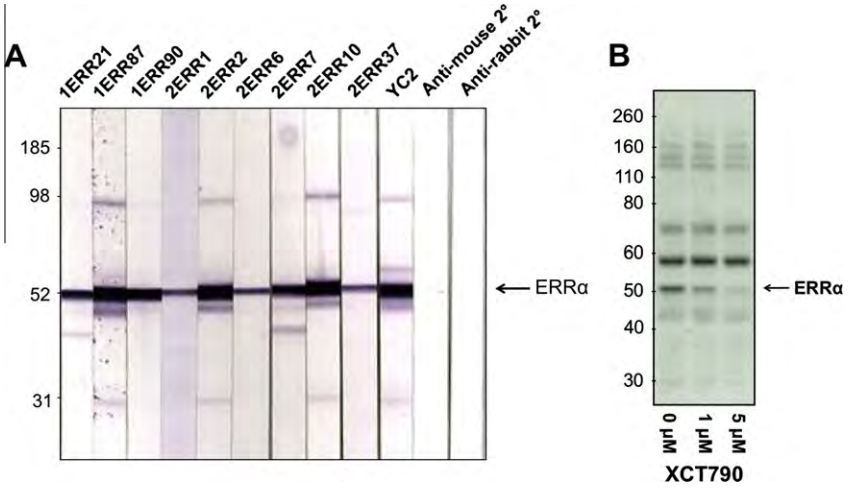

Fig. 2. mAb detection of ERRα in Western blots. (A) Full-length ERRα was overexpressed in MCF-7 cells and whole-cell lysates were prepared. Approximately 300 μg of total protein was loaded into a single-lane 4–12% gel and separated by SDS–PAGE. Reactivities of anti-ERRα mAbs were assessed by Western blot analyses. The anti-ERRα rabbit polyclonal antibody YC2 [4] was included as a positive control. Blots were probed with mouse and rabbit secondary (2°) antibody alone to test their reactivities with lysate components. Molecular weight in kDa is indicated on the left. (B) BT-474 cells were treated for three hours with 0, 1, or 5 μM XCT790, an inverse agonist of ERRα which selectively targets the protein for degradation. Whole-cell lysates were prepared and separated by SDS–PAGE on a 4–12% gel (30 μg lysate per lane). Endogenous ERRα was detected by Western blot using mAb 2ERR10. Molecular weight in kDa is indicated on the left.

formed bacteria were cultured in 500 mL of Luria–Bertani (LB) medium containing 30 μg/mL kanamycin and 35 μg/mL chloramphenicol and grown overnight at 37 °C with shaking. Once the cultures reached an $O.D._{600\ nm}$ of 0.6, they were induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and grown for an additional 2.5 h, after which the bacterial pellets were harvested and stored at −80 °C. The pellet (1 g wet weight of cells) was resuspended in 8 mL 1× His binding buffer (20 mM Tris–HCl, pH 7.9; 500 mM NaCl; 5 mM imidazole), then sonicated and centrifuged. The soluble fraction was applied to a 2-mL equilibrated nickel–nitrilotriacetic acid (Ni–NTA) column (Qiagen, Valencia, CA), washed, then eluted sequentially with binding buffer containing 80 mM, 100 mM, and 200 mM imidazole. The total protein in each eluted fraction was determined by Bradford assay, using bovine serum albumin (BSA) as a protein standard. Fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) to determine purity, then pooled and dialyzed against TENG buffer (50 mM Tris–HCl, pH 7.9; 0.1 mM EDTA; 100 mM NaCl; 20% glycerol).

Hybridoma generation and mAb production

Four female Balb/c ByJ mice (Jackson Labs, Bar Harbor, ME) were injected with purified human recombinant ERRα every fourteen days; each injection administered was approximately 50 μL subcutaneously and 50 μL intraperitoneally. The first injection contained 5 μg purified ERRα in Freund's complete adjuvant (Sigma–Aldrich, St.Louis, MO). The second and third injections contained 10 μg and 20 μg ERRα, respectively, in Freund's incomplete adjuvant (Sigma–Aldrich). The mice were bled on day 56, and the sera were assayed by ELISA and Western blot. Sera from all four mice showed a titer of at least 1:32,000 in the ELISA and reacted with overexpressed ERRα by Western blot analyses. Three days before the fusion and at least thirty days after the last injection in incomplete adjuvant, the selected mouse was injected with 50 μg of ERRα in phosphate-buffered saline (PBS), administered intraperitoneally. Hybridomas were prepared from isolated splenocytes by standard hybridoma methods [27], using NS1 myeloma cells as the fusion partner. Twelve days after the fusion, cell culture media

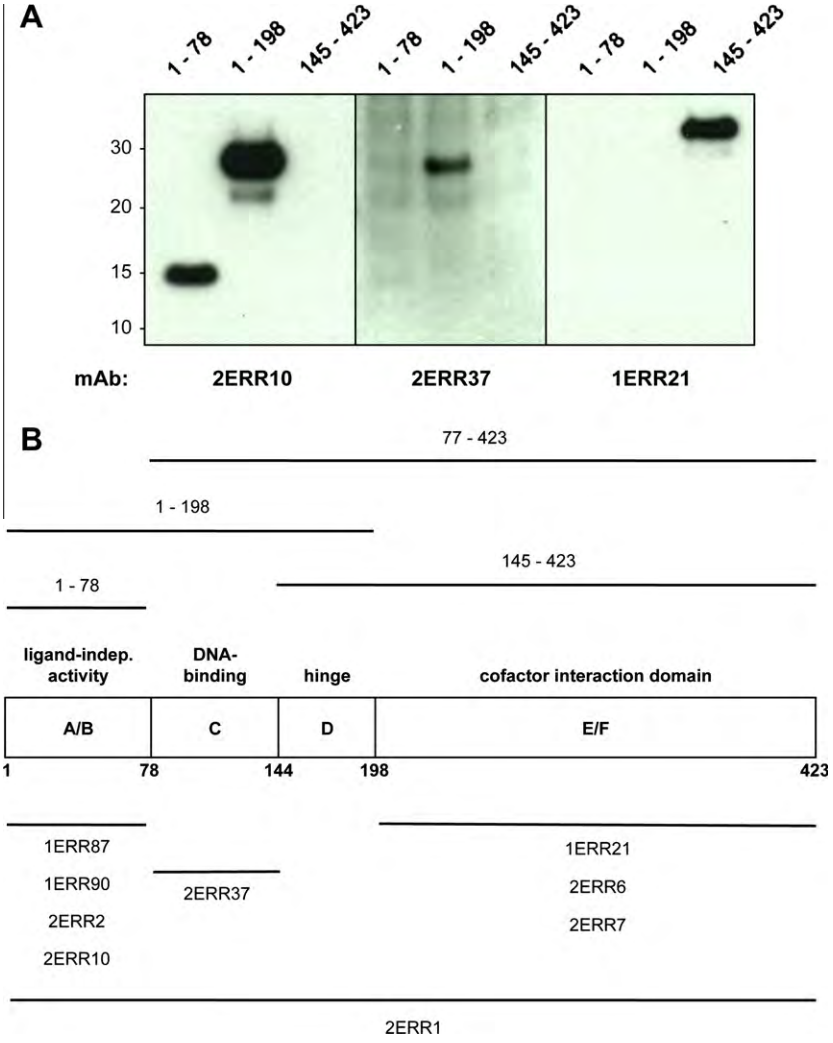

Fig. 3. Epitope mapping for anti-ERRα mAbs. (A) Human embryonic kidney 293T cells were transiently transfected with constructs expressing ERRα truncation mutants. The regions represented by these constructs are indicated by amino acid (aa) position in relation to full-length ERRα: aa 1–78, aa 1–198, and aa 145–423. We also used a construct encoding 77–423 in our epitope mapping studies (data not shown). Whole-cell extracts expressing ERRα fragments were analyzed by Western blot. Each lane is labeled with the construct expressed, numbered according to amino acid position. Equal concentrations of purified mAb were used to probe the Western blots. Molecular weight in kDa is shown on the left. (B) Summary of epitope mapping. The schematic depicts full-length ERRα protein, with domain locations indicated by numbers representing amino acid (aa) position. The N-terminal A/B domain (aa 1–78) is typically involved in ligand-independent functions of nuclear receptors. The C region (aa 79–144) contains the DNA-binding domain and is adjacent to the D domain (aa 145–198), also known as the hinge region. The C-terminal E/F region (aa 199–423) contains LXXLL motifs which interact with cofactors. The regions represented by truncation mutants used in epitope mapping studies are indicated by lines shown above the ERRα schematic, numbered by amino acid (aa) position: 1–78, 1–198, 77–423, and 145–423. Mapped mAb binding regions are shown below full-length ERRα. 2ERR1 did not react with any of the truncation mutants in Western blots and is depicted as interacting with full-length ERRα.

were assayed by standard ELISA for the presence of ERRα-reactive antibodies. Cells from the ELISA-positive wells were cloned at least twice by limiting dilution. All animal protocols were approved by the University of Wisconsin-Madison School of Medicine and Public Health Animal Use and Care Committee.

mAb purification

Antibody-producing hybridomas were grown in ascites fluid or Celline flasks (IBS Integra Biosciences, Chur, Switzerland) according to manufacturer instructions [28], and mAbs were purified as follows. To remove albumin from IgG_1 antibodies, samples were precipitated with saturated ammonium sulfate (45% w/v), mixed on ice for twenty minutes, then incubated at 4 °C for eighteen hours. Samples were collected by centrifugation, resuspended in antibody buffer (50 mM Tris–HCl, 25 mM NaCl, pH 6.9), and dialyzed into antibody buffer for eighteen hours at 4 °C. Samples were applied to 4-mL equilibrated DE52 diethylaminoethyl cellulose columns (Whatman, Maidstone, England); purified mAbs were collected in the flowthrough fraction. For IgG_{2a} and IgG_{2b} mAbs, samples were diluted twofold with PBS and applied to protein A-conjugated agarose columns (Repligen, Waltham, MA). Columns were washed with PBS, after which mAbs were eluted with 0.75 M acetic acid. Eluted mAbs were neutralized by the addition of 2 M Tris–HCl pH 7.9, and mAbs were dialyzed against PBS.

ELISA and ELISA-elution

ELISA and ELISA-elution assays were performed as previously described [29–33]. Microtiter plates (96-well) were coated with 100 ng purified ERRα per well, then blocked in BLOTTO (1% dried nonfat milk in PBS) overnight at 4 °C. Wells were washed with 0.1% Tween-20 in PBS (PBS-T), then mAbs were added to wells and incubated for one hour at 23 °C. For ELISAs, plates were washed with PBS-T, then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (American Qualex, San Clemente, CA). Bound antibody was detected using hydrogen peroxide substrate and o-phenylenediamine indicator in citrate buffer. For ELISA-elution experiments, polyol elution buffers containing various concentrations of propylene glycol and ammonium sulfate in TE buffer (50 mM Tris–HCl and 0.1 mM EDTA, pH 7.9) were added to each well and incubated at room temperature for twenty minutes. Plates were washed with PBS-T, after which any bound primary antibody was detected by reacting with secondary antibody and substrate as described above. Colorimetric analysis of ELISA and ELISA-elution was performed using an SLT Spectra plate reader.

Mammalian cell lines and transfections

Human embryonic kidney 293T cells (HEK 293T) and BT-474 mammary carcinoma cells were grown in high-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Thermo Scientific, Hudson, NH). MCF-7: WS8 (derived from the MCF-7 human mammary carcinoma cell line, kind gift from Dr. V. Craig Jordan, Georgetown University) and SK-BR-3 mammary carcinoma cells were grown in DMEM/F-12 medium supplemented with 10% FBS, 100 μM non-essential amino acids, and 6 ng/mL insulin. All cells were grown at 37 °C in a humidified 5% CO_2 atmosphere. To express ERRα fragments suitable for epitope mapping, 293T cells were transiently transfected with pcDNA3.1 plasmid constructs encoding ERRα truncation mutants, designated by amino acid position: 1–78, 1–198, 77–423, and 145–423 (kind gift of Richard Kraus, UW-Madison). For crossreactivity studies, 293T cells were transiently transfected with human cytomegalovirus promoter-driven CMX expression plasmid encoding ERα, ERβ, ERRα, ERRβ, or ERRγ (kind gifts of Dr. Wei Xu, UW-Madison). For all 293T cell transfections, 25-kDa polyethyleneimine (PEI) was used as a transfection reagent (40 μg PEI per 2.5 μg DNA per 10-cm dish of cells). To overexpress full-length ERRα in mammalian cells, MCF-7 cells were transiently transfected with CMX-ERRα or CMX empty vector, using *Trans*IT-LT1 transfection reagent (Mirus). All transfected cells were grown at 37 °C with 5% CO_2 for forty-eight hours, after which they were lysed in Freedman buffer [50 mM Tris, pH 7.5; 150 mM NaCl; 1% Nonidet P-40; 10% glycerol; 2 mM ethylenediaminetetraacetic acid (EDTA); 50 mM NaF; 1 mM phenylmethanesulfonyl fluoride (PMSF)] to generate whole-cell extracts [34].

SDS–PAGE and Western blot, and silver staining

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), using 4–12% Bis–Tris NuPAGE gels (Invitrogen) in 2-(*N*-morpholino)ethanesulfonic acid (MES) running buffer. For initial Western blots detecting ERRα overexpressed in MCF-7 cells, all blocking and antibody incubation steps were performed in BLOTTO. For these studies, we used an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Millipore, Billerica, MA) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) reagent as substrate. In all other Western blots, horseradish peroxidase (HRP)-conjugated light chain-specific goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA) or HRP-conjugated donkey anti-rabbit IgG secondary antibody (GE Healthcare, Piscataway, NJ) was used in concert with a chemiluminescent substrate (Thermo Scientific, Rockford, IL) for detection. In these assays, blocking and antibody incubation steps for the majority of the mAbs were performed in 5% nonfat dry milk in a Tris-buffered solution containing 0.1% Tween-20 (TBS-T); blocking and incubations for mAbs 2ERR1, 2ERR6, and 2ERR37 were performed in 3% BSA in TBS-T. For detection of endogenous ERRα by mAb 2ERR10, BT-474 cells were treated for three hours with increasing amounts of XCT790, an inverse agonist of ERRα which selectively targets the protein for degradation [43].

Electrophoretic mobility supershift assay (EMSA)

Electrophoretic mobility supershift assays were performed as previously described [4,8,35,36]. For overexpression studies, MCF-7 cells were transiently transfected with pCMX-ERRα; for examination of endogenous ERRα, BT-474 cells were used. Cells were lysed in Freeman buffer and lysate was incubated with mAb and 1 ng double-stranded radiolabeled ERRE probe (5′-AGCAGTGGCGATTTGTCAAGGTCACACAGT-3′; ERRE underlined) in a solution containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4; 1 mM dithiothreitol (DTT); 100 mM NaCl; 10% glycerol (v/v); 3 μg BSA; and 4 μg poly(dI–dC). Samples were loaded onto non-denaturing 5% polyacrylamide gels and electrophoresed at 200 V for two hours at 4 °C. Gels were transferred to Whatman paper, dried at 80 °C for one hour, then analyzed with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation (IP)

For all immunoprecipitation assays, protein G agarose (Millipore) was washed in dialysis buffer (50 mM Tris–HCl, pH 8.1; 2 mM EDTA), then blocked overnight at 4 °C with 0.5 mg/mL bovine serum albumin (BSA) and 0.5 mg/mL herring sperm DNA (Promega, Madison, WI). Resin was washed and resuspended in an equal volume of dialysis buffer. For each IP, mAb was pre-bound to blocked protein G agarose in dilution buffer (16.7 mM Tris–HCl, pH 8.1; 1.2 mM EDTA; 167 mM NaCl; 1.1% Triton ×-100; 0.01%

SDS; 10 mM sodium pyrophosphate). The antibody-resin complexes were washed twice with dilution buffer to remove excess antibody, then incubated overnight at 4 °C with whole-cell extract from MCF-7 cells overexpressing ERRα (1 mg total protein per IP). Resins were briefly washed three times in dilution buffer, then eluted in 2× Laemmli SDS loading buffer. Immunoprecipitated ERRα was detected by Western blot analyses, using mAb 2ERR10 as the primary antibody and a light chain-specific anti-mouse IgG secondary antibody as described above.

Chromatin immunoprecipitation (ChIP) and PCR

For ChIP assays, BT-474 cells were treated with formaldehyde to crosslink DNA and associated proteins, then lysed as described [37,38]. Chromatin was sheared to an average size of 500 bp by sonicating samples three times for ten seconds at 9 W output using a Fisher Scientific Sonic Dismembrator 100. After sonication, chromatin was precleared with blocked protein G agarose. Each antibody was prebound to protein G agarose resin, then incubated with precleared lysate in dilution buffer overnight at 4 °C. ChIP samples were washed and eluted, after which crosslinks were reversed. DNA was purified by using QiaQuick PCR purification spin columns (Qiagen). Pulldown of endogenous DNA-bound ERRα was checked by PCR, using primers designed to amplify the *ESRRA* promoter, which contains a characterized ERRα binding site [46]. PCR amplification was performed for 33 cycles using Go Taq reagents (Promega). Primer sequences were as follows: forward: 5-GCAGTGACCTTGAGC TTTCTCC-3; reverse: 5-GAACCGTAGACCCAGTAGCC-3.

Immunofluorescence

For immunofluorescent detection of overexpressed ERRα, MCF-7 mammary carcinoma cells were grown on round glass coverslips in 12-well plates, then transiently transfected with the pCMX-ERRα expression plasmid described above. At forty-eight hours post-transfection, cells were fixed in 3% paraformaldehyde, then permeabilized in 0.2% Triton ×-100. Permeabilized cells were incubated with purified anti-ERRα mAbs at a concentration of 20 μg/mL (twofold dilution of cell supernatants for mAbs 2ERR2 and 2ERR6) in 1.5% goat serum (Jackson ImmunoResearch) overnight at 4 °C. The cells were then incubated with Alexa^{488}-conjugated goat anti-mouse IgG secondary antibody (Invitrogen) and 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain (Invitrogen) for one hour at room temperature. Imaging was performed using a Leica DM5000 fluorescence microscope; all settings were conserved within each experiment. For detection of endogenous ERRα by mAb 2ERR10, cells were treated for twenty-four hours with 0.1% DMSO vehicle control or 5 μM XCT790, an inverse agonist of ERRα which selectively targets the protein for degradation [43]; all immunocytochemistry steps were performed as described above. Imaging of endogenous ERRα was performed using a Bio-Rad Radiance 2100 MP Rainbow confocal/multiphoton microscope, and all settings were conserved within each experiment.

mAb conjugation to Sepharose resin

Antibody-conjugated Sepharose was prepared as described previously [39]. Purified 2ERR1 antibody was dialyzed into coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) overnight at 4 °C. Cyanogen bromide-activated Sepharose (GE Healthcare) was swollen in 1 mM HCl and rinsed with coupling buffer. Dialyzed 2ERR1 antibody (6 mg) was conjugated to 0.5 g dry weight Sepharose at 23 °C for two hours and collected on a sintered glass filter. 2ERR1-Sepharose was mixed with blocking agent (1 M ethanolamine in coupling buffer, pH 8.0) for two hours at 23 °C, followed by four sets of washes alternating coupling buffer and acetate buffer (0.1 M sodium acetate, 0.5 M NaCl, pH 4.0). 2ERR1-Sepharose was stored in TE +0.02% NaN_3 at 4 °C; resin can be stored for six months and can be used multiple times if treated as described [32,40].

Immunoaffinity purification of ERRα using polyol-responsive mAbs

2ERR1-Sepharose was blocked with BSA and herring sperm DNA as described above, then equilibrated in Freedman buffer. Resin was incubated at 4 °C for one hour with cell lysate, then washed with TE. A third wash was performed with TE +250 mM ammonium sulfate, after which ERRα was gently eluted from the column with polyol elution buffer (TE +0.75 M ammonium sulfate +40% propylene glycol). Proteins were visualized by silver staining and Western blot analyses.

Results and discussion

Monoclonal antibody (mAb) production and initial screening

His_6-tagged human ERRα protein was expressed in *E. coli* and purified (Fig. 1), then used to immunize mice for mAb production. ELISAs were used to screen sera for reactivity to purified ERRα. Spleen cells from immunoreactive mice were fused with myeloma cells to produce immortal antibody-producing hybridoma cell lines; two separate fusions generated nine high-affinity mAbs which reacted with His_6-ERRα in ELISAs. All nine antibodies were able to detect ERRα overexpressed in MCF-7 cells by Western blot analyses (Fig. 2A). For mAbs 2ERR6 and 2ERR37, performing all blocking and antibody incubation steps in 3% bovine serum albumin (BSA) increased signal intensity. At least four of the mAbs (1ERR87, 1ERR90, 2ERR2, 2ERR10) were useful for detecting endogenous ERRα in 40 μg BT-474 whole-cell extract by Western blot analyses. A representative Western blot showing detection of endogenous ERRα by mAb 2ERR10 is shown in Fig. 2B. In this figure, BT-474 cells were treated with XCT790, a compound which specifically targets the ERRα protein for degradation [13,41–43]. The major form of ERRα is a 52-kDa protein which is shown here to be degraded with increasing amounts of XCT790. In these Westerns, bands larger than 52 kDa likely indicate splice variants [4,7], post-translationally modified forms of the protein [2,7], or strongly-associated ERRα complexes [47]. The properties of the ERRα mAbs are summarized in Table 1.

Isotyping and epitope mapping

Antibody isotyping was performed using an ELISA-based kit (HyClone, Logan, UT), and all nine mAbs were determined to be IgG subtypes. 1ERR21, 1ERR87, 1ERR90, 2ERR10, and 2ERR37 were found to be IgG_1 molecules; 2ERR1 and 2ERR2 are IgG_{2a} mAbs, and 2ERR6 and 2ERR7 belong to subisotype IgG_{2b} (summarized in Table 1). Next, we performed epitope mapping to determine which region of the ERRα protein is recognized by each mAb. Human embryonic kidney 293T cells were transiently transfected with plasmids encoding ERRα truncation mutants representing different regions of the protein, as indicated by amino acid position in full-length ERRα: 1–78, 1–198, 77–423, and 145– 23. Cell lysates were separated by SDS–PAGE and analyzed by Western blot. Antibodies 1ERR87, 1ERR90, 2ERR2, and 2ERR10 reacted with epitopes in the N-terminal A/B domain, while mAbs 1ERR21, 2ERR6, and 2ERR7 recognized epitopes in the C-terminal E/F domain. 2ERR37 was the only mAb which reacted with region C, which contains the DNA-binding domain (DBD). Antibody 2ERR1 was able to detect overexpressed full-length ERRα in Western blots, but its epitope could not be mapped because it did not react with any of the ERRα truncation mutants. Overall, these mAbs appear to offer broad cov-

Table 1
Summary of ERRα mAb properties.

mAb	Isotype	Epitope	Western blot [a]	IP [b]	EMSA [c]	ChIP [d]	Immuno-fluorescence [e]	Polyol-responsive [f]
1ERR21	IgG_1	198–423	+	+	–	+	–	+
1ERR87	IgG_1	1–78	++	++	+	+	+	–
1ERR90	IgG_1	1–78	++	++	+	–	+	–
2ERR1	IgG_{2a}	nyd	+	++	+	+	+	++
2ERR2	IgG_{2a}	1–78	++	++	nyd	+	+	–
2ERR6	IgG_{2b}	198–423	+(BSA)	–	–	+	–	++
2ERR7	IgG_{2b}	198–423	+	+	–	++	–	–
2ERR10	IgG_1	1–78	++	++	+	+	++	+
2ERR37	IgG_1	79–144	+(BSA)	–	–	–	+	++

nyd = not yet determined.
[a] + indicates reactivity with overexpressed ERRα; ++ indicates ability to detect endogenous ERRα in 40 μg total protein in BT-474 whole-cell extract. Performing Western blot incubations in 3% bovine serum albumin (BSA) increased signal for 2ERR6 and 2ERR37.
[b] Antibodies 1ERR21 and 2ERR7 could immunoprecipitate ERRα, but not as well as those mAbs marked ++.
[c] All mAbs marked + could shift both overexpressed and endogenous ERRα.
[d] 2ERR7 gave a significantly more robust pulldown than the other single mAbs (indicated by ++). We found that mixtures of mAbs 1ERR21 and 1ERR87 with mAb 2ERR1 or 2ERR10 gave a pulldown comparable to that of 2ERR7.
[e] 2ERR10 was able to detect both overexpressed and endogenous ERRα (as indicated by ++).
[f] Polyol-responsiveness indicates the ability of each mAb to release ERRα under gentle eluting conditions as determined by ELISA-elution.

erage of the entire ERRα protein. Fig. 3A shows representative epitope mapping for mAbs 2ERR10, 2ERR37, and 1ERR21 by Western blot analyses. A schematic showing ERRα truncation mutants and mAb reactivity with regions of ERRα can be seen in Fig. 3B.

mAb reactivities with ER and ERR proteins

To test mAb reactivities with ERRα-related proteins, the known estrogen receptor and ERR isoforms were overexpressed and mAb crossreactivities were analyzed by Western blot. Human embryonic kidney 293T cells were transiently transfected with plasmids encoding empty vector or full-length ERα, ERβ, ERRα, ERRβ, or ERRγ. Cell lysates were separated by SDS–PAGE and overexpression of each isoform was confirmed by Western blot using antibodies specific to the individual ER and ERR proteins (Fig. 4A). The same lysates were subjected to Western blot analyses using the individual mAbs to determine their crossreactivities; representative data are shown in Fig. 4B. None of the mAbs reacted with any ER and ERR proteins other than ERRα.

Immunoprecipitation of ERRα

We overexpressed full-length ERRα in MCF-7 cells and generated whole-cell extracts for use in immunoprecipitation (IP) stud-

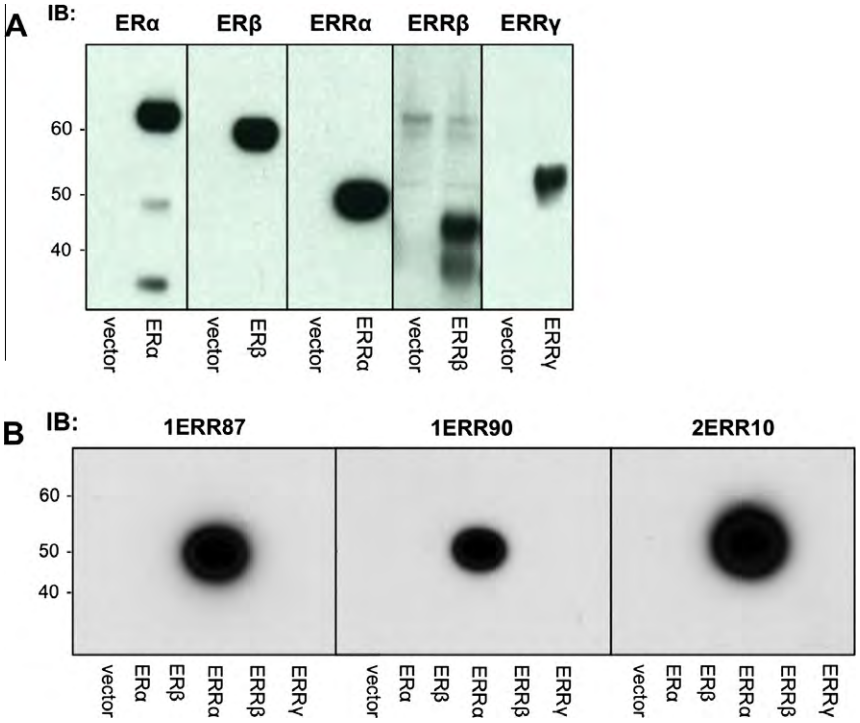

Fig. 4. mAb reactivity with ER and ERR proteins. (A) Human embryonic kidney 293T cells were transiently transfected with plasmid constructs encoding one of the estrogen receptors (ERα or ERβ) or ERRs (ERRα, ERRβ, or ERRγ). Whole-cell lysates were separated by SDS–PAGE, with 375 ng total protein loaded per lane as determined by BCA assay. Overexpression of proteins was confirmed by Western blot analysis using antibodies specific to ERα (Santa Cruz Biotechnology, Santa Cruz, CA), FLAG tag (for detection of FLAG-tagged ERβ; Gentaur, Kampenhout, Belgium), ERRα (mAb 2ERR10), ERRβ (kind gift of Dr. Jeremy Nathans, Johns Hopkins University School of Medicine), and ERRγ (Perseus Proteomics, Tokyo, Japan). Molecular weight in kDa is indicated on the left. The apparent molecular weights of the overexpressed proteins (as detected by Western blot) are as follows: ERα, 65 kDa; ERβ, 60 kDa; ERRα, 50 kDa; ERRβ, 45 kDa; ERRγ, 55 kDa. (B) mAb crossreactivity with ER and ERR proteins was analyzed by Western blot. The same lysates used in Fig. 4A were separated by SDS–PAGE (375 ng total protein loaded per lane), and equal concentrations of purified mAb were used to probe blots. The data shown in this figure are representative; none of the mAbs crossreacted with proteins other than ERRα.

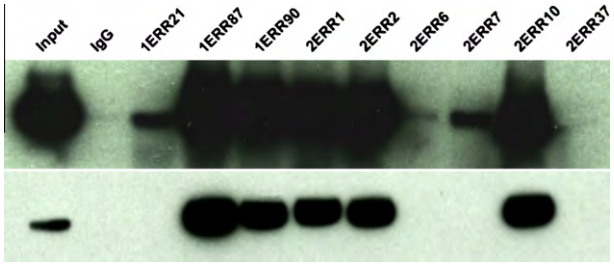

Fig. 5. Immunoprecipitation of ERRα by mAbs. The ability of the mAbs to immunoprecipitate ERRα was tested. Blocked protein G agarose (Millipore) was pre-incubated with mAb for one hour at room temperature, then whole-cell lysates from MCF-7 cells overexpressing ERRα (1 mg total protein per IP) were incubated with the antibody-resin complexes overnight. The resins were washed, then eluted in SDS loading buffer. The top blot was loaded with 20 μL IP sample per lane (20 μg total protein for the input sample); the bottom blot was loaded with 2 μL IP sample per lane (2 μg total protein for the input sample). Western blots were probed with mAb 2ERR10 followed by a light chain-specific anti-mouse IgG secondary antibody to avoid detection of any mAb heavy chain present in the IP samples.

ies. Antibody subisotype is an important consideration when choosing resins for IP applications; since protein G binds all mouse IgG subisotypes, we used protein G-conjugated agarose in all IP experiments. Those antibodies reacting with N-terminal epitopes (1ERR87, 1ERR90, 2ERR2, and 2ERR10) as well as polyol-responsive mAb 2ERR1 were able to pull down significant quantities of ERRα (Fig. 5). Antibodies 1ERR21 and 2ERR7 did not work as well in this assay but were able to immunoprecipitate detectable amounts of ERRα. 2ERR6 and 2ERR37 IPs gave no detectable signal. It is worth noting that these four weakly- or non-immunoprecipitating mAbs (1ERR21, 2ERR6, 2ERR7, and 2ERR37) interact with the E/F region of the protein; it may be that these C-terminal epitopes are inaccessible when ERRα is in its native conformation.

EMSA supershift of DNA-bound ERRα

Each antibody was tested for its ability to react with DNA-bound ERRα in electrophoretic mobility supershift assays (EMSAs). Lysates from MCF-7 cells overexpressing ERRα were incubated with mAb and a ^{32}P-labeled estrogen-related response element (ERRE) DNA probe, after which samples were run on a non-denaturing gel. N-terminal IgG_1 antibodies (1ERR87, 1ERR90, and 2ERR10) and mAb 2ERR1 were able to supershift DNA-bound ERRα, as shown in Fig. 6. These four antibodies could also supershift endogenous ERRα in BT-474 whole-cell lysates (Fig. 6B).

Chromatin immunoprecipitation (ChIP) of endogenous DNA-bound ERRα

Next, we evaluated antibody effectiveness in pulling down endogenous DNA-bound ERRα in chromatin immunoprecipitation (ChIP) assays. For these studies, we used the BT-474 mammary carcinoma cell line, which endogenously expresses high levels of ERRα. Conditions for the assay were optimized based on established protocols [37,38]. We tested each of the antibodies at various concentrations and found that the majority of mAbs could immunoprecipitate chromatin-bound ERRα as determined by PCR amplification of the *ESRRA* promoter (Fig. 7). It is of interest to note that those mAbs capable of supershifting DNA-bound ERRα in EMSAs were not necessarily useful in the ChIP assay and *vice versa*. 2ERR7 gave the best pulldown, while mAbs 1ERR90 and 2ERR37 did not appear to work well in this assay. We investigated the possibility that using a "cocktail" consisting of several mAbs recognizing different epitopes might improve pulldown efficiency. We tested various combinations of these antibodies and determined that mAbs 1ERR21 and 1ERR87, combined with either 2ERR1 or 2ERR10, enhanced pulldown of ERRα in the ChIP assay. Using several mAbs in concert may be advantageous because it could permit isolation of certain ERRα complexes which cannot be detected by a

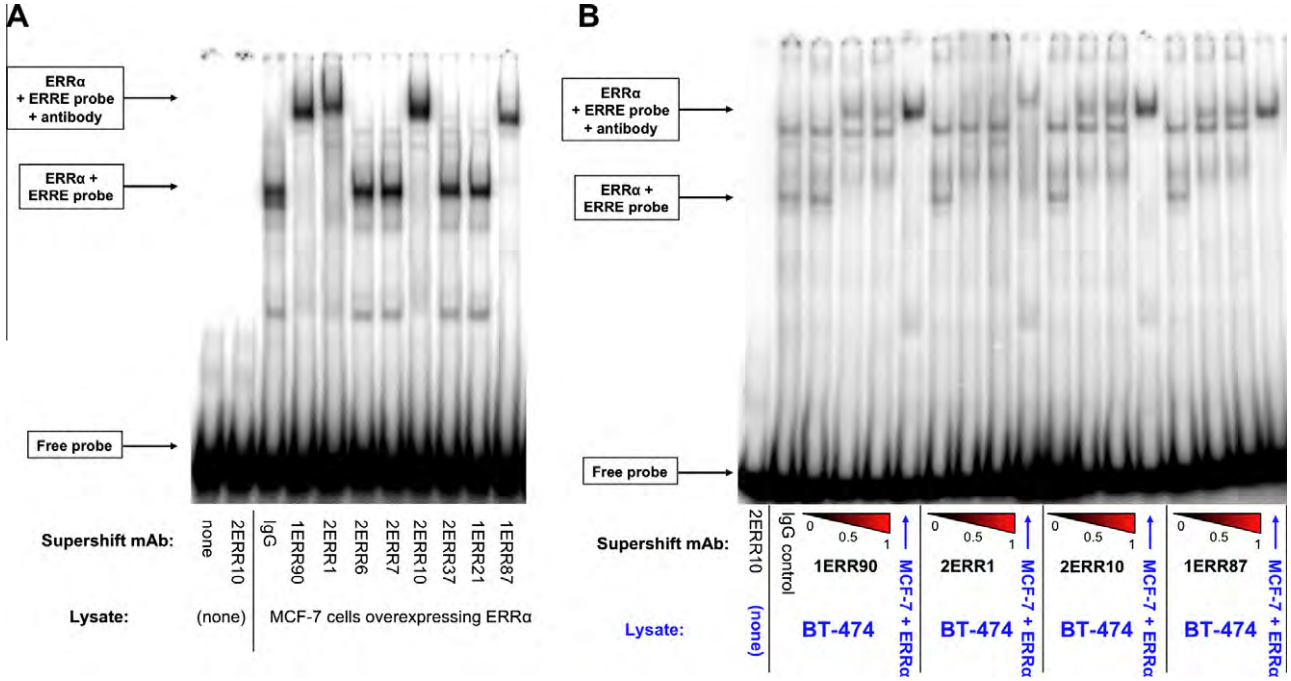

Fig. 6. mAb-mediated supershift of DNA-bound ERRα. Antibodies were assayed for their abilities to interact with DNA-bound ERRα. (A) ERRα was overexpressed in MCF-7 cells, and whole-cell lysates were incubated with mAbs and radiolabeled ERRE probe (5′-AGCAGTGGCGATTTGTCAAGGTCACACAGT-3′). The ability of the mAbs to shift the ERRα/DNA complex was analyzed by non-denaturing gel electrophoresis. (B) For detection of endogenous ERRα, BT-474 whole-cell lysates were incubated with radiolabeled ERRE probe and increasing amounts of mAb in ascites fluid. The approximate amount of antibody in micrograms is given above the mAb name (0, 0.5, and 1 μg mAb). For each antibody, a positive control of MCF-7 cells overexpressing ERRα was included (lanes marked "MCF-7 + ERRα"). Supershifts were analyzed as described above.

Fig. 7. Chromatin immunoprecipitation of endogenous ERRα by mAbs. Antibodies were tested for their capacities to bind endogenous DNA-bound ERRα in cell lysates. BT-474 cells were formaldehyde-fixed to crosslink protein-DNA complexes, then lysed. Lysates were sonicated to fragment chromatin, then incubated with Sepharose-bound mAbs. Protein-chromatin complexes bound to the resin were washed and eluted, after which crosslink reversal and DNA purification steps were performed. Pulldown by mAbs was assessed by PCR amplification of the proximal promoter of the *ESRRA* gene, an established ERRα binding site [46]. Cocktail #1 consisted of mAbs 1ERR21, 1ERR87, and 2ERR1; cocktail #2 consisted of mAbs 1ERR21, 1ERR87, and 2ERR10.

single mAb. A paper describing the use of these antibodies in ChIP-chip is currently in preparation.

Immunofluorescent detection of ERRα

We performed immunofluorescence assays in MCF-7 cells overexpressing ERRα to determine the usefulness of these antibodies in detecting ERRα. Six of the nine mAbs – 1ERR87, 1ERR90, 2ERR1, 2ERR2, 2ERR10, 2ERR37 – were able to detect overexpressed ERRα in the nuclei of formalin-fixed cells with high signal intensity and low background (Fig. 8A). mAb 2ERR10 could also detect endogenous ERRα. In order to test the specificity of 2ERR10 for endogenous ERRα, we cultured three different mammary carcinoma cell lines (BT-474, MCF-7, and SK-BR-3) in the presence or absence of XCT790. We found that XCT790-mediated knockdown of ERRα significantly diminished nuclear staining, indicating that 2ERR10 specifically detects endogenous ERRα in this assay (Fig. 8B). This mAb may prove to be useful in clinical applications for the detection of ERRα in formalin-fixed human tissue samples.

Determination of mAb polyol-responsiveness by ELISA-elution

Our lab has discovered that certain high-affinity antibodies possess the ability to release antigen in the presence of a low-molecular-weight polyhydroxylated compound (polyol) and nonchaotropic salt. This property can be employed to specifically and efficiently isolate proteins which retain native structure and biological activity [32,40]. Polyol-responsive mAbs (PR-mAbs) have been utilized to isolate protein complexes of sufficient purity and yield for crystallization and for identification of novel interacting partners [31,33,39,44,45].

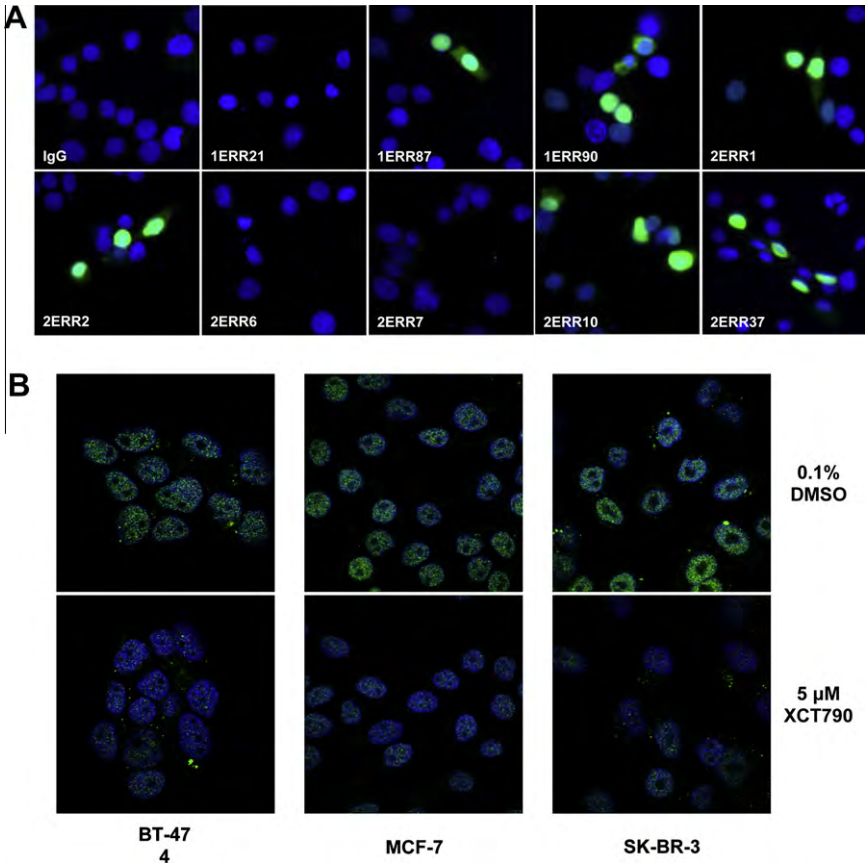

Fig. 8. Immunofluorescent detection of ERRα by mAbs. Antibodies were assayed for their abilities to detect overexpressed and endogenous ERRα in immunofluorescent applications. Cells were fixed and permeabilized, then incubated with anti-ERRα mAbs. Alexa^{488}-conjugated goat anti-mouse IgG secondary antibody was used to detect ERRα-bound mAb (in green); nuclei were visualized with DAPI nuclear counterstain (in blue). Microscope settings were conserved for all samples within each experiment. (A) MCF-7 cells were transiently transfected to overexpress ERRα. Imaging was performed using a Leica DM5000 fluorescence microscope at 40x magnification. (B) MCF-7, BT-474, and SK-BR-3 mammary carcinoma cells were treated for twenty-four hours with 0.1% DMSO vehicle control or 5 μM XCT790, an inverse agonist of ERRα which selectively targets the protein for degradation. 2ERR10 was used as the primary antibody. Imaging was performed using a Bio-Rad Radiance 2100 MP Rainbow confocal/multiphoton microscope at 60× magnification.

We estimate that about 10% of mAbs are polyol-responsive; the mechanism and mAb characteristics contributing to this property remain unclear.

We performed modified enzyme-linked immunosorbent assays (ELISA-elutions) to screen for mAbs that gently release ERRα in the presence of 40% propylene glycol and 0.75 M ammonium sulfate. Five mAbs showed varying degrees of polyol-responsiveness in this assay (Fig. 9A). 1ERR21 and 2ERR10 were weakly polyol-responsive, retaining about 70% signal when compared with the TE control. Our lab has determined that even weakly polyol-responsive mAbs can perform efficiently in immunoaffinity applications (unpublished data). Antibodies 2ERR1, 2ERR6, and 2ERR37 are strongly polyol-responsive, showing significantly reduced signal in the presence of polyol elution buffer when compared with TE vehicle control. Of these, we focused on 2ERR1 for further investigation because it reacts strongly with ERRα in a variety of assays, including immunoprecipitation. The affinity of 2ERR1 for ERRα is affected somewhat by the presence of propylene glycol or ammonium sulfate alone; however, the combination of salt and polyol has a synergistic effect, causing more than 80% of the mAb to release its antigen in 40% propylene glycol at a relatively low concentration of salt. A detailed analysis of 2ERR1 responsiveness to increasing concentrations of polyol and salt is shown in Fig. 9B.

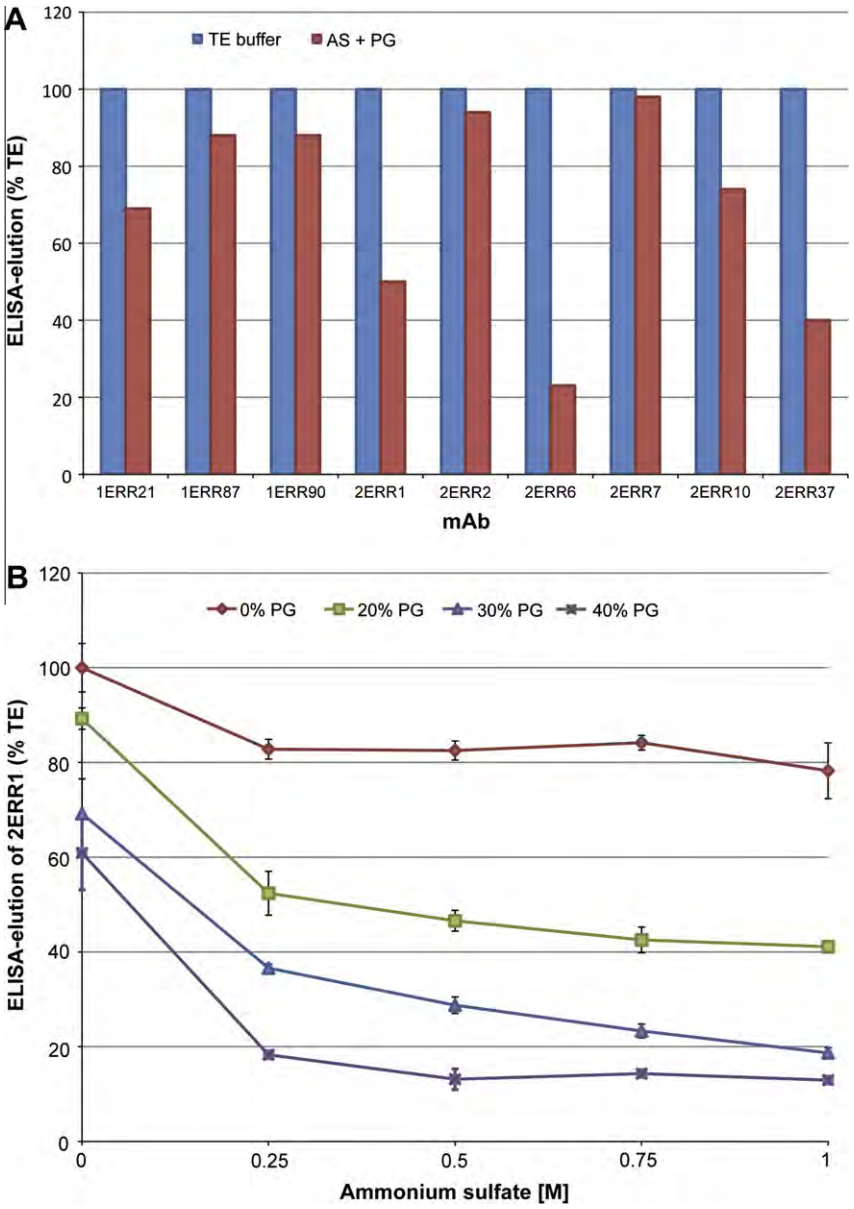

Fig. 9. Polyol-responsiveness of anti-ERRα mAbs. ELISA-elution assays were performed to identify polyol-responsive mAbs (PR-mAbs). Antibodies were bound to ERRα-coated wells in 96-well plates, then incubated in TE control buffer or salt-polyol buffer containing ammonium sulfate (AS) and propylene glycol (PG). Bound mAb was detected with HRP-conjugated anti-mouse secondary antibody and peroxide substrate, and colorimetric analysis was performed. Polyol-responsivity was determined by comparing signal in the presence and absence of salt-polyol, given as a percentage of TE control buffer; PR-mAbs have lower affinity for antigen in salt-polyol buffer, which results in diminished signal. Antibodies are considered polyol-responsive if they show greater than 50% reduction in signal when compared to TE buffer control, as indicated in Table 1. (A) Antibodies were bound to ERRα, then incubated in TE control buffer or salt-polyol buffer containing 0.75 M ammonium sulfate (AS) and 40% propylene glycol (PG). Signal reduction resulting from elution of mAbs in the presence of salt and polyol is shown as a percentage of TE buffer control. (B) The ability of mAb 2ERR1 to bind ERRα in response to increasing concentrations of ammonium sulfate and propylene glycol (PG) was tested. Each condition was assayed in triplicate within an experiment. Data are shown the average of these technical replicates within one experiment, and error bars represent the standard deviation of these. Data are representative of three independent experiments.

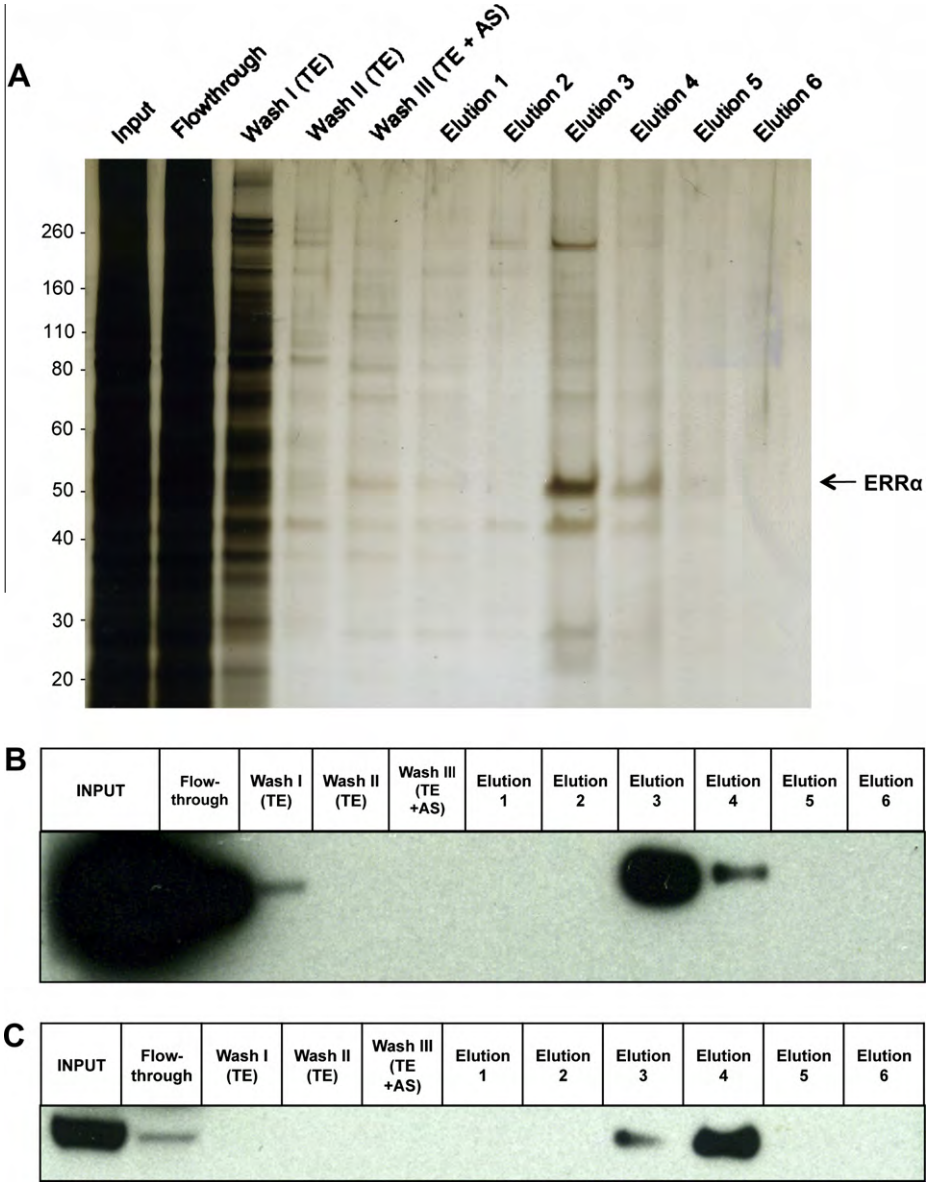

Fig. 10. Purification of ERRα by gentle immunoaffinity chromatography (IAC). 2ERR1-conjugated Sepharose was incubated with whole-cell lysate, then washed. ERRα was gently eluted from the column with polyol elution buffer (TE + 0.75 M ammonium sulfate +40% propylene glycol). IAC fractions were loaded on polyacrylamide gels in proportion to fraction volumes, separated by SDS–PAGE, then prepared for silver staining or Western blot. Western blots were probed with mAb 2ERR10 followed by a light chain-specific anti-mouse secondary antibody to avoid detection of mAb heavy chain present in the samples. (A) A 2-mL column was loaded with lysate containing 8 mg total protein harvested from MCF-7 cells overexpressing ERRα. Samples were loaded on a polyacrylamide gel in proportion to fraction volumes. Molecular weight (in kDa) is indicated at left. TE designates Tris–EDTA buffer; AS signifies 0.25 M ammonium sulfate. (B) The sample set in Fig. 10A was subjected to Western blot analysis to confirm the presence of ERRα in IAC fractions. (C) For purification of endogenous ERRα, a 1-mL column was loaded with BT-474 cell lysate containing 15 mg total protein. ERRα was detected by Western blot.

Gentle immunoaffinity chromatography (IAC) purification of overexpressed and endogenous ERRα

Immunoaffinity chromatography (IAC) is a powerful tool which can isolate proteins rapidly and specifically in a single purification step; however, this approach usually requires harsh eluting conditions which can denature proteins, rendering them inactive. Gentle IAC using PR-mAbs facilitates recovery of active proteins in native conformations and complexes. In order to purify ERRα using gentle IAC, we covalently attached 2ERR1 to cyanogen bromide-activated Sepharose and used this column to purify ERRα from mammalian cell lysates in a single chromatographic step. Figs. 10A and B show IAC fractionation of lysate from MCF-7 cells overexpressing ERRα (silver-stained polyacrylamide gel and Western blot, respectively). As expected, ERRα remained bound to the 2ERR1 resin until eluted with salt-polyol buffer. The eluates show a significant purification of ERRα, which is the major band in the fraction as determined by silver-staining. The other bands present in this fraction likely consist of ERRα-interacting proteins. In addition to isolating overexpressed ERRα, we were able to use gentle immunoaffinity chromatography to purify endogenous ERRα from BT-474 cell lysate (Fig. 10C).

Conclusions

These characterization studies demonstrate that high-affinity anti-ERRα mAbs are suitable for the detection and purification of ERRα in a variety of biochemical assays. Of the nine monoclonal

antibodies investigated, mAbs 1ERR87, 2ERR1, and 2ERR10 seem to have the highest affinities for ERRα and were effective in all assays tested. We found that using mAb "cocktails" – mixtures of mAbs which recognize different epitopes – had a synergistic effect in ChIP, and this approach may be applicable to other assays. Sequential use of different mAbs in tandem purifications could be useful for sorting ERRα subpopulations associated with splice variants, post-translational modifications, or interacting partners. We determined that 2ERR1 is strongly polyol-responsive and that immunoprecipitated ERRα can be gently eluted in the presence of polyol and salt. 2ERR1-mediated gentle immunoaffinity chromatography could be useful for crystallization of ERRα, mass spectrometric analyses of post-translational modifications and ERRα-interacting proteins, and other purification-based strategies. We anticipate that these high-affinity immunological reagents will be useful in a range of novel research and clinical applications.

Author contributions

Conceived and designed the experiments: AME, NET, JAL, JEM, and RRB. Performed the experiments: AME, NET, and JAL. Analyzed the data: AME and NET. Wrote the manuscript: AME and NET. Edited the manuscript: AME, NET, JAL, JEM, and RRB.

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The monoclonal antibodies described in this paper are available from Santa Cruz Biotechnologies.

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