

The catalytic subunit of the proteasome is engaged in the entire process of estrogen receptor-regulated transcription

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The ubiquitin–proteasome system plays an important role in a variety of cellular functions by means of its proteolytic activity. Interestingly, recent studies have indicated that the proteasome components are also integral parts of transcription complexes. In genome-wide screening for steroid receptor coactivator (SRC)-interacting proteins using yeast two-hybrid system, we found that the 20S proteasome β subunit LMP2 (Low Molecular mass Polypeptide 2) interacts directly with the SRC coactivators. We showed that LMP2 is required for estrogen receptor (ER)-mediated gene transcription and for estrogen-stimulated cell cycle progression. We found that LMP2-associated proteasome is recruited to the entire sequence of ER target genes, implicating a role for the proteasome in both transcription initiation and elongation. We demonstrated that the recruitment of LMP2 by SRC coactivators is necessary for cyclic association of ER-regulated transcription complexes on ER targets. These results revealed a mechanism by which the proteasome machinery is recruited in ER-mediated gene transcription. Our experiments also provided evidence implicating SRC coactivators in gene transcription elongation.

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Introduction

The ubiquitin–proteasome system has emerged as a major player in a variety of regulatory processes in the cell. The 26S proteasome is the primary machinery of the ubiquitin–proteasome system, which represents one of the most important degradation systems in a cell, typically for destruction of short-lived and regulatory proteins (Hershko and Ciechanover, 1998; Pickart, 2001b). The protein degradation involves protein ubiquitination and the subsequent protea-

somal destruction. Protein ubiquitination is operated by the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s) (Hershko and Ciechanover, 1998; Pickart, 2001a). Ubiquitination provides a signal for recognition of the target protein by proteasome, and subsequent degradation of the protein is a function of the 26S proteasome, which is composed of a 19S regulatory complex and a 20S core catalytic complex (Pickart, 2001a). The regulation of protein substrate turnover by the ubiquitin–proteasome system is essential for a variety of normal cellular functions, such as gene regulation, antigen presentation, endocytosis, and cell stress responses (Pickart, 2001b; Lipford and Deshaies, 2003). In addition, the ubiquitin–proteasome system is also implicated in an array of pathological processes such as carcinogenesis and the onset of skeletal muscle atrophy (Zhang *et al.*, 1998; Schwartz and Ciechanover, 1999; Sandri *et al.*, 2004).

Although its roles in cell cycle regulation and signal transduction are well established, the importance of the ubiquitin–proteasome pathway in nuclear receptor-regulated gene transcription has been recognized only recently. Nuclear receptors are degraded by the ubiquitin–proteasome both in the presence or absence of their cognate ligands (Nawaz *et al.*, 1999a; Kopf *et al.*, 2000; Lonard *et al.*, 2000; Wijayarathne and McDonnell, 2001; Reid *et al.*, 2002, 2003; Nonclercq *et al.*, 2004; Tateishi *et al.*, 2004; Callige *et al.*, 2005; Dennis *et al.*, 2005; Horner-Glister *et al.*, 2005; Laios *et al.*, 2005; Valley *et al.*, 2005; Callige and Richard-Foy, 2006). In addition, both coactivators and corepressors of nuclear receptors are also targeted to the ubiquitin–proteasome for degradation (Lonard *et al.*, 2000; Yan *et al.*, 2003; Hoang *et al.*, 2004; Lonard *et al.*, 2004; Li *et al.*, 2006). It is believed that the ubiquitin–proteasome functions in promoting the turnover of transcription complexes, thereby facilitating proper gene transcription (Reid *et al.*, 2002; Lipford and Deshaies, 2003; Muratani and Tansey, 2003; Baker and Grant, 2005). More recently, it has become evident that nuclear receptor degradation and nuclear receptor-dependent transcription are interdependent processes (Lonard *et al.*, 2000; Ferdous *et al.*, 2001; Stenoien *et al.*, 2001; Reid *et al.*, 2003; Lonard and O'Malley, 2005), and a number of ubiquitin pathway enzymes and components of the proteasome have been found to act as modulators of nuclear receptor function, and the enzymes and components of the proteasome are recruited to the promoters of nuclear receptor-responsive genes (Nawaz *et al.*, 1999b; Verma *et al.*, 2004).

Estrogen exhibits a broad spectrum of physiological functions ranging from regulation of the menstrual cycle and reproduction to the modulation of bone density, brain function, and cholesterol mobilization. Estrogen is also associated, pathologically, with tumorigenesis of human mammary gland and uterine body (McDonnell and Norris, 2002). The biological function of estrogen is believed to be largely

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mediated by estrogen receptors (ERs), ER α and ER β (McDonnell and Norris, 2002). ERs belong to the nuclear hormone receptor superfamily, which regulates downstream target genes through recruitment of a variety of coactivator proteins such as steroid receptor coactivators (SRCs) (Mangelsdorf *et al*, 1995; Lemon and Freedman, 1999; Glass and Rosenfeld, 2000; McDonnell and Norris, 2002; McKenna and O'Malley, 2002; Metivier *et al*, 2003). SRC family, also referred to as p160 family, consists of three distinct but related members including SRC-1/NCoA1, GRIP1/TIF2/SRC-2/NCoA2, and pCIP/ACTR/AIB1/RAC3/TRAM1/SRC-3/NCoA3 (Onate *et al*, 1995; Hong *et al*, 1996; Anzick *et al*, 1997; Chen *et al*, 1997; Li *et al*, 1997; Takeshita *et al*, 1997; McKenna and O'Malley, 2002), which enhance the nuclear receptor-mediated gene transcription through direct and/or indirect recruitment(s) of other cofactors as well as through their chromatin remodeling capabilities (Mangelsdorf *et al*, 1995; Lemon and Freedman, 1999; Glass and Rosenfeld, 2000; McDonnell and Norris, 2002; McKenna and O'Malley, 2002; Metivier *et al*, 2003). Previously, we demonstrated that the SRC family is both necessary and sufficient to initiate ER-mediated gene transcription (Shang *et al*, 2000) and that the members of the SRC family, although structurally and biochemically similar, exhibit differential functions in gene transcription (Shang and Brown, 2002; Zhang *et al*, 2004; Wu *et al*, 2005).

In this report, we showed the 20S proteasome β subunit, LMP2 (Low Molecular mass Polypeptide 2), directly interacts with the members of the SRC family and enhanced ER-mediated gene transcription. We demonstrated the recruitment of LMP2 not only on the promoter but also on the entire sequence of ER target genes, indicating that the proteasome is engaged in the entire process of ER-mediated gene transcription.

Results

Identification of LMP2 as an SRC-interacting protein by yeast two-hybrid screening

The SRC coactivators share several important functional domains (Figure 1A). The nuclear receptor binding domain, which contains nuclear receptor boxes, is located in the middle region of these proteins, and, in the carboxy (C)-terminus, these proteins harbor domains that interact with other coactivators such as p300 and CARM1 (Chen *et al*, 1999; Leo and Chen, 2000). In the amino (N)-terminus, SRC-1, GRIP1, and AIB1 all contain basic-helix-loop-helix (bHLH) and Per-Arnt-Sim (PAS) domains that are implicated in specific DNA binding and/or heterodimerization with other bHLH-PAS-containing proteins (Belandia and Parker, 2000; Kim *et al*, 2003). As the nuclear receptor-interacting domain and the C-terminal coactivator-interacting domain are well characterized, in an effort to search for proteins that interact with different members of SRC family to define differential biological activities of these proteins, we utilized the N-terminal fragments of the SRC proteins, which include the bHLH-PAS domain, to screen for new potential proteins that might be differentially associated with members of the SRC family. GAL4-based yeast two-hybrid system 3 from Clontech was used with yeast GAL4 BD fusion constructs of pGBKT7-SRC-1-N, pGBKT7-GRIP1-N, and pGBKT7-AIB1-N to screen a human breast cDNA library (Clontech) in the yeast

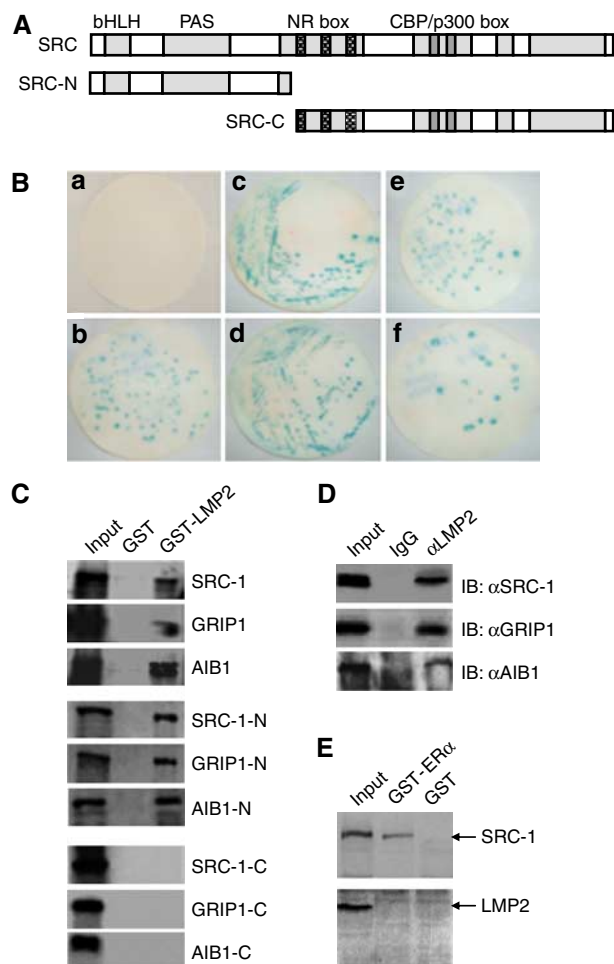


Figure 1 Identification of LMP2 as an SRC-interacting protein. (A) Schematic representation of SRC proteins and their domains and deletions. (B) Yeast two-hybrid screening. (a) Negative control: cotransformation of yeast AH109 cells with pGBKT7-Lam and GADT7-T plasmids; (b) positive control: cotransformation of yeast AH109 cells with pGBKT7-53 and GADT7-T plasmids; (c) and (d) positive clones from the primary selection of mammary library using pGBKT7-SRC-1-N or pGBKT7-GRIP1-N, respectively; (e) and (f) confirmation of the interaction between LMP2 and SRC-1 or between LMP2 and GRIP1 by cotransformation of the yeast AH109 cells with isolated pACT2-LMP2 and pGBKT7-SRC-1-N or pGBKT7-GRIP1-N, respectively. (C) GST pull-down assays with ^{35}S -labeled full-length SRC coactivators or their C- or N-terminal mutants and GST-LMP2 fusion protein. (D) Co-immunoprecipitation experiments. ECC-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) to 95% confluence and cellular nuclear extracts were prepared and immunoprecipitated with antibodies against LMP2. The immunoprecipitated materials were subjected to Western blotting analysis with antibodies against SRC-1, GRIP1, or AIB1. (E) GST pull-down assays with ^{35}S -labeled full-length LMP2 and GST-ER α fusion protein. SRC-1 is included as a positive control.

strain AH109. After four-round screening of 1×10^6 clones with each of the three BD fusion proteins, positive results were found for LMP2 with both SRC-1-N and GRIP1-N BD fusion proteins (Figure 1B). To confirm the interaction of LMP2 with SRC-1 or GRIP1, LMP2 cDNA clone was rescued from the library and re-transformed into yeast cells together with pGBKT7-SRC1-N or pGBKT7-GRIP1-N constructs. The back hybridization experiments confirmed the interaction between LMP2 and SRC-1 or GRIP1 (Figure 1B). In addition,

similar experiments in yeast cotransformed with LMP2-AD and pGBKT7-AIB1-N BD plasmids indicated that LMP2 is also capable of interacting with AIB1.

In vitro and in vivo interaction of LMP2 with SRC proteins

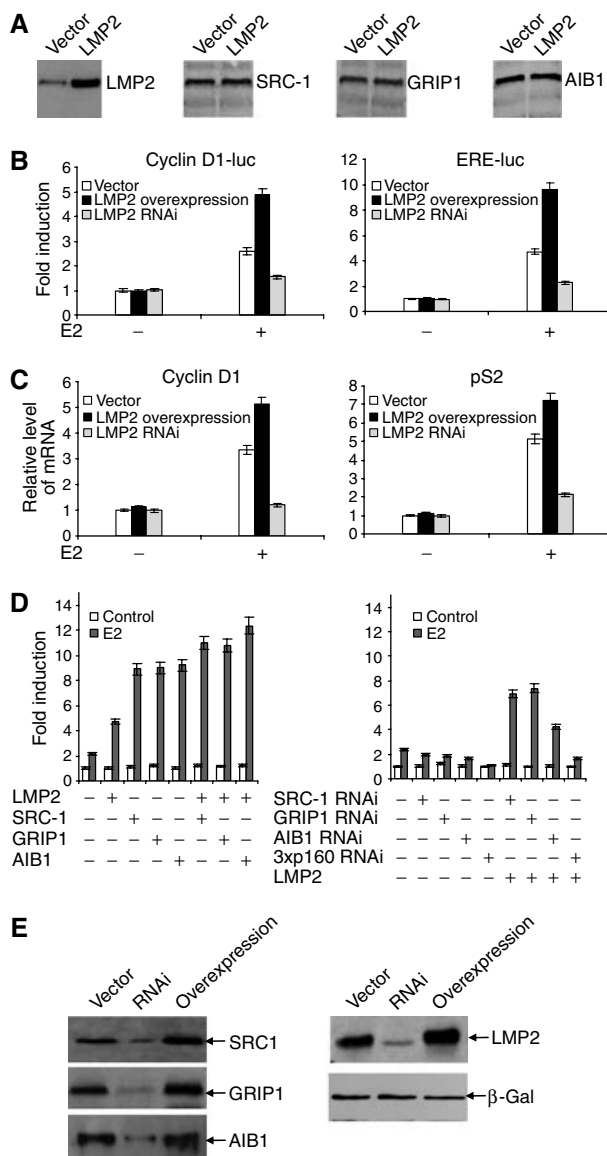
To further validate the physical interaction between LMP2 and the SRC proteins, we performed GST pull-down assays with GST-fused LMP2 and *in vitro*-transcribed/translated and ³⁵S-labeled SRC proteins. The results of the experiment indicated that LMP2 directly interacted with full-length SRC-1, GRIP1, and AIB1 (Figure 1C). GST pull-down assays were also performed separately with the N- and C-terminal fragments of the SRC proteins. The results from these assays showed that interaction of LMP2 was detected only with the N-terminal fragments of the SRC proteins (Figure 1C), further validating the data obtained from yeast two-hybrid screening. In addition, co-immunoprecipitation assays with cellular extracts from ECC-1 endometrial carcinoma cells also revealed the existence of an LMP2/SRC protein complex *in vivo* (Figure 1D). GST pull-down assays were also performed with GST-fused ER α and radiolabeled LMP2, and the results indicated that LMP2 does not interact directly with ER α (Figure 1E).

LMP2 enhances ER-regulated gene transcription

LMP2 is a component of the 20S proteasome complex. The observation that LMP2 interacted with SRC proteins may simply be a reflection of a mechanism by which SRC proteins undergo ubiquitin-mediated degradation. However, LMP2 overexpression was not linked to any significant changes in the expression level of the SRC proteins (Figure 2A). Alternatively, the interaction of LMP2 with the SRC transcrip-

tional coactivators would suggest that LMP2 may be involved in the regulation of gene transcription. In order to investigate this possibility, we transfected ECC-1 cells with an LMP2 expression construct or an siRNA construct to knock down the expression of LMP2 together with a luciferase reporter whose expression is driven by either estrogen responsive elements (EREs) or by the promoter of an estrogen target gene, cyclin D1, and the reporter activity was measured with or without 17 β -estradiol (E2) stimulation. The results of these experiments indicated that LMP2 overexpression resulted in a significant enhancement of E2-dependent activation of ER target genes, and that knockdown of the expression of LMP2 by RNA interference (RNAi) led to a significant attenuation of the reporter gene activity (Figure 2B). Similar results were also obtained in mammary carcinoma cell line, MCF-7 (Supplementary Figure 1). Furthermore, analysis by real-time reverse transcription-PCR (RT-PCR) of the endogenous expression of cyclin D1 and pS2 indicated that the expression level of endogenous cyclin D1 and pS2 was also elevated with overexpression of LMP2 and decreased with LMP2 knock-

Figure 2 LMP2 enhances ER-mediated transcription. (A) LMP2 overexpression did not result in any significant changes in SRC protein expression. ECC-1 cells were transfected with an empty vector or an LMP2 expression vector. Seventy-two hours after transfection, cellular extracts were prepared and Western blotting analysis was carried out to analyze the expression of SRC-1, GRIP1, and AIB1. (B) LMP2 enhanced ER-mediated transcription. ECC-1 cells were transfected with cyclin D1 promoter-driven luciferase construct (cyclin D1-luc) or ERE-tk-luciferase (ERE-luc) reporter plasmid together with pcDNA3.1-LMP2 or pSUPER-LMP2-siRNA constructs. Forty-eight hours after transfection, cells were treated with 100 nM of E2 and reporter activity was measured. The firefly luciferase data for each sample were normalized to *Renilla* luciferase activity. Each bar represents the mean \pm s.d. for triplicate experiments. (C) LMP2 enhanced the expression of endogenous ER target genes. The expression of ER target genes cyclin D1 and pS2 was measured by real-time RT-PCR in ECC-1 cells transfected with either LMP2 expression construct or LMP2 siRNA construct. Each bar represents the mean \pm s.d. for triplicate experiments. (D) Collaboration of LMP2 and SRC coactivators in enhancement of ER target transcription. ECC-1 cells were transfected with the ERE-luciferase reporter plasmid together with pcDNA3.1-LMP2 or SRC expression plasmids or pSUPER vector-based RNAi constructs. Forty-eight hours after transfection, cells were treated with 100 nM of E2 and the cells were harvested and firefly luciferase and *Renilla* activities were measured. Each bar represents the mean \pm s.d. for triplicate experiments. 3xp160 RNAi means transfection of a combination of three p160 RNAi constructs for silencing the expression of all SRC-1, GRIP1, and AIB1. (E) Western blotting analysis of the protein expression in ECC-1 cells that were transfected with the indicated plasmids. Transfection efficiency was monitored by co-transfection with the *E. coli lacZ* construct.



down (Figure 2C). These experiments clearly indicated that LMP2 is required for the maximal activity of ER-mediated gene transcription.

In previous studies, we showed that the SRC proteins are both necessary and sufficient for ER-mediated gene transcription (Shang *et al*, 2000) and that SRC proteins, although share a high degree of structural and biochemical similarity, exert differential functions in nuclear receptor-regulated gene transcription (Shang and Brown, 2002; Zhang *et al*, 2004; Wu *et al*, 2005). In order to further investigate the mechanisms underlying the enhancement of ER-mediated gene transcription by LMP2, we next determined whether LMP2 could cooperate with p160 coactivators in gene transcriptional activation. For this purpose, LMP2 was cotransfected into ECC-1 cells with expression constructs or siRNA constructs for each member of the SRC coactivators together with the ERE-luc reporter, and the reporter activity was measured. As shown in Figure 2D, overexpression of LMP2 and a member of SRC proteins together resulted in a greater reporter activity compared with overexpression of LMP2 or a member of SRC proteins alone. On the other hand, in ECC-1 cells that overexpressed LMP2, knockdown of the expression of either member of the p160 coactivators by RNAi led to a significant reduction of the reporter activity, and knockdown of the expression of all three members of the p160 coactivators abolished the reporter activity, even under LMP2 overexpression (Figure 2D, right panel). The overexpression and knockdown of the proteins were analyzed by Western blotting (Figure 2E). Taken together, these data suggest that the recruitment of LMP2 by the p160 family of coactivators is required for the maximal activity of ER-mediated gene transcription.

Recruitment of LMP2 on the entire sequence of transcriptionally active pS2 gene

To further investigate the mechanism by which LMP2 enhances ER-mediated gene transcription, chromatin immunoprecipitation (ChIP) assays were performed to examine the recruitment of LMP2 on the promoter as well as on the coding region of an ER target gene, pS2, under E2 stimulation. Primers that cover the ER binding site on the pS2 promoter and span different genomic regions of the pS2 gene were used in these experiments (Figure 3A). The results indicated that E2 treatment resulted in the recruitment of ER α , SRC-1, AIB1, and GRIP1 on the pS2 promoter (Figure 3B, primer a), as reported previously (Shang *et al*, 2000; Metivier *et al*, 2003; Zhang *et al*, 2004). Interestingly, E2 treatment also led to the recruitment of LMP2 on the pS2 promoter. Moreover, ChIP and ChIP re-immunoprecipitation (Re-IP) experiments demonstrated that ER α , SRC-1, LMP2, and RNA polymerase II (pol II) exist in the same complex on pS2 promoter (Figure 3D). Examination of the coding region of pS2 revealed that LMP2 was present on the entire sequence of the pS2 gene, whereas the ER α and p160 coactivators occupied only the promoter of pS2 gene (Figure 3B). A similar recruitment pattern of these proteins was observed on the promoter and coding sequence of another ER target gene, EBAG9 (data not shown). These data suggest that LMP2 might be recruited on pS2 gene not only during transcription initiation but also during transcription elongation.

The elongation of mRNA transcription by RNA pol II is regulated by various elongation factors such as TFIIs, pTEFb,

and Elongins. There are three members in the Elongin family, Elongin A, B, and C (Shilatifard *et al*, 2003). In order to further investigate the involvement of LMP2 in ER-mediated gene transcription, we next performed ChIP and ChIP Re-IP assays to examine the association of LMP2 with transcription elongation complex on pS2 gene. The results of the experiments indicated that LMP2 coexisted with Elongin A and pol II in the same transcription complex on the coding region of pS2 gene, further supporting the idea that LMP2 is involved in both transcription initiation and elongation (Figure 4).

The recruitment of LMP2 by SRC coactivators is required for cyclic association of ER-mediated transcription complexes on pS2 gene

As shown by us and other investigators, ER-mediated formation of transcriptional complexes on gene promoters is ordered and involves sequential association and dissociation of ER and other cofactors in a cyclic fashion (Shang *et al*, 2000; Metivier *et al*, 2003; Reid *et al*, 2003). These studies also indicated that the cyclic association of the transcription complex on gene promoter is required for, and/or is a reflection of, efficient transcription elongation. In order to further explore the functional connection between SRC coactivators and LMP2 in ER-mediated gene transcription, we next investigated the requirement of SRC coactivators for the recruitment of LMP2 and for subsequent function of LMP2 in transcription elongation as well as the effect of LMP2 recruitment by SRC coactivators on cyclic association of ER-regulated transcription complexes on pS2 gene promoter. For these purposes, ECC-1 cells were transfected with siRNA constructs to knock down the expression of SRC coactivators or LMP2. To rescue the function of LMP2, 2 days after the transfection of cells with an LMP2 siRNA construct, the cells were transfected again with a mouse LMP2 expression plasmid. After all these transfections, the cells were grown for 3 days in estrogen-depleted media followed by treatment with α -amanitin for 2 h to deplete the background activity of the pS2 promoter. This generates a synchronized population of cells in which the pS2 promoters are devoid of *trans*-acting factors and whose local histones are not acetylated (Shang *et al*, 2000; Metivier *et al*, 2003; Reid *et al*, 2003). Following α -amanitin treatment, the cells were washed and treated with E2 and collected at a 5 min interval for chromatin preparation. The occupancy of different protein factors on pS2 gene was then measured by quantitative ChIP. As shown in Figure 5, ER α , SRC-1, LMP2, and pol II were recruited to pS2 gene promoter (primer a) in an ordered and cyclic fashion with a cycling frequency of \sim 45 min (Figure 5A). The association of Elongin A with the coding region of pS2 gene (primer b) was also cyclic. These results agree well with the previous observations (Shang *et al*, 2000; Metivier *et al*, 2003; Reid *et al*, 2003). However, in cells in which the expression of all SRC-1, GRIP1, and AIB1 was silenced, the recruitment of ER α was severely affected and the recruitment of LMP2, pol II, and Elongin A was almost abrogated, so was the cyclic pattern of their association. A similar effect on the recruitment of pol II and Elongin A and their cyclic association with pS2 gene was observed in cells in which LMP2 expression was silenced. These results are consistent with the effect of SRC coactivators and LMP2 on ER-mediated gene transcription, described in Figures 2 and 5C. Interestingly, the recruitment of pol II and Elongin A

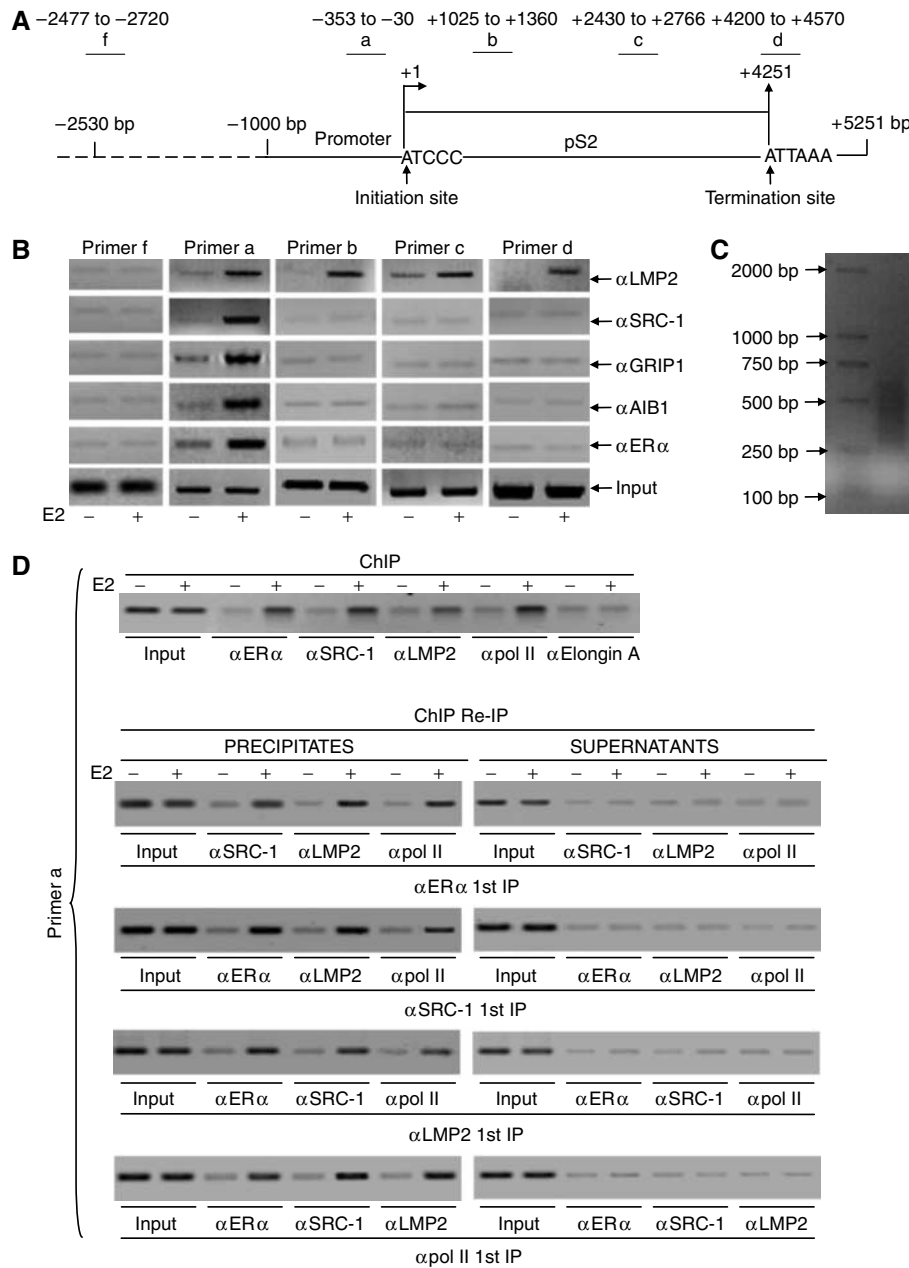


Figure 3 LMP2 is recruited on the entire sequence of pS2 gene. (A) A map of the pS2 gene showing the regions that were amplified by PCR in ChIP experiments. (B) The recruitment of LMP2, ER α , and SRC coactivators on promoter as well as on other regions of pS2 gene. EEC-1 cells were grown in the absence of estrogen for 3 days and treated with E2 for 45 min. The cells were then collected and ChIP experiments were performed with different primer pairs described in Materials and methods. (C) The size range of the DNA fragments used in ChIP experiments. (D) The recruitment of ER α , SRC-1, LMP2, Elongin A, and pol II on pS2 gene promoter. EEC-1 cells were grown in the absence of estrogen for 3 days and treated with E2 for 45 min. The cells were then collected and ChIP and ChIP Re-IP experiments were performed with primer a described in Materials and methods.

and their cyclic association with pS2 gene could be rescued by the expression of mouse LMP2 in the presence of the expression of SRC coactivators, whereas in cells in which the expression of SRC coactivators was silenced, the expression of mouse LMP2 could not restore the recruitment of pol II and Elongin A and their cyclic association with pS2 gene. The protein expression under these experimental conditions was analyzed by Western blotting (Figure 5B). The restoration of pol II and Elongin A recruitment on pS2 gene by mouse LMP2 expression was functionally significant as indicated by reporter assays under these experimental conditions

(Figure 5C). These experiments strongly support the requirement of SRC recruitment of LMP2 in ER-mediated gene transcription, implicating SRC coactivators in transcription elongation.

Functional association of LMP2 with proteasome in gene transcription

As LMP2 is a subunit of proteasome, we wished to know if its transcriptional activation activity of ER target genes was a function of LMP2 itself or through the proteasome complex. For this purpose, we cotransfected ECC-1 cells with the ERE-

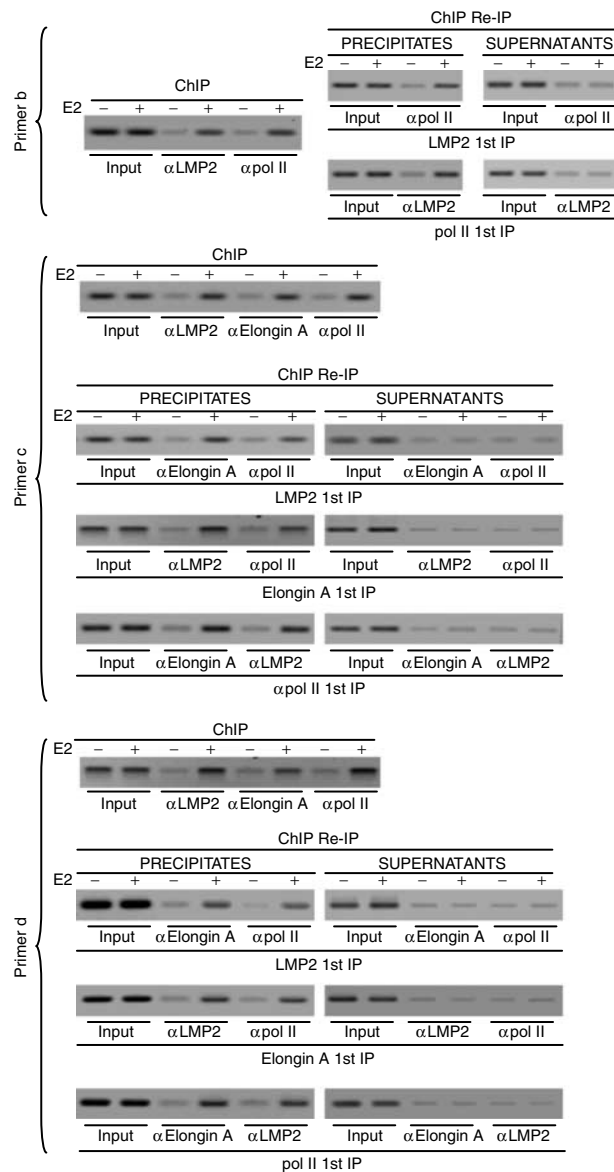


Figure 4 The recruitment of LMP2, Elongin A, and pol II on pS2 gene. EEC-1 cells were grown in the absence of estrogen for 3 days and treated with E2 for 45 min. The cells were then collected and ChIP and ChIP Re-IP experiments were performed with different primer pairs described in Materials and methods.

luc and the LMP2 expression vector, and the cells were then treated with E2 in the presence or absence of proteasome inhibitor MG132. As shown in Figure 6A, MG132 treatment almost totally abolished the enhancement effect of LMP2 on E2-induced transcription, suggesting that the function of LMP2 in gene activation was linked to proteasome activity.

In order to further support the argument that the involvement of LMP2 in gene transcription is associated with the proteasome complex, we next performed ChIP experiments to examine the recruitment of SUG1/Rpt6, a subunit of the 19S regulatory particle, along with the recruitment of LMP2 on pS2 gene. ChIP results showed that SUG1/Rpt6 along with pol II was also recruited on the entire sequence of pS2 gene upon E2 stimulation. On the other hand, knockdown of the expression of LMP2 resulted in not only undetected LMP2 recruitment on pS2 gene, but also diminished recruitment of SUG1/

Rpt6 and pol II. Collectively, these data suggest that LMP2-associated proteasome complex is engaged in both initiation and elongation of ER-mediated gene transcription.

LMP2 promotes estrogen-stimulated cell cycle progression

Although LMP2 is not a new gene, in the light of the observation that it enhanced ER-mediated gene transcription, it is important to investigate LMP2's physiological relevance in the context of estrogen actions. Thus, we next examined the effect of LMP2 on estrogen-stimulated cell cycle progression. In these experiments, ECC-1 cells with LMP2 overexpression or LMP2 knockdown were grown in the absence of estrogen for 3 days and then left untreated or treated with E2 for 12 h. The cells were then collected and cell cycle profile was analyzed by cell flow cytometry. As shown in Figure 7, under estrogen deprivation, ECC-1 cells were arrested in G₀/G₁ phase, and E2 treatment resulted in G₁-S transition of the ECC-1 cells. The proliferation was enhanced for ECC-1 cells overexpressing LMP2, whereas in ECC-1 cells with a silenced expression of LMP2, the cell proliferation was significantly inhibited. These experiments indicate that LMP2 could indeed influence the estrogen actions.

Discussion

Although the ubiquitin-proteasome system was initially perceived as a graveyard for proteins, recent advances in molecular biology have redefined its role as a regulatory system that influences the fate of many cellular processes, such as cell proliferation, apoptosis, and gene transcription (Muratani and Tansey, 2003; Nawaz and O'Malley, 2004; Baker and Grant, 2005).

The nuclear receptor superfamily describes a diverse array of transcription activators functioning in a ligand-dependent manner and exerting actions by regulating the expression of specific subsets of genes. However, it emerged recently that proteasomal degradation is inextricably linked to nuclear receptor-mediated gene transcription, and the mechanistic insight into the interdependence between proteasomal degradation and nuclear receptor-mediated gene transcription has been intensively investigated (Lonard *et al*, 2000; Reid *et al*, 2003). In the current study, we identified the proteasome β subunit LMP2 that interacts with the N-terminus of the SRC proteins, a family of coactivators that are both necessary and sufficient to initiate ER-mediated gene transcription (Shang *et al*, 2000). We demonstrated that overexpression of LMP2 resulted in enhanced transcription activation of ER target genes and that knockdown of the expression of LMP2 attenuated ER-mediated gene transcription, indicating that LMP2 functions as a positive transcriptional cofactor for ER-mediated gene transcription. In support of this, we detected an estrogen-dependent LMP2 recruitment on the promoter of pS2 gene together with ER α , SRC-1, AIB1, and GRIP1. Surprisingly, however, the recruitment of LMP2 was detected in the entire sequence of the pS2 gene, whereas the recruitment of ER α , SRC-1, AIB1, and GRIP1 was detected only on the pS2 promoter, suggesting that LMP2 participates in both initiation and elongation of pS2 transcription. In supporting the argument that LMP2 also participates in pS2 elongation, we demonstrated that LMP2 existed with pol II and Elongin A in the same complex on the sequence of pS2 gene.

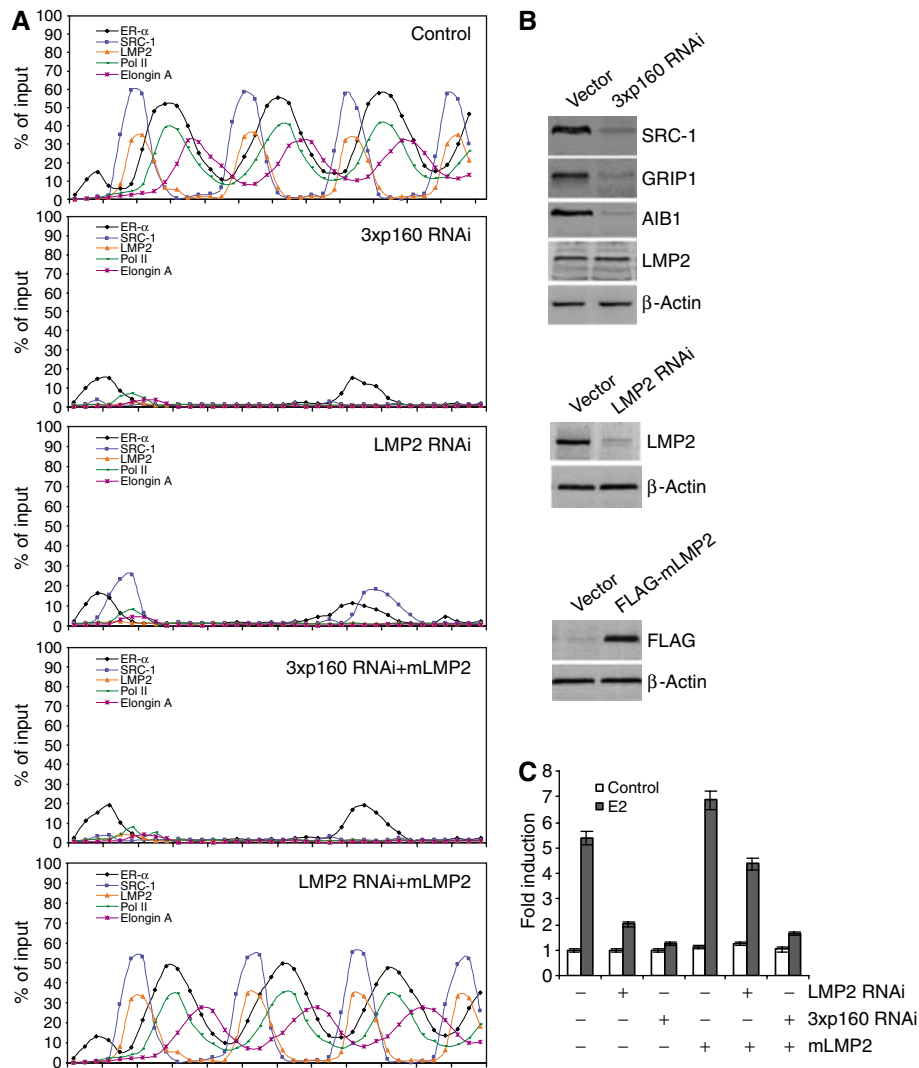


Figure 5 The dynamic recruitment of ER α , SRC-1, LMP2, Elongin A, and pol II on pS2 gene. **(A)** EEC-1 cells were transfected with RNAi constructs to silence the expression of SRC-1, GRIP1, and AIB1 (3xp160 RNAi) or the expression of LMP2. Forty-eight hours after transfection, the cells were transfected again with a mouse LMP2 (mLMP2) expression construct. The cells then were switched to estrogen-depleted media for 3 days and treated with E2 for different periods of time. The cells were then collected at a 5-min interval and quantitative ChIP PCR was performed with primer a (for ER α , SRC-1, LMP2, and pol II) or primer b (for Elongin A). **(B)** Western blotting analysis of the protein expression in ECC-1 cells treated as above. **(C)** ECC-1 cells treated as above were transfected with ERE-luc reporter. After addition of E2 for 12 h, the cells were harvested and reporter activity was measured. The firefly luciferase data for each sample were normalized to *Renilla* luciferase activity. Each bar represents the mean \pm s.d. for triplicate experiments.

Previously, several laboratories have also shown that components of the ubiquitin-proteasome system act as nuclear receptor coactivators. For example, ubiquitin-conjugating enzymes (UbcH7) (Verma *et al*, 2004) and ubiquitin ligases (E6-AP) (Nawaz *et al*, 1999b) have been shown to interact with nuclear receptors and their coactivators and are implicated in nuclear receptor-dependent transcription. In our experiments, we showed that the interaction with SRC proteins, the recruitment on ER target gene, and the transcriptional activation activity of LMP2 are not a function of LMP2 itself but a function of its associated proteasome. In supporting this concept, we demonstrated that the SUG1/RPT6, a 19S proteasome regulatory subunit, was also recruited to pS2 gene sequence with a similar pattern of LMP2 recruitment. Therefore, it appears that proteasome complex is recruited to ER-mediated transcription initiation complex via an interaction between LMP2 and the SRC

proteins on target gene promoter. After disassembly of the initiation complex and commence of transcription elongation, the proteasome complex was in association with the transcription elongation complex. Our data indicated the requirement of SRC recruitment of LMP2 in the cyclic association of the transcription complex on ER target gene and in ER-mediated gene transcription, thus implicating SRC coactivators in transcription elongation.

It has been proposed that degradation by the ubiquitin-proteasome pathway might provide an efficient mechanism for regulating the cyclic interaction of nuclear receptor with the promoter (Shang *et al*, 2000; Metivier *et al*, 2003; Reid *et al*, 2003). Alternatively, the ubiquitin-proteasome pathway might serve to clear out corepressors and/or coactivators so that other coregulators can subsequently bind (Dennis and O'Malley, 2005). Moreover, it has been demonstrated that TBL1 and TBLR1, which are components of E3 ubiquitin

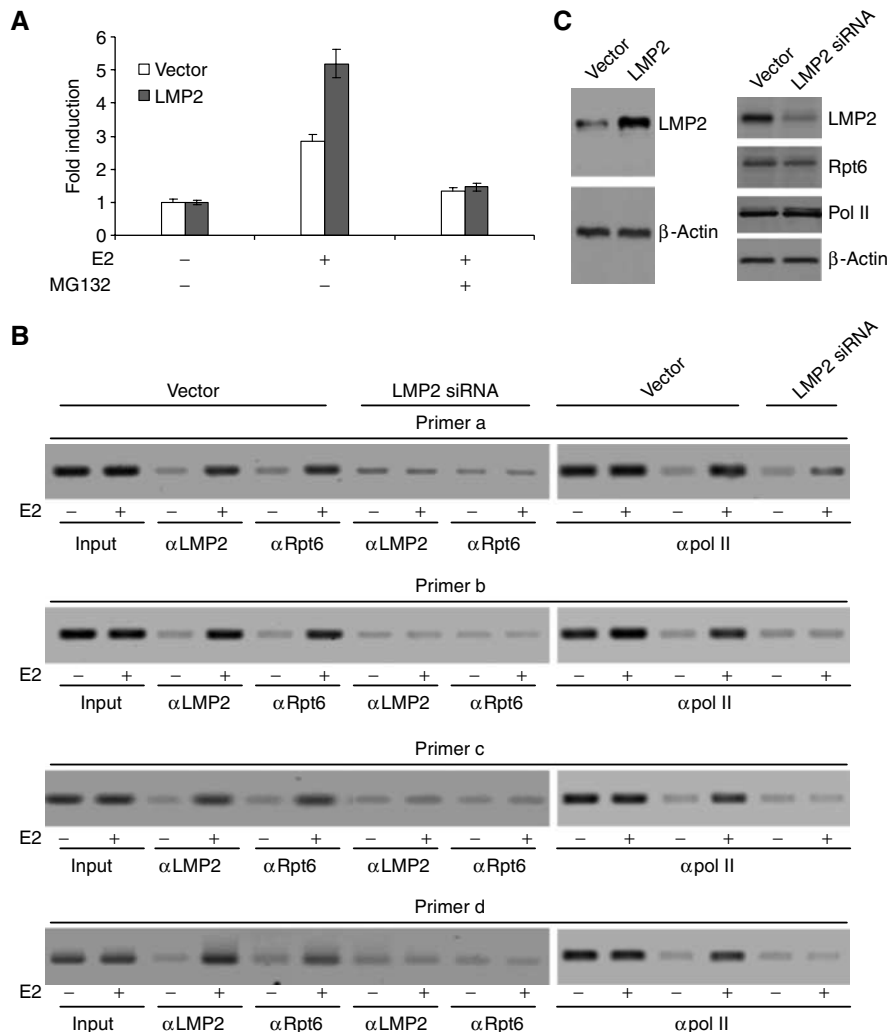


Figure 6 The association of LMP2 with proteasome on pS2 gene. **(A)** Inhibition of ER-mediated transactivation by proteasome inhibitor MG132 in ECC-1 cells. ECC-1 cells were seeded in DMEM medium supplemented with 10% charcoal-dextran-stripped FBS and transfected with either vector or pcDNA3.1-LMP2 together with the reporter construct ERE-luc. Forty-eight hours after transfection, cells were treated with E2 in the absence or presence of proteasome inhibitor MG132 for 12 h and luciferase activities were assayed. Each bar represents the mean \pm s.d. for triplicate experiments. **(B)** Co-recruitment of LMP2 with Rpt6 and pol II on pS2 gene. ECC-1 cells were grown in phenol red-free DMEM medium supplemented with 10% charcoal-dextran-stripped FBS and transfected with the LMP2 siRNA construct. Forty-eight hours after transfection, the cells were left untreated or treated with 100 nM of E2 for 45 min. CHIP assays were performed using specific antibodies against Rpt6, pol II, and LMP2 and with primers for different regions of the pS2 gene. **(C)** Western blotting analysis of the expression of LMP2, Rpt6, and pol II.

ligase complexes, function as adaptor molecules for the recruitment of the proteasome and the degradation of the N-CoR/SMRT corepressors (Perissi *et al*, 2004). Such a process could facilitate the exchange of corepressor for coactivator complexes. Proteasomal degradation of nuclear receptors and their coregulators could also promote disassembly of the initiation complex, facilitating the transition to a productive elongation complex (Arndt and Winston, 2005; Muratani *et al*, 2005), and proteasome involvement in transcription elongation, in turn, could sustain an efficient elongation process as well as other related transcription events including 5'-capping, polyadenylation, and pre-mRNA splicing. Our experiments provided evidence to support this concept.

Finally, there is growing evidence for a pathological connection between the ubiquitin-proteasome pathway and gene transcription regulation. Our experiments indicated that LMP2 is able to promote estrogen-stimulated cell cycle pro-

gression. It is conceivable that the level of LMP2 in a particular tissue could influence the enrichment and/or the activity of specific proteins that are critical for the proliferation and even malignant transformation of the cell. Future studies are needed to investigate this hypothesis.

Materials and methods

Plasmids and protocol for yeast two-hybrid screening

The N-terminal fragments of SRC proteins were fused to BD plasmid, which contains SRC-1 N-terminal fragment (1-1896 nt) (PCR primers: forward, 5'-gggaattccatatgatgagtggtgctcgaggacagttc-3', and reverse, 5'-acgcgtcgactgtctccatctgattgtctgtc-3'); GRIP1 N-terminal fragment (1-1917 nt) (PCR primers: forward, 5'-ggaattccatgatggatgggagaaaatcctc-3', and reverse, 5'-acgcgtcgactgtctcatgcagctgtctctg-3'); or AIB1 N-terminal fragment (1-1955 nt) (PCR primers: forward, 5'-gggaattccatatgatgagtggtgattaggagaaaacttg-3', and reverse, 5'-acgcgtcgacctcaactgtctctcttacttc-3'). The resultant plasmids were named pGBKT7-SRC1-N, pGBKT7-GRIP1-N, or pGBKT7-AIB1-N.

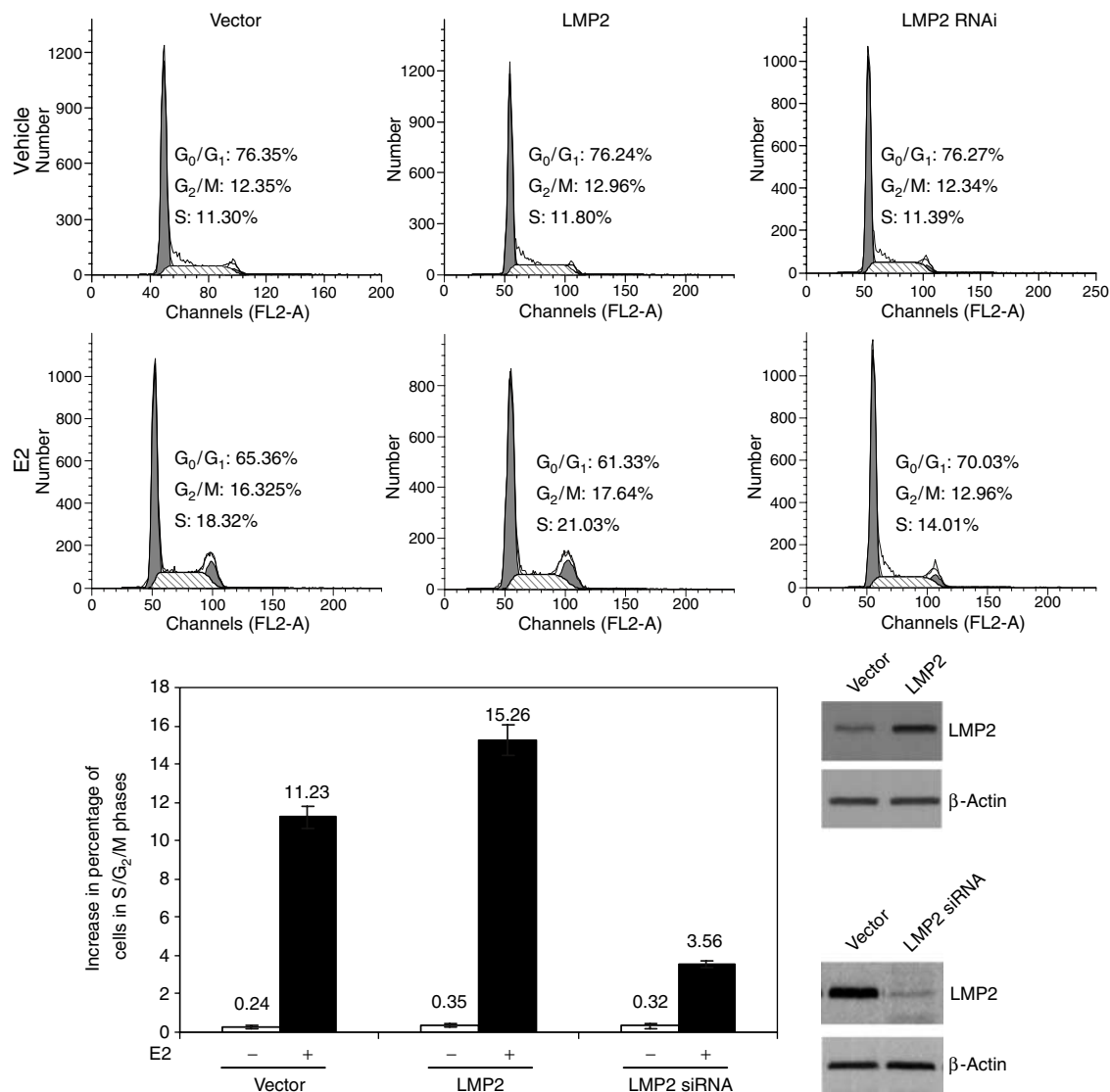


Figure 7 LMP2 promotes estrogen-stimulated cell cycle progression. ECC-1 cells were cotransfected with GFP and LMP2 expression constructs or with GFP and LMP2 RNAi constructs and were grown in phenol red-free DMEM supplemented with 10% charcoal-dextran-stripped FBS for 3 days before treatment with 100 nM of E2 for another 12 h. Cells were then collected for cell flow cytometry analysis. The expression of LMP2 was analyzed by Western blotting and is shown in the lower right.

Human mammary gland library (Clontech, Palo Alto, CA) was screened for proteins that interacted with the N-terminal region of the p160 family by using the Matchmaker GAL4 Two-hybrid System 3 (Clontech protocol PT3247-1). The bait plasmids pGBKT7-SRC1-N, pGBKT7-GRIP1-N, and pGBKT7-AIB1-N were transformed into yeast strain AH109 and about 2×10^3 transformants were screened. The transformants were assayed for MEL1 activation by selecting on the high-stringency medium plates: SD/-Ade/-His/-Leu/-Trp/X- α -gal. Yeast colonies were assayed for β -galactosidase activity using colony-lift filter as follows: colonies were transferred to 3 MM filter papers, permeabilized by brief immersion in liquid nitrogen, and incubated on a filter paper saturated with Z-buffer containing 1 mg/ml X-GAL at 30°C for 0.5–8 h. AD plasmids were recovered from positive yeast clones and re-transformed into native AH109 yeast with the bait construct to re-test these interactions, and finally confirmed by DNA sequencing.

The FLAG-tagged LMP2 coding region was obtained from the human mammary cDNA library (Clontech) by PCR with primers 5'-ataagaatgcggccgccaccatgattacaaagatgacgacgataagatctgcccggcggaagaatccac-3' (forward) and 5'-ggaattctcatcactcatcagattttggcag-3' (reverse) and cloned into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) to generate plasmid pcDNA3.1-FLAG-LMP2. For GST fusion protein construction, the entire coding region of the LMP2 was amplified and subcloned into the pGEX-4T-3 vector (Amersham Biosciences, Piscataway, NJ).

Cell culture, transfection, and luciferase reporter assay

ECC-1 cells were maintained in DMEM supplemented with 10% FBS. Transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Luciferase activity was measured using a dual luciferase kit (Promega, Madison, WI) according to the manufacturer's protocol. The firefly luciferase intensity for each sample was normalized based on transfection efficiency measured by *Renilla* luciferase activity.

GST pull-down assays

GST-LMP2 fusion constructs were expressed in BL21 *Escherichia coli* cells, and crude bacterial lysates were prepared by sonication in TEDGN (50 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 1 mM dithiothreitol (DTT), 10% (vol/vol) glycerol, 0.4 M NaCl) in the presence of the Complete[®] protease inhibitor mixture (Roche Molecular Biochemicals, Germany). *In vitro* transcription and translation experiments were performed with rabbit reticulocyte lysate (TNT systems, Promega) and L-methionine (Amersham Biosciences) according to the manufacturer's recommendations. In GST pull-down assays, about 10 μ g of the appropriate GST and GST fusion proteins was mixed with 5–8 μ l of the *in vitro*-transcribed/translated products and incubated in binding buffer (75 mM NaCl, 50 mM HEPES, pH 7.9) at room temperature (RT) for 30 min. The binding

reaction was then added to 30 μ l of glutathione-Sepharose beads and mixed at 4°C for 2 h. The beads were washed three times with binding buffer, boiled in 30 μ l of 2 \times SDS-PAGE loading buffer, and resolved on 8–10% gels. The gels were then fixed in 50% methanol and 10% acetic acid for 30 min and dried. Protein bands were detected by autoradiography at –80°C for 4–16 h.

Immunoprecipitation and Western blotting

ECC-1 cell lysates were prepared by incubating cells in the lysis buffer (20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5% glycerol) for 20 min at 4°C, followed by centrifugation at 14 000 *g* for 15 min at 4°C. For immunoprecipitation, 500 μ g of protein was incubated with specific antibodies (1–2 μ g) for 2 h at 4°C with constant rotation; 70 μ l of 50% protein A and G agarose bead mixtures was then added, and the incubation was continued for an additional 2 h. Beads were then washed three times using the lysis buffer. Between washes, beads were collected by centrifugation at 3000 *g* for 30 s at 4°C. The precipitated proteins were eluted from beads by resuspending the beads in 2 \times SDS-PAGE loading buffer and heating at 75°C for 10 min. The resultant materials from immunoprecipitation or cell lysates were resolved using 6 or 12% SDS-PAGE and transferred onto nitrocellulose membranes. For Western blot analysis, membranes were incubated with appropriate antibodies for 1 h at RT or overnight at 4°C followed by incubation with a secondary antibody. Immunoreactive bands were visualized using ECL Plus reagents according to the manufacturer's recommendation (Amersham Biosciences).

Vector-based RNAi

Vectors used for RNAi were constructed by inserting a synthesized 64-mer oligonucleotide containing a specific sequence for LMP2 mRNA into pSUPER vector (Brummelkamp *et al*, 2002). The synthesized oligonucleotide sequences were 5'-gatccccctggagaaacctccattgttcaagagacaagtggaggctcctccagttttggaaa-3' (forward) and 5'-gatccccctgcagttatacacagattcaagagatactgtataaactgcagttttggaaa-3' (reverse). The sequences used for silencing the expression of SRC coactivators have been described elsewhere (Zhang *et al*, 2004). The oligos were resuspended in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM acetate) and heated to 95°C for 4 min, 70°C for 10 min, and then cooled to room temperature to generate double-stranded DNA. The double-stranded DNA was then phosphorylated and cloned into the *Bgl*III/*Hind*III-digested pSUPER vector. The vector was then transfected into cells with the Lipofectamine 2000 Reagent (Invitrogen). Transfection efficiency was monitored by cotransfection with an *E. coli lacZ* construct.

Real-time quantitative PCR

The ABI PRIZM 7700 Sequence Detector and the TaqMan EZ RT-PCR Kit (Applied Biosystem, Foster City, CA) were used to quantitate gene expression with the expression of GAPDH as an internal control. The primers and probes used were as follows:

cyclin D1 forward primer: caccgcagacctctggt; *cyclin D1* reverse primer: gcggattggaaatgaactca; and *cyclin D1* probe: 6FAM-cctctgtgcacagat-TAMRA. *pS2* forward primer: aatggccaccatggagaa; *pS2* reverse primer: cgaggccagcatgga; and *pS2* probe: 6FAM-tgcgcccctggtcc-TAMRA.

ChIP/ChIP Re-IP

ChIP experiments were performed according to the procedure described previously (Shang *et al*, 2000; Zhang *et al*, 2004). The following primer pairs were used for *pS2* gene sequence: primer a (–353 to –30), 5'-ggccatctcactatgaatcactctg-3' (forward) and 5'-ggcaggctctgtttgcttaagagcg-3' (reverse); b (+1025 to +1360), 5'-ctccagccactcttgatc-3' (forward) and 5'-caaagacgctctgagcctt-3' (reverse); c (+2430 to +2766), 5'-gagatggagcttctctg-3' (forward) and 5'-caggtagagagaccagaa-3' (reverse); d (+4200 to +4570), 5'-gctcacaacacagattgactg-3' (forward) and 5'-caagatgctcttggactg-3' (reverse); and f (–2477 to –2720), 5'-ggaaatcattataaatgatcc-3' (forward) and 5'-gcacagtggctcacacttgaatc-3' (reverse). For ChIP Re-IP assays, protein-DNA complexes were eluted from primary immunoprecipitation by incubation with 10 mM DTT at 37°C for 30 min and diluted 1:50 in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) followed by Re-IP with second antibodies. ChIP Re-IPs of supernatants were performed essentially as for the primary IPs. Quantitative ChIP was performed using SybrGreen (Molecular Probes, Eugene, Oregon) as a marker for DNA amplification on the ABI PRIZM 7700 Sequence Detector.

Flow cytometry

ECC-1 cells were cotransfected with GFP and LMP2 expression constructs or with GFP and LMP2 siRNA constructs and were grown in phenol red-free DMEM supplemented with 10% charcoal-dextran-stripped FBS for 3 days. The cells were then treated with 100 nM of E2 for another 12 h and collected and resuspended in PBS with 2% glucose and 3% paraformaldehyde. After permeabilization with ethanol, cells were stained with propidium iodide solution (69 μ M propidium iodide, 38 mM sodium citrate). Cell cycle data were collected with FACScan (Becton Dickinson Immunocytometry System) and analyzed with ModFit LT (Verity Software House Inc., Topsham, ME).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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