Reciprocal Recruitment of DRIP/Mediator and p160 Coactivator Complexes in Vivo by Estrogen Receptor*

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Two functionally distinct classes of coactivators are recruited by liganded estrogen receptor, the DRIP/Mediator complex and p160 proteins, although the relative dynamics of recruitment is unclear. Previously, we have shown a direct, estriadiol-dependent interaction between the DRIP205 subunit of the DRIP complex and the estrogen receptor (ER) AF2 domain. Here we demonstrate the in vivo recruitment of other endogenous DRIP subunits to ER in response to estrogen treatment in MCF-7 cells. To explore the relationship between DRIP and p160 coactivators, we examined the kinetics of coactivator recruitment to the ER target promoter, pS2, by chromatin immunoprecipitation. We observed a cyclic association and dissociation of coactivators with the promoter, with recruitment of p160s and DRIPs occurring in opposite phases, suggesting an exchange between these coactivator complexes at the target promoter.

The actions of estradiol are mediated by two isoforms of the estrogen receptor (ER),1 ERα and ERβ, which function as ligand-regulated transcription factors. The liganded ER homodimer binds the promoters of target genes and interacts with coactivators to facilitate transcriptional activation (1). A number of coactivators interact with the C-terminal activation domain (AF2) in a ligand-dependent manner and have been implicated in ER-mediated transcription. One class of coactivators, collectively termed the p160 family, includes SRC1/NCoA-1, TIF2/GRIP1/NCoA-2, and pCIP/ACTR/AIB1 (re-

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† The abbreviations used are: ER, estrogen receptor; E2, 17β-estradiol; ChIP, chromatin immunoprecipitation; RT, reverse transcription; PMSF, phenylmethylsulfonyl fluoride; CATD, cathepsin D, CBP, CREB-binding protein (where CREB is CAMP-response element-binding protein); pol II, RNA polymerase II; TBP, TATA-binding protein.

viewed in Refs. 16 and 23). The p160 coactivators not only possess weak histone acetyltransferase activity but also recruit CBP/p300 (2), presumably leading to the generation of an open chromatin structure at the promoter (3). A second distinct class of coactivators, alternatively called DRIP, ARC, or TRAP (4, 5), comprises a multi-protein complex that interacts with liganded nuclear receptors, including ERα and ERβ, via the DRIP205/TRAP220 subunit (6–10). The DRIP complex shares several subunits with the mammalian Mediator complex, suggesting that it functions in the direct recruitment of RNA polymerase II to the promoter (11).

It is evident that both p160s and DRIP205 play a key role in ER-mediated transcription (12–15), and despite the functional distinction between the p160 coactivators and the DRIP complex, the molecular determinants of the interactions between these coactivators and ER appear to be very similar. Both classes of coactivators interact with the receptor AF2 via LXXL signature motifs (16), and the same residues in the ERα-AF2 are critical for interactions with both p160s and DRIP205 (6), raising the question of whether these complexes are utilized by the receptor dimer simultaneously or sequentially. It has been reported that ACTR can be acetylated by CBP/p300, leading to the dissociation of p160 coactivator complexes from the promoter-bound ER (17), suggesting a mechanism whereby coactivator exchange may take place. Recently, spectroscopic methods have been used to demonstrate that the stoichiometry of the SRC1/ERα/E2 complex is one coactivator molecule per ERα dimer, supporting a sequential model of coactivator recruitment (18). Conversely, chromatin immunoprecipitation (ChIP) experiments reported by Shang et al. (19) favor a combinatorial model.

Here we show the recruitment of multiple DRIP complex subunits by liganded ER both in vitro and in vivo, suggesting that the entire DRIP complex is utilized by ER. Using ChIP, we have examined the kinetics of coactivator recruitment to an endogenous estrogen-regulated promoter. We observe a cycling of coactivators on and off the promoter, and strikingly, there is an inverse relationship between DRIP205 and p160 promoter occupancy, suggesting an exchange between the two coactivator complexes at the promoter and supporting a sequential model of coactivator recruitment.

EXPERIMENTAL PROCEDURES

BV-FLAG-ER Pull-downs—BV-FLAG-ER (α or β) was purified from SF9 cells (20) and pull-downs performed as described (21). Immobilized proteins were extracted with SDS buffer, separated, and Western blotted with the specified antibodies.

RT-PCR and ChIP Assays—MCF-7 cells, grown in phenol red-free Dulbecco’s modified Eagle’s medium with 10% dextran-charcoal stripped fetal bovine serum, were treated with 100 nM E2 as indicated. For RT-PCR, 5 μg of total RNA (Trizol, Invitrogen) was reverse transcribed (Superscript™ Preamplification System, Invitrogen), and 10% of the RT product was PCR-amplified (pS2 (+571 to +1236) and β-actin (+16 to +696)). For ChIP, cells were cross-linked with 1% formaldehyde at 37 °C for 30 min then quenched with glycine to 125 mM. The cells were washed with phosphate-buffered saline and collected into 100 mM Tris·HCl (pH 9.4), 10 mM dithiothreitol. The cell pellet was resuspended (10 mM Tris·Cl (pH 8.0), 0.25% Triton X-100, 0.5% Nonidet P-40, 10 mM EDTA, 0.5 mM EGTA, 1 mM PMSF) and incubated on ice for 10 min. Nuclei were collected by centrifugation, washed (10 mM Tris·Cl (pH 8.0), 0.2 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF) and resuspended in the same buffer without NaCl. Samples were sonicated (10 × 10 s), centrifuged, and 0.10 volume of 10× radioimmunoprecipitation buffer (10% Triton X-100, 1% sodium deoxycholate, 1.4 M NaCl) was added to...
ERα and ERβ interact with multiple DRIP subunits as well as with p160 coactivators in an agonist-dependent manner. A, expression and purification of ERα and ERβ. Purified baculovirus expressed FLAG-ERα and FLAG-ERβ were analyzed by SDS-PAGE with visualization by Coomassie staining. B, interaction of ER with endogenous coactivators. Immobilized FLAG-ER was incubated with HBL100 nuclear extract (1 mg) in the absence (-) or presence of $10^{-8}$ M E$_2$ (Sigma) or 4-hydroxytamoxifen (OHT, Sigma). ER-interacting proteins were detected by Western blot with indicated antibodies. Nuclear extract equivalent to 5% of input (NE) is shown. C, interactions between endogenous proteins. ER-interacting proteins were immunoprecipitated from whole cell extracts of MCF-7 cells treated with $10^{-8}$ M E$_2$ for 1 h or left untreated (-) as described (6): antibodies to hERα/H11002 or hERβ/H9252 elution buffer (1% SDS, 0.1 M NaHCO$_3$) and reverse-cross-linked at 871 °C. DNA fragments were purified (QIAquick Spin Kit, Qiagen) and PCR-amplified (pS2 promoter region (65-1256)) (Fig. 3B, panel ii). For DRIP205, using two different antibodies, we detected two peakss of recruitment. This might be explained in part by the different promoters used in each study; pS2 here compared with cathepsin D (CATD) in the previous report. One significant difference was in the kinetics of CBP/p300 recruitment to the promoter. The first (45 min) and last (150 min) peaks of CBP recruitment in our study coincide with those observed by Shang et al. (19); however, we detected an additional peak of CBP binding at 150 min (panel ii). Moreover, we consistently observed p300 throughout the time course of E$_2$ treatment, unlike the transient appearance of p300 at 30 min reported previously. This might reflect the use of different anti-p300 antibodies in each study. The appearance of histone H4 acetylation within 30 min is consistent with the kinetics of ER and CBP/p300 recruitment to the promoter (panel ii).

Results and Discussion

ER Interacts with Multiple DRIP Complex Subunits—We reported previously that the DRIP205 subunit of the DRIP coactivator complex interacts with ERα and ERβ in a ligand-dependent manner (6); we failed, however, to observe the corecruitment of other DRIP subunits from nuclear extracts by ER. We have re-addressed this question using a modified assay. Full-length baculovirus-expressed FLAG-tagged ERα and ERβ were affinity-purified (Fig. 1A) and used to pull down ER-interacting proteins from nuclear extracts of the human breast-derived cell line, HBL100. In addition to DRIP205 we were able to detect several DRIP subunits, including DRIP150, DRIP130 (Fig. 1B), and DRIP240 (data not shown), as well as two members of the p160 family, SRC1 and ACTR. These factors all interacted with ER in an agonist-dependent manner, and this interaction was abolished in the presence of an antagonist, 4-Hydroxytamoxifen (Fig. 1B). Importantly, these interactions were also detected among endogenous proteins. DRIP205, DRIP150, DRIP130 and SRC1 were commmunoprecipitated from MCF-7 cell lysates with both ERα and ERβ in a ligand-dependent manner (Fig. 1C). These results suggest that both ER isoforms recruit the whole DRIP complex in response to ligand.

Kinetics of Recruitment of ERα, Coactivators, and Components of the Transcription Machinery to an Estrogen-regulated Promoter in Vivo—A series of ChIP experiments were performed to examine the *in vivo* recruitment of DRIPs and p160 coactivators by liganded ER to the endogenous pS2 promoter in MCF-7 cells. Initially, sequential dilutions of the coprecipitated DNA template and input DNA were PCR-amplified to determine the linear range of the assay (data not shown); all subsequent analyses used 5 μl of DNA template, which lies within the linear range. To establish an appropriate time course of ligand treatment, we examined the kinetics of E$_2$-induced pS2 gene expression by RT-PCR (Fig. 2A). pS2 mRNA increased above control levels within 30 min of treatment, reaching a maximum at 45 min, after which it remained elevated through to the last time point at 165 min.

The recruitment of several classes of transcription factors to the pS2 promoter was examined over a 165-min time course of E$_2$ treatment (Fig. 2B). Within 30 min of E$_2$ treatment we observed a significant increase in ER occupancy at a region of the pS2 promoter containing a single, well characterized estrogen response element (22) (panel i). In accordance with a previous report (19), we observed a cycling of cofactors on and off the promoter over the time course of estradiol addition to cells, although with some minor differences in the absolute timing of peaks of recruitment. This might be explained in part by the different promoters used in each study; pS2 here compared with cathepsin D (CATD) in the previous report. One significant difference was in the kinetics of CBP/p300 recruitment to the promoter. The first (45 min) and last (150 min) peaks of CBP recruitment in our study coincide with those observed by Shang et al. (19); however, we detected an additional peak of CBP binding at 150 min (panel ii). Moreover, we consistently observed p300 throughout the time course of E$_2$ treatment, unlike the transient appearance of p300 at 30 min reported previously. This might reflect the use of different anti-p300 antibodies in each study. The appearance of histone H4 acetylation within 30 min is consistent with the kinetics of ER and CBP/p300 recruitment to the promoter (panel ii).

The recruitment of two members of the p160 family of coactivators, SRC1 and ACTR, is shown in panel iii. The level of SRC1 at the promoter increased over 30–45 min, reaching a peak at 60 min, with a second less pronounced peak at 150 min. This kinetics parallels that of CBP/p300, consistent with the fact that CBP/p300 is recruited to nuclear receptors through its interaction with p160 coactivators (23). ACTR was detected at 30 min, after which we observed reduced levels of this protein at the promoter until 165 min, when there was a second pronounced peak. We cannot distinguish whether the difference in the kinetics of SRC1 and ACTR is due to functional differences between these related coactivators or because of intrinsic differences in the antibodies used in the assays.

Using ChIP we found that at least three subunits of the DRIP complex, DRIP205, DRIP150, and DRIP130, are brought to the pS2 promoter following ligand addition to MCF-7 cells (panel iv). These three DRIP subunits are also recruited to the E$_2$-regulated c-Myc promoter in this cell line (data not shown). For DRIP205, using two different antibodies, we detected two distinct peaks of recruitment, a transient peak at 45 min and a broader peak at 120 min after E$_2$ addition. Interestingly, in the context of both the pS2 and c-Myc promoters, DRIP130 and DRIP150 were corecruited with DRIP205 only during the second peak, suggesting a stabilization of DRIP205 at the promoter in the presence of other subunits. It is possible that an intact DRIP complex that includes DRIP205, DRIP150, and DRIP130 may act at a later stage in E$_2$-dependent gene activation and that free DRIP205, or an alternate DRIP205-containing subcomplex, might be recruited at earlier time points.

Finally, we examined the recruitment of components of the basal transcription machinery to the pS2 promoter (panel v). The level of RNA pol II remained steady over the time course of treatment. TBP was also detected on the promoter throughout the time course, reaching a maximal level at 45 min, which correlates with the induction of pS2 gene expression.

From these experiments it is evident that both DRIPs and p160 coactivators are involved in ER-mediated transcription in vivo.
Reciprocal Binding of DRIP and p160 Coactivators to ER

vivo. Strikingly, however, there appears to be an inverse relationship between DRIP205 and p160 coactivator recruitment to the pS2 promoter. When we compared the promoter occupancy of DRIP205 with either ACTR or SRC1 over the time course of hormone treatment, we observed a clear reciprocal relationship (Fig. 2C). This was also true at the c-Myc promoter (data not shown). It should be noted that this reciprocal pattern of recruitment of DRIP205 and p160 coactivators is in contrast to the study of Shang et al. (19), where AIB1 (ACTR) and PBP (DRIP205) were detected simultaneously at the CATD promoter.

**FIG. 2.** Recruitment of ERα transcription complex to the pS2 promoter in vivo. A, schematic representation of the pS2 locus. PCR primers used in ChIP are indicated by arrows. B, recruitment of ERα and coactivators to the pS2 promoter, and histone H4 acetylation, in response to E2 treatment as assayed by ChIP using primers from the pS2 promoter (panel i) and coding region (panel ii) as a control. Antibodies were the same as for Fig. 2B except for anti-SRC1 (antipeptide) and an additional anti-DRIP205 antibody (TRAP220, Santa Cruz). C, the intensities of PCR bands from SRC1 and DRIP205 (NR2) chromatin IPs (from B) were quantitated and plotted as a percentage of the maximum intensity over the time course of E2 treatment.

**FIG. 3.** Early kinetics of recruitment of the ERα transcription complex to the pS2 promoter in vivo. A, schematic representation of the pS2 locus. PCR primers used in ChIP are indicated by arrows. B, recruitment of ERα and coactivators to the pS2 promoter, and histone H4 acetylation, in response to E2 treatment as assayed by ChIP using primers from the pS2 promoter (panel i) and coding region (panel ii) as a control. Antibodies were the same as for Fig. 2B except for anti-SRC1 (antipeptide) and an additional anti-DRIP205 antibody (TRAP220, Santa Cruz). C, the intensities of PCR bands from SRC1 and DRIP205 (NR2) chromatin IPs (from B) were quantitated and plotted as a percentage of the maximum intensity over the time course of E2 treatment. DRIP205 (2H) is compared with both SRC1 and ACTR.
Early Kinetics of ERα and Coactivator Recruitment—As our ChIP data indicated that ER and most transcriptional cofactors are already recruited to the pS2 promoter at the first time point following estradiol addition to cells (i.e. 30 min), we looked at earlier time points in the hope that we might elucidate the order of recruitment of coactivator complexes. MCF-7 cells were treated with E2 for a short time course (2.5–30 min), and ChIP was performed using a subset of the antibodies used previously. The results, presented in Fig. 3B, show that ERα is recruited to the promoter by the earliest time point (2.5 min) after E2 stimulation. We also observed the recruitment of CBP and p300 at 2.5 min, followed by the appearance of acetylated histone H4. Two different antibodies, anti-ACTR and anti-p300 at 2.5 min, followed by the appearance of acetylated H4. Two different antibodies, anti-ACTR and anti-SCC1, were used to assess the recruitment of p160 coactivators to the promoter within 2.5 min after E2 addition. Remarkably, three peaks (2.5, 10, and 30 min) were detected with the anti-SCC1 antibody, although this cycling was not as apparent when the anti-ACTR antibody was used. Using three independent antibodies to DRIP205, we also observed its recruitment to the promoter within 2.5 min following addition of ligand, with peaks at 5 and 15 min. As with the longer time course, this pattern is the reciprocal of that seen for the p160 coactivators (Fig. 3C), suggesting an exchange between the two coactivator complexes when recruited to ER.

Coactivator Exchange on the pS2 Promoter—We believe that the early time points shown in Fig. 3 represent the limit of resolution of the ChIP technique. However, the use of fluorescently tagged receptors and stably integrated hormone response element arrays have enabled the visualization of receptor-cofactor dynamics at the scale of seconds in living cells. Using this approach, it appears that ligand-occupied glucocorticoid and estrogen receptors undergo rapid (within seconds) exchange with DNA targets; moreover, rapid coregulator exchange also occurs (24, 25). The peaks of coactivator-chromatin association that we observed by ChIP may represent the average of multiple peaks of oscillation that take place within a time point interval. Thus the limited sensitivity of the ChIP assay makes it impossible to unambiguously determine the order of coactivator recruitment in vivo. Nevertheless, the most striking observation from our ChIP assays was a clear reciprocal relationship between DRIP205 and p160 coactivator recruitment to the pS2 promoter following ligand addition (Figs. 2C and 3C), strongly suggesting that the receptor does not bind both coactivator complexes at the same time, but rather exchanges one bound complex for another. We believe that this is consistent with a sequential model of coactivator utilization whereby one coactivator complex, such as p160/CBP, is recruited to ER and acts at one level of transcription initiation, i.e. chromatin remodeling and/or modification. This complex would then dissociate from the receptor allowing recruitment of a second, distinct complex, such as DRIP/Mediator, which in turn would facilitate a functional interaction with RNA pol II (11).

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Addendum—While this manuscript was in preparation, the recruitment of the whole DRIP complex by liganded ER was reported (26), further supporting the results presented herein.

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