Endogenous Purification Reveals GREB1 as a Key Estrogen Receptor Regulatory Factor


SUMMARY

Estrogen receptor-α (ER) is the driving transcription factor in most breast cancers, and its associated proteins can influence drug response, but direct methods for identifying interacting proteins have been limited. We purified endogenous ER using an approach termed RIME (rapid immunoprecipitation mass spectrometry of endogenous proteins) and discovered the interactome under agonist- and antagonist-liganded conditions in breast cancer cells, revealing transcriptional networks in breast cancer. The most estrogen-enriched ER interactor is GREB1, a potential clinical biomarker with no known function. GREB1 is shown to be a chromatin-bound ER coactivator and is essential for ER-mediated transcription, because it stabilizes interactions between ER and additional cofactors. We show a GREB1-ER interaction in three xenograft tumors, and using a directed protein-protein approach, we find GREB1-ER interactions in half of ER+ primary breast cancers. This finding is supported by histological expression of GREB1, which shows that GREB1 is expressed in half of ER+ cancers, and predicts good clinical outcome. These findings reveal an unexpected role for GREB1 as an estrogen-specific ER cofactor that is expressed in drug-sensitive contexts.

INTRODUCTION

Estrogen receptor-α (ER) is the key transcription factor that drives gene expression programs in ER+ luminal breast cancers (Ali and Coombes, 2002). ER+ breast cancers constitute the majority of breast tumors, and these are generally treated with ER antagonists, although clinical response varies significantly (Ali and Coombes, 2002). ER-mediated transcription is determined by the associated cofactors and interacting transcription factors that form the ER complex (Shang et al., 2000). Increased levels of ER-associated cofactors have been shown to correlate and contribute to drug resistance (Anzick et al., 1997; Torres-Arzayus et al., 2004), highlighting the importance of these cofactors in mediating ER transcriptional activity. Despite the fact that there are many known ER-associated factors (Métilier et al., 2003; Okada et al., 2008), it is clear that critical regulators are still being identified, as highlighted by the recent discovery of ER-associated pioneer factors such as FoxA1, PBX-1, and AP-2γ (Carroll et al., 2005; Magnani et al., 2011; Tan et al., 2011), via enrichment of their DNA consensus sequence within ER binding domains. Direct experimental approaches for finding interacting proteins usually involve exogenous-tagged methods or require very large-scale cell line culture (Malovannaya et al., 2010, 2011; Selbach and Mann, 2006) and are nonexistent in primary tissue.

We develop an endogenous protocol for systematic analysis of protein-protein interactions and protein-DNA binding events. We have combined several robust methods including formaldehyde crosslinking and on-bead digestion, permitting rapid and sensitive purification of endogenous-interacting proteins. Crosslinking with formaldehyde is well established in chromatin immunoprecipitation (ChIP) and tissue fixation. Its size (~2 Å) and ability to permeate membranes of intact living cells have two important implications: only amino acids in close proximity (2.3–2.7 Å) will be crosslinked, and nonspecific interactions by abundant proteins are minimized by the cells own architecture (Sutherland et al., 2008). This approach, termed RIME (rapid immunoprecipitation mass spectrometry of endogenous proteins), identifies ER-associated transcriptional networks and ligand-specific interactions.
GREB1 is shown to be the most estrogen-specific ER interactor, and endogenous associations between ER, GREB1, and additional factors are verified both in vitro and in vivo. Very little is known about the function of GREB1, but GREB1 is one of the highest estrogen-induced genes (Ghosh et al., 2000) and correlates well with changes in ER activity following breast cancer treatment (Dunbier et al., 2010). In addition, it is known that GREB1 is required for growth of ER+ breast cancer cells (Rae et al., 2005), and overexpression can increase colony formation (Liu et al., 2012). However, it was not known what the mechanism is that allows GREB1 to mediate these effects. We now provide functional insight into how GREB1 mediates ER transcriptional activity and show that it is an independent predictor of clinical outcome in patients with breast cancer.

RESULTS

Purification of ER-Associated Proteins Using RIME

Endogenous ER was purified from $1 \times 10^7$ asynchronous MCF-7 breast cancer cells using RIME. The validation of the method is provided in Figure S1. In addition, RIME has been successfully utilized for purification of numerous other factors (an additional example [E2F4] is shown in Figure S2). We only considered ER-associated proteins that occurred in three out of three independent replicates and excluded any protein that appeared in any one of five IgG control RIME. This resulted in 108 ER-associated proteins (Figure 1A). The most confident protein identified from the ER RIME was ER. Also included within the ER interaction list are recently characterized ER pioneer factors FoxA1, TLE1, and AP2-γ (Carroll et al., 2005; Holmes et al., 2012; Tan et al., 2011), putative pioneer factors such as GATA3 (Theodorou et al., 2013), known cofactors including RIP140 (NRIP1), AIB1 (NCOA3), p300, CBP, and CARM1, plus RAR family members that were recently implicated as ER-associated protein (Hua et al., 2009; Ross-Innes et al., 2010). Peptide coverage of specific associated proteins is shown in Figure 1B. We also find a number of repressors, including N-CoR, SMRT, and HDAC2, which are known to be recruited with estrogen-ER to repressed genes such as cyclin G2 (Stossi et al., 2006). A full list of ER-interacting proteins is provided in Figure S3.

More than 25% of the 108 ER-associated proteins were previously validated ER interactors (Figure S3). In addition, six previously uncharacterized ER-associated proteins were selected for validation by standard coimmunoprecipitation (coIP) (no crosslinking) and ChIP sequencing (ChIP-seq) in MCF-7 cells (Figure S4).

ER RIME was repeated in asynchronous SILAC-labeled cells following 3 hr of estrogen treatment or 3 hr of treatment with tamoxifen, the clinical ER antagonist. Two biological replicates were conducted. A number of proteins were predominantly bound with ER following treatment of either the agonist (estrogen) or the antagonist (tamoxifen) (Figure 1C). The core ER-DNA complex, composed of ER and the putative pioneer
factors FoxA1, GATA3, and TLE1, is found under both ligand conditions (Figure 1C). GREB1 was the most markedly induced estrogen-ER-interacting protein, based on SILAC ratio. Information on the spectra is provided in Figure S5. These data support the hypothesis that GREB1 is an ER-associated protein only recruited under estrogenic conditions.

**GREB1 Is a Chromatin-Bound Protein Required for ER-Mediated Transcription**

GREB1 is a highly estrogen-induced gene (Ghosh et al., 2000) with almost no known function. ChIP-seq of GREB1 in estrogen-rich asynchronous MCF-7 cells revealed 8,420 GREB1 binding regions that occurred in two independent replicates (Figure 2A). Almost all (~95%) of the GREB1 binding events were shared by ER (Robinson et al., 2011) (Figure 2A). GREB1 binding was dependent on ER because pretreatment with ICI 182780 (an ER degrader) reduced GREB1 binding, as assessed by ChIP-seq (Figure 2B), despite GREB1 protein levels remaining the same at this short treatment time point (Figure S6). The GREB1 binding to ER cis-regulatory elements was validated by standard ChIP (Figure S6) and with an independent antibody (Figure S6). The interaction between ER and GREB1 was further validated by performing genome-wide ER and GREB1 re-ChIP-seq, confirming global co-occupancy of these two proteins to the same genomic loci (data not shown).

Because GREB1 was found to be the most estrogen-enriched ER interactor, we investigated the functional role of this poorly understood protein. MCF-7 cells were hormone deprived, transfected with control small interfering RNA (siRNA) or a siRNA to GREB1 (Figure 2C), and treated with estrogen or vehicle control for 6 hr for microarray analysis. Differential gene expression analysis revealed 739 genes that were estrogen regulated (p < 0.01), and almost half of these were no longer differentially expressed when GREB1 was specifically silenced (Figure 2D). In addition, silencing of GREB1 significantly impaired the ability of MCF7 breast cancer cells to form colonies in soft agar, suggesting that it is required for their transformed phenotype (Figure 2E).

We overlapped GREB1 binding events with p300 or CBP binding events previously mapped in MCF-7 cells (Zwart et al., 2011) and found that most GREB1 binding events were also co-occupied by the coactivators p300 and CBP (Figure 2F). GREB1 possesses two LXXLL motifs, used by cofactors to interact with nuclear receptors (Heery et al., 1997), and as such, we assessed whether GREB1 may be involved in modulating binding between p300/CBP and ER. GREB1 was silenced with siRNA, and ChIP of ER, p300, and CBP was assessed at shared cis-regulatory elements. Specific silencing of GREB1 did not affect ER binding (data not shown), but p300 and CBP binding was decreased on all the tested regions (Figure 2F), suggesting that GREB1 may stabilize the interaction with the classic, enzymatically active coactivators at specific ER binding regions.

**Purification of Associated Proteins In Vivo**

ER RIME was conducted from three independent MCF-7 xenografts, implanted in immunocompromised mice (data not shown). In all three xenografts, ER-GREB1 interactions were observed (relative to matched IgG controls) (Figure S7), providing evidence that ER-GREB1 associations occur in solid tumor models. In addition to proteomic analysis, ER and GREB1 ChIP-seq was conducted on one of the MCF7 xenografts. Unexpectedly, MCF7 cells grown as a xenograft have a different ER binding profile compared to MCF7 cell growth in vitro (Figure 3A). In the xenografts, ER and GREB1 binding overlapped significantly, confirming that GREB1 binding paralleled the ER-DNA interactions observed in vivo (Figures 3B and 3C). We recently established a method for transcription factor mapping in primary human breast cancer samples (Ross-Innes et al., 2012), enabling genomic interrogation of ER binding properties. We sought to establish a method for protein–protein assessment in primary human tumors, to complement the genomic mapping approaches with proteomic analyses. Because these primary tumors were very small, potentially degraded, and heterogeneous, we opted for a targeted approach by coupling RIME with selective reaction monitoring (RIME-SRM), a method for sensitive and quantitative assessment of specific peptides of interest. A schematic of the approach taken is shown in Figure 3D. Seven primary frozen breast cancers were used, including six ER+ tumors and one ER− control tumor (tumor details are provided in Figure S7). Each tumor was crosslinked and split into ER or IgG RIME-SRM. We assessed multiple peptides that represent ER or some of its interactors identified from the cell line experiments, including GREB1, RXRα, TLE1, CBP, p300, and NRP1. The data represent the average of all peptides for a specific protein (all peptides are provided in Figure S8), and the enrichment was normalized to the matched IgG control. We could successfully identify ER in all six ER+ tumors, but not the ER− tumor (Figures 3E and S7). Interestingly, we could also find several ER-interacting proteins in some of the ER− tumors (Figure 3E). We confirmed the identity of peptide precursors from tumor lysates by fragmentation (MS/MS) using a parallel platform (AB Sciex 5500MS) (data not shown). GREB1-ER interactions were not found in the ER− tumor or in any of the IgG controls and were observed in three out of the six ER RIME-SRMs from ER+ tumors (Figure 3E), suggesting that not all ER+ tumors have ER-GREB1 interactions.

**GREB1 Expression in Breast Cancer**

Given the observation that ER-GREB1 interactions could be detected in three out of six ER+ breast cancers by ER RIME-SRM (Figure 3E), we assessed GREB1 expression in a larger cohort (n = 419) of patients with breast cancer by immunohistochemistry. An antibody for GREB1 was assessed in cell lines (Figure S9), and examples of GREB1 staining are shown in Figure 4A. This antibody has been previously validated for immunohistochemistry (Hnatyszyn et al., 2010). GREB1 expression was seen in 46.0% of all breast cancers, and of these, 96.3% were ER+ breast cancers (Figure 4B), implying that GREB1 expression is mostly limited to ER+ disease. Of all ER+ breast cancers, 55% were GREB1 positive, slightly more than the 40% previously observed by Hnatyszyn et al. (2010). Although GREB1 is an estrogen-induced gene, and GREB1 expression is limited to ER+ cancers, ER mRNA levels did not increase with increasing levels of GREB1 mRNA levels (Figure S9). Within ER+ breast cancers, GREB1 positivity (Allred >2) correlated with a good clinical outcome (Figure 4C). Although GREB1 is estrogen induced,
Figure 2. GREB1 Is an Estrogen-Mediated ER-Interacting Protein Required for ER Function

(A) Venn diagram showing overlap of GREB1 binding events and ER binding events, as determined by ChIP-seq. Almost all GREB1 binding events are shared with ER.

(B) ER ChIP-seq was conducted in asynchronous MCF-7 cells. GREB1 ChIP-seq was conducted in asynchronous cells treated with control or ICI 182780 (an ER downregulator). An example of a binding region is shown.

(C) MCF-7 cells were transfected with siControl or siGREB1, and western blotting confirmed effective knockdown of GREB1.

(D) Hormone-deprived MCF-7 cells were transfected with siControl or siGREB1 (four replicates) and treated with vehicle or estrogen for 6 hr. Microarray analysis was conducted, and estrogen-mediated differential gene changes (in siControl conditions) were determined. The heatmap represents all genes selected as differentially expressed following estrogen treatment relative to vehicle, in either siControl or siGREB1-transfected cells.

(E) MCF-7 cells were transfected with siControl or siGREB1, and soft agar assays were conducted. **p < 0.001, Student’s t test. The error bars represent SD.

(F) Overlap of GREB1 binding with the coactivators p300 and CBP. MCF-7 cells were transfected with siControl or siGREB1, and p300 or CBP ChIP was performed followed by real-time PCR of known ER regulatory regions. In the absence of GREB1, p300 and CBP coactivators cannot bind to enhancer elements. The error bars represent SD.

Additional relevant information is provided in Figure S6.
this correlation with good outcome may also reflect the functional role of GREB1 as a component of the ER complex. In support of this, GREB1 was found to be a prognostic factor independent of the Nottingham prognostic index (NPI) (multivariate model GREB1 HR = 0.49 [0.26–0.90], p = 0.021; NPI HR = 2.3 [1.7–3.1], p < 0.001) (Figure S9), and in combination with the classic prognostic marker PR, GREB1 predicted an additive clinical benefit, when compared to PR alone (Figure S9). These findings place GREB1 as a key estrogen-specific ER-associated protein, where it is functionally linked with the transcriptional output of the ER complex. We observed that GREB1 is not expressed in a tamoxifen-resistant (Tam-R) breast
cancer model (Figure S10), but interestingly, re-expression of GREB1 in these Tam-R cells results in decreased cell growth in the presence of tamoxifen. As such, there may be pressure to inactivate or downregulate GREB1 in endocrine-resistant cells, potentially explaining why GREB1 expression is restricted to the 50% of ER+ breast cancer with a good clinical outcome.

**DISCUSSION**

We have established a rapid protocol for the identification of endogenous-interacting proteins from limited starting material, which enables assessment of endogenous protein interactions in tumor material. By applying RIME to ER, the major driving transcription factor in luminal breast cancer, we have identified protein networks and functional components of this transcription complex. These include unexpected transcriptional associations between ER and COUP-TFII (COT2), KLF4, TLE3, and GREB1 (Figure S4). In addition to ER, we have successfully used this approach to identify associated complexes for numerous transcription factors. An additional example is shown in Figure S2, where E2F4 is purified, and the components of the core E2F4 complex are found by RIME in two independent treatment conditions. Our approach provides a cost-effective, sensitive, and rapid method for discovery of endogenous-interacting proteins. We exploit the size and permeability of formaldehyde to preserve bona fide protein-protein interactions, avoiding nonspecific interactions that would be anticipated in any postlysis procedure. Furthermore, the low-affinity interactions between protein constituents of transcription factor complexes (interactions with rapid on-off rates) that would in any other protocol be missed with even moderate wash stringencies are maintained using RIME. This is further controlled for by the use of parallel IgG controls in all experiments and quantitative SILAC approaches to confirm specificity. It is also possible that adjacent transcription complexes that occupy similar DNA binding domains may be crosslinked, yet digestion of DNA did not greatly affect the ER interactome identified (Figure S1). The sensitivity of RIME from limited material enables directed approaches for identification of protein-protein interactions in primary material, such as breast cancer tissue used in this study.

The most confident estrogen-enriched ER-associated protein discovered using RIME was the poorly characterized protein GREB1, a gene with little known function. The interaction between ER and GREB1 was observed in cell lines, xenograft tumors, and in several primary human ER+ breast cancer samples. We show that GREB1 is recruited with ER to transcriptionally active cis-regulatory regions, where it functions as an essential factor, stabilizing associations with additional cofactors. This is of particular significance given the potential of GREB1 as a clinical biomarker (Dunbier et al., 2010). Our findings suggest that GREB1 expression functions as both a readout of ER transcriptional activity and as a contributing factor to that transcriptional potential. Interestingly, GREB1 has been shown to be important for growth of ER+ breast cancer cells (Rae et al., 2005) and AR+ prostate cancer cells (Rae et al., 2006), suggesting that it might also be involved in AR function in the prostate cancer.

Our observations suggest that GREB1 is expressed in half of ER+ tumors, where it correlates with positive clinical outcome (Figure 4C). In this subset of ER+ cancers, GREB1 is induced by the ER complex and is functionally linked with ER transcriptional activity. The absence of GREB1 in the remaining 50% of ER+ breast cancer (Figure 4B) may reflect a nonfunctional ER complex, where GREB1 is no longer ER induced. Our data would suggest (Figure S10) that GREB1 loss may be a contributing factor to the changes in hormonal responses and acquisition of
antiestrogen resistance. This is supported by the observation that GREB1 expression is downregulated in multiple endocrine-resistant cell line models (McCune et al., 2010; Oyama et al., 2011; Shen et al., 2011) (Figure S10) and that re-expression of GREB1 results in abrogation of the tamoxifen resistance (Figure S10). In drug-sensitive cells, GREB1 functions as a contributor toward a functional ER complex, whereas in drug-resistant cells, where tamoxifen-ER complex mediates cell growth, GREB1 is inhibitory. Given the findings that GREB1 is the least tamoxifen-enriched ER-associated protein (Figure 1C), tamoxifen-ligated ER complex may acquire dependence on other upregulated cofactors and may not be able to drive gene expression in the presence of GREB1. Although it is currently unclear why GREB1 needs to be downregulated for acquisition of endocrine resistance, our findings place GREB1 as one of the central estrogen-specific ER cofactors, where it functions to mediate interactions between the ER and additional proteins.

EXPERIMENTAL PROCEDURES

Detailed methods are available in Extended Experimental Procedures. All animal work and human tissue work were performed with the appropriate ethics, as described in Extended Experimental Procedures.

ACCESSION NUMBERS

All microarray data have been deposited in the GEO database with the accession number GSE37386. All ChIP-seq data have been deposited in the GEO database with the accession number GSE41561. All proteomic data have been deposited in the PRIDE database with the accession numbers 27371–2738.

SUPPLEMENTAL INFORMATION

Supplemental information includes ten figures, one table, and Extended Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.01.010.

LICENSING INFORMATION

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