Cell

A Nuclear Function of β-Arrestin1 in GPCR Signaling: Regulation of Histone Acetylation and Gene Transcription

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SUMMARY

Chromatin modification is considered to be a fundamental mechanism of regulating gene expression to generate coordinated responses to environmental changes, however, whether it could be directly regulated by signals mediated by G protein-coupled receptors (GPCRs), the largest surface receptor family, is not known. Here, we show that stimulation of delta-opioid receptor, a member of the GPCR family, induces nuclear translocation of β-arrestin 1 (βarr1), which was previously known as a cytosolic regulator and scaffold of GPCR signaling. In response to receptor activation, βarr1 translocates to the nucleus and is selectively enriched at specific promoters such as that of *p27* and *c-fos*, where it facilitates the recruitment of histone acetyltransferase p300, resulting in enhanced local histone H4 acetylation and transcription of these genes. Our results reveal a novel function of βarr1 as a cytoplasm-nucleus messenger in GPCR signaling and elucidate an epigenetic mechanism for direct GPCR signaling from cell membrane to the nucleus through signal-dependent histone modification.

INTRODUCTION

Beta-arrestins (βarrs, consisting of βarr1 and βarr2) are cytosolic proteins. Activation of G protein-coupled receptors (GPCRs), the largest family of cell-surface receptors, induces the translocation of βarrs from the cytoplasm to cell membrane and the interaction of βarrs with the activated recep-

tor, and hence results in receptor endocytosis and attenuation of receptor signaling (Claing et al., 2002). βarrs were initially known merely as negative regulators of GPCRs, but new roles of βarrs in receptor trafficking and signaling have been discovered recently (Lefkowitz and Whalen, 2004). βarrs also serve as scaffolds and adapters in receptor endocytosis and signal transduction. They recruit endocytic proteins including AP-2, clathrin, ARF6, and NSF to the receptors and associate with various signaling molecules, connecting the receptors to various cytoplasmic effector pathways such as MAPK cascades (Shenoy and Lefkowitz, 2003). Recent studies from Lefkowitz and our laboratories demonstrate that βarrs bind to IκBα in the cytoplasm in an agonist-dependent manner and regulate NF-κB signaling (Gao et al., 2004; Witherow et al., 2004). These studies reveal important roles of βarrs acting as key scaffold proteins to guide the receptor signals from cell membrane to various target cascades and thus to different destinations in cell.

Receptor-mediated extracellular signals are transmitted through the cytoplasm to the nucleus by a complicated signaling network through a series of protein-protein interactions and protein kinase cascades. Agonist-stimulated receptor phosphorylation and internalization have been long thought to be solely a negative feedback regulatory mechanism of this process. However, recent evidence suggests that these receptor-activation-dependent signal regulatory processes such as endocytosis may also be directly involved in the signaling functions and serve as an important pathway to transmit signals from the cell membrane to the cytoplasm and the nucleus (Benmerah, 2004; Shi and Massague, 2003). A very recent work of Miaczanska and colleagues has shown that endocytic protein Rab5 interacts with APPL proteins shuttling between the cytoplasm and the nucleus. Upon activation of Rab5 by extracellular stimuli, APPL1 translocates from the membranes to the nucleus where it interacts with the nucleosome remodeling and histone deacetylase multiprotein complex NuRD/MeCP1, an established regulator of chromatin structure and gene expression (Miaczynska et al., 2004). Previous studies from Benmerah and our groups reveal that βarrs, mediators of endocytosis of seven membrane-spanning receptors, are also able to shuttle between
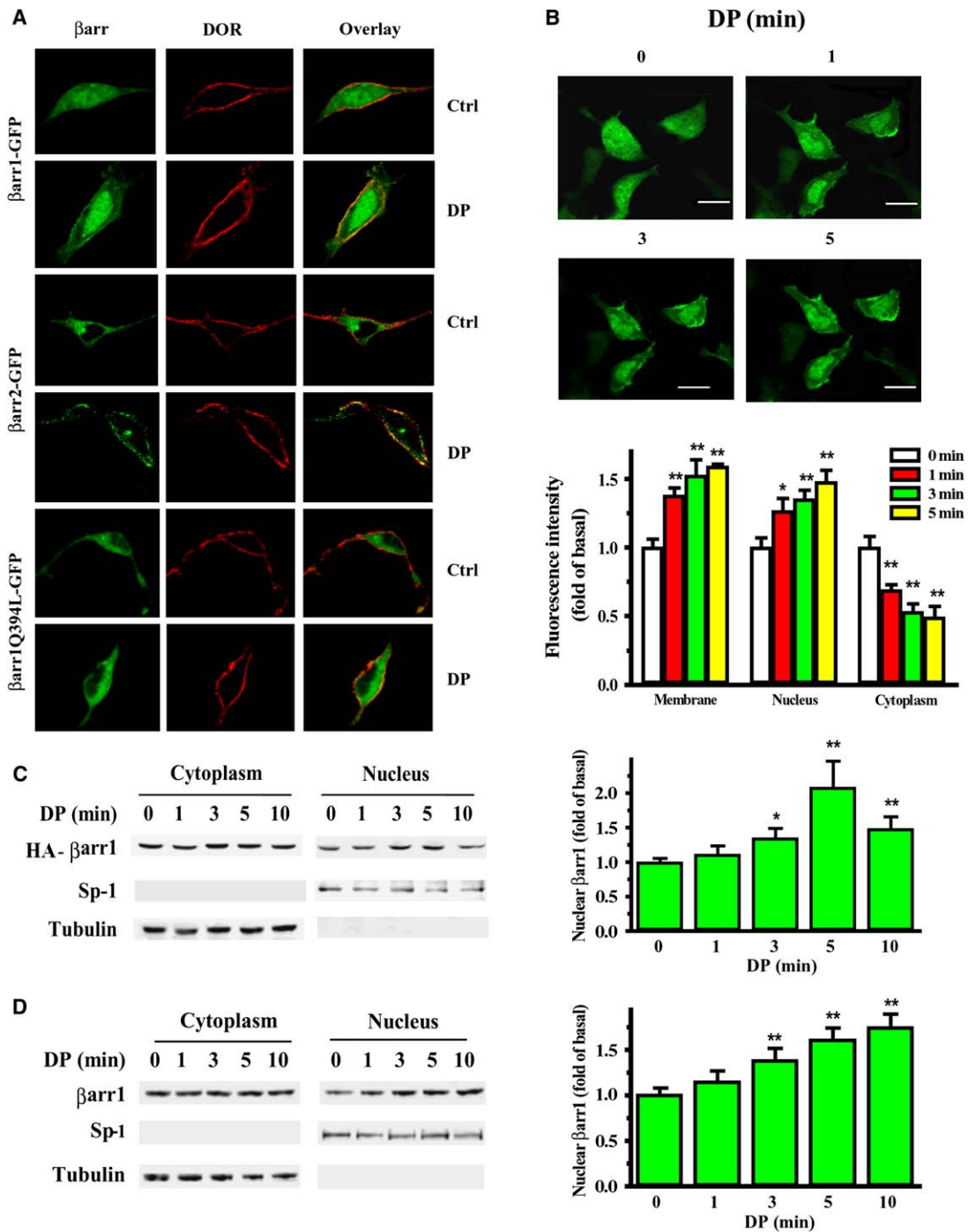
Figure 1. Activation of DOR Induces Translocation of βarr1 to the Nucleus

(A) Confocal visualization of HA-DOR and βarr1-GFP, βarr2-GFP or βarr1Q394L-GFP in HEK293 cells incubated without (Ctrl) or with 1 μM DPDPE (DP) for 5 min before fixation.

(B) Confocal real-time visualization of βarr1-GFP distribution in living HEK293 cells incubated with 1 μM DP. The pictures shown are representative of five independent experiments. The scale bars represent 20 μm. Values are expressed as the mean ± SD, *p < 0.05, **p < 0.01 versus the 0 min group.

(C and D) HEK293 cells (C) expressing exogenous HA-βarr1 and DOR and HeLa cells (D) expressing endogenous βarr1 and exogenous DOR were incubated with 1 μM DP for different intervals and βarr1 content in nuclear extracts was analyzed by Western blotting and infrared fluorescence imaging. The tubulin and Sp-1 were also detected to show, if any, crosscontamination in the cytosolic and nuclear fractions. The bands of βarr1 in nuclear fractions were quantified and normalized to Sp1, and the data shown are the means ± SD of three independent experiments, *p < 0.05, **p < 0.01 versus the 0 min group.

the cytoplasm and the nucleus, and βarr1 is present in both the nucleus and the cytoplasm at steady state (Scott et al., 2002; Wang et al., 2003b). These observations suggest that βarr may have an important, yet unknown function in the nucleus, most likely, to regulate gene transcription through a novel mechanism.

GPCRs transduce a diverse array of signals to the interior of cells via cascades that lead to appropriate gene and cellular responses to the environmental changes. Growing evidence suggests that epigenetic alteration of chromatin may serve as a fundamental mechanism of regulating gene expression to integrate environmental signals and generate a coordinated transcriptional response (Levenson and Sweatt, 2005). However, whether epigenetic events could be directly regulated by GPCR-mediated signal transduction remains unknown.

The current study explored the effects of activation of GPCRs on nuclear distribution of βarr1 and reported that stimulation of delta-opioid receptor (DOR), a member of GPCR family, induces nuclear translocation of βarr1 and βarr-dependent histone H4 acetylation. We further demonstrated that nuclear translocation of βarr1 leads to its accumulation and H4 hyperacetylation at the *p27* and *c-fos* promoter regions, stimulating transcription of these genes. These results highlight an epigenetic mechanism for GPCR signaling from the cell membrane to the nucleus through signal-dependent histone modification and a novel function of βarr in the nucleus as a GPCR cytoplasmic-nuclear messenger to control transcription of the target genes.

RESULTS

DOR Activation Induces Translocation of βarr1 to the Nucleus

HEK293 cells transiently expressing DOR and βarr-GFP were challenged with DPDPE, a specific agonist of DOR, and the effect of DOR activation on the level of βarr in the nucleus was examined by confocal microscopy. As shown in Figure 1A, before agonist stimulation, βarr1-GFP fluorescence was distributed in both the nucleus and cytoplasm, while βarr2-GFP fluorescence was mainly distributed in the cytoplasm. DPDPE stimulation induced trafficking of βarr1-GFP and βarr2-GFP to the cell membrane. However, an apparent accumulation of βarr1-GFP, but not βarr2-GFP, in the nucleus was also observed under the same conditions. Introduction of the nuclear export signal of βarr2 into βarr1 by a single point (Q394L) mutation eliminated nuclear distribution of βarr1, which is consistent with what was previously observed (Scott et al., 2002; Wang et al., 2003b), and also abolished DPDPE-induced βarr1 nuclear accumulation (Figure 1A). DOR activation-stimulated βarr nuclear translocation was confirmed by monitoring real-time βarr1-GFP fluorescence in living cells during agonist treatment. As shown in Figure 1B, accumulation of βarr1-GFP in the nucleus of HEK293 cells could be observed within 1 min of DPDPE challenge. Significant increase of βarr1-GFP fluorescence in the nucleus was observed in 51% of the βarr1-GFP expressing cells challenged with DPDPE (102 out of 200 cells analyzed). Western analysis of HA-βarr1 exogenously ex-

pressed in HEK293 cells and βarr1 endogenously expressed in HeLa cells indicated that concentration of βarr1 in the nuclear fraction increased 50%–100% after 5 min of DPDPE incubation (Figures 1C and 1D), confirming the results of fluorescence microscopy and suggesting that agonist-stimulated nuclear translocation of βarr1 is not an artifact of GFP-fusion or overexpression of βarr1. Activation of κ-opioid receptor (KOR) also induced nuclear translocation of βarr1, however, activation of μ-opioid receptor (MOR) or β₂-adrenergic receptor (β₂AR) only caused βarr1 translocation to the plasma membrane but not to the nucleus (Figures S1A and S1B in the Supplemental Data available with this article online). These data indicate that activation of certain GPCRs induces βarr1 trafficking into and accumulation in the nucleus.

DOR Activation Stimulates βarr1-Mediated *p27* and *c-fos* Transcription

Our preliminary microarray analysis using Affymetrix gene-chip U133A showed that inhibition of expression of βarr1 by βarr1 siRNA in HeLa cells downregulated transcription of apoptosis- or cell cycle-related genes such as *p27* and *c-fos*. Thus, the effect of βarr1 on the expression of *p27*, *c-fos*, *c-jun*, *cyclin A*, and *cyclin D1* genes, which play important roles in regulation of cell proliferation, was examined. As shown in Figures 2A and S2A, overexpression of βarr1 (by ∼8-fold) resulted in a statistically significant increase in *p27* mRNA and protein levels in HeLa cells, whereas overexpression of βarr2 or βarr1Q394L (by ∼8-fold over endogenous βarr2 or βarr1) had no such effect. Furthermore, expression of βarr1 siRNA, but not βarr2 siRNA (both reduced the level of the corresponding isoform by 80%), significantly decreased mRNA levels of *p27* and *c-fos* and protein level of p27 (Figures 2A and S2A). The effect of βarr1 siRNA on c-Fos protein level was not observed in Western assays, possibly due to the instability and rapid degradation of c-Fos protein (Curran et al., 1985). In contrast, expression of βarr1 or its siRNA had no influence on the mRNA level of *c-jun* (Figure 2A), *cyclin A*, or *cyclin D1* (data not shown). The effect of βarr1 on gene transcription was also examined in βarr1 and βarr2 double knockout ($βarrs^{-/-}$) MEF cells (Kohout et al., 2001). Consistent with what observed in HeLa cells, expression of βarr1 significantly increased *p27* mRNA and protein levels, but expression of βarr2 or βarr1Q394L failed to cause any change in *p27* transcription (Figures 2B and S2B). These results indicate that βarr1 can regulate expression of *p27* and *c-fos* genes and this function of βarr1 is apparently correlated with its presence in the nucleus. Coincidently, DPDPE treatment, which induces accumulation of βarr1 in the nucleus, significantly increased the expression of *p27*, but not that of *c-jun* (Figure 2C). The effect of DPDPE on *p27* transcription could be blocked by either DOR antagonist naltridole or βarr1 siRNA (Figure 2C), but not by inhibitors of Gi/Go (pertussis toxin), PI3K (wortmannin), p38 (SB203580), JNK (SP600125), or ERK (PD98059) (data not shown). These results indicate that activation of DOR can affect gene expression at transcription level and such an effect is mediated by βarr, likely via receptor-activation-induced βarr1 nuclear translocation.
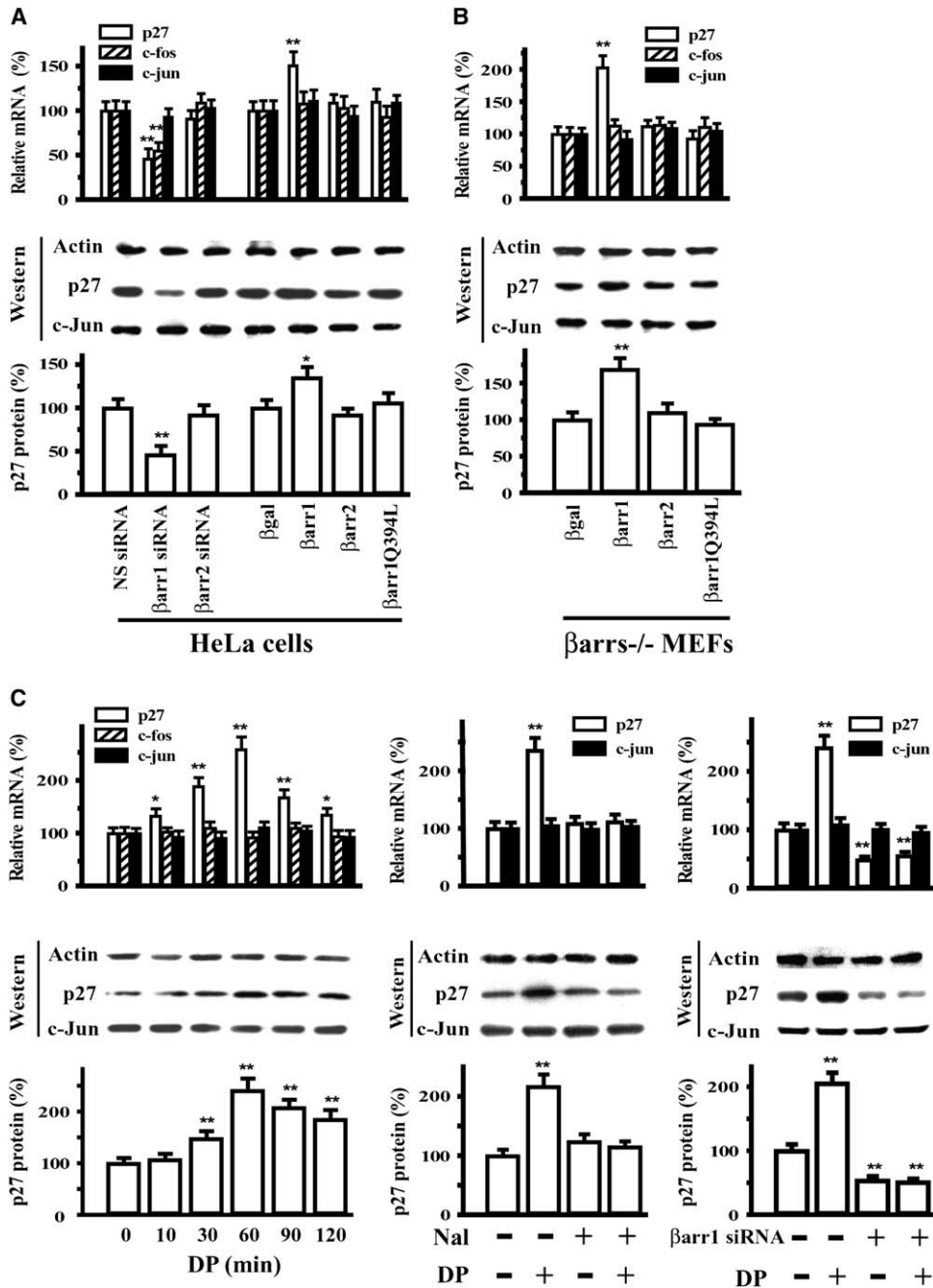
Figure 2. DOR Activation Stimulates βarr1-Mediated *p27* and *c-fos* Transcription

(A and B) HeLa (A) and *βarrs*^{−/−} MEF (B) cells transiently expressing the indicated plasmids were subjected to RT-qPCR and Western blotting for detection of *p27*, *c-fos*, and *c-jun* expression.

(C) HeLa cells transiently expressing DOR or DOR and βarr1 siRNA (as indicated) were pretreated with or without 100 nM naltridole (Nal) for 5 min, incubated with 1 μM DP for the times as indicated (left) or 60 min (middle and right), and subjected to RT-qPCR and Western analysis. Hypoxanthine phosphoribosyl transferase (*HPRT*) was used as a control in RT-qPCR. The protein levels of p27 and c-Jun were quantified and normalized to actin protein in Western. Data shown are means ± SD of three independent experiments, *p < 0.05, **p < 0.01 versus the corresponding control.

Nuclear Accumulation of βarr1 Promotes Acetylation of Histone H4 at *p27* and *c-fos* Promoters

Epigenetic regulation, especially acetylation modification of histones, has been found to play critical roles in regulation of eukaryotic gene transcription (Grewal and Moazed, 2003; Grunstein, 1997). Thus the potential influence of βarr1 on histone acetylation was investigated. The Western data (Figure S3A) showed that the acetylation of histone

H4, but not H3, increased in HeLa cells following overexpression of βarr1 and decreased after βarr1 siRNA application. Out of the four conserved lysine residues (Lys-5, Lys-8, Lys-12, and Lys-16) susceptible to acetylation in histone H4, βarr1 selectively affected the acetylation of Lys-12 and Lys-16. Moreover, the acetylation of H4 at Lys-12 and Lys-16 in $βarrs^{-/-}$ MEFs was significantly lower than that in the wild-type MEFs, and reintroduction of βarr1 in $βarrs^{-/-}$ MEFs significantly increased the acetylation of H4 at these sites (Figure S3B). As expected, no significant difference was observed in acetylation of H3 or H4 after expression of βarr2, βarr1Q394L, or βarr2 siRNA in HeLa or $βarrs^{-/-}$ MEF cells (Figure S3). These data suggest that nuclear βarr1 regulates acetylation level of H4 and may thus affect transcription of a number of genes including $p27$ and c-fos.

Transcription of a particular gene is dependent on the status of histone acetylation in close proximity to this gene, especially within its promoter region (Fry and Peterson, 2002; Vermaak et al., 2003). As shown by chromatin immunoprecipitation (ChIP), the acetylation levels of histone H4 in the $p27$ and c-fos promoter regions were decreased by expression of βarr1 siRNA and increased by overexpression of βarr1 in HeLa cells (Figure 3A). Reintroduction of βarr1 in $βarrs^{-/-}$ MEFs also significantly increased the amount of acetylated H4 in the $p27$ and c-fos promoter regions. Whereas, overexpression of βarr2 or βarr1Q394L had no effect on acetylation of the histones associated with any of the promoters tested (Figure 3A), suggesting that nuclear accumulation of βarr1 is necessary for the altered H4 acetylation at these promoters. Expression of βarr1 siRNA or overexpression of βarr1 did not show any significant effect on the acetylation of H4 at the site of c-jun, $cyclin A$, or $cyclin D1$ promoter and acetylation of H3 around all five promoters tested (Figure 3A, and data not shown for $cyclin A$, or $cyclin D1$), indicating that βarr1 induces a gene-specific H4 acetylation. Coincidently, DPDPE treatment led to a βarr1- and receptor-activation-dependent increase of H4 acetylation at $p27$ (Figures 3B and 3C) and c-fos (data not shown) promoters. Our data from various βarr1 mutants indicate that their abilities to promote H4 acetylation at $p27$ promoter and to stimulate $p27$ transcription correlate well (Figure 3E), suggesting that H4 acetylation alteration is probably one of the mechanisms for βarr1-mediated regulation of gene transcription.

Protein-chromatin binding assay was used to explore the potential association of βarr1 and chromatin and the mechanism of regulation on histone acetylation by βarr1. Similar to the distribution pattern of histone H4 and p300 (Figure 3D), the endogenous and exogenous βarr1 could be detected in the crude chromatin pellet (lane 3), the supernatant after micrococcal nuclease (MNase) digestion (lane 4), and the pellet after ultra-centrifugation of the supernatant of MNase digestion (lane 7), indicating the binding of βarr1 with chromatin. The accumulation of βarr1 at $p27$ and c-fos promoter regions was also shown by ChIP assay, where βarr1-specific antibody could immunoprecipitate the genomic DNA fragments containing the promoter sequences of $p27$ and c-fos, but not that of c-jun (Figure 3A), $cyclin A$, or $cyclin D1$ (data not shown) in HeLa cells. As expected, βarr1 antibody

could not recover any DNA fragments containing the tested promoter sequences in $βarrs^{-/-}$ MEFs (Figure 3A and data not shown for $cyclin A$ and $cyclin D1$). Consistent with the stimulation effect of DOR activation on H4 acetylation, accumulation of βarr1 at $p27$ and c-fos promoters was also significantly increased in response to DOR activation (Figure 3B and data not shown for c-fos). These results indicate a possible linkage between βarr1 enrichment and the elevated H4 acetylation at these promoter regions.

βarr1 Recruits p300 to $p27$ and c-fos Promoter Regions

The level of histone acetylation is regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC). Our in vitro acetylation and deacetylation assays using purified GST-βarr1 and immunoprecipitated HA-βarr1 showed that βarr1 possesses neither the activity of HAT nor the capability to affect the catalytic activity of HATs or HDACs in vitro (Figures S4A–S4C). Furthermore, trichostatin A (TSA) and sirtinol, the specific inhibitor of Class I, II, and III HDACs, respectively (Grozinger et al., 2001; Yoshida et al., 1990), had no significant effect on βarr1-induced H4 hyperacetylation in $p27$ and c-fos promoter regions (Figure S4D). These results suggest that βarr1 may promote H4 acetylation through recruiting HAT proteins to the specific genomic regions.

p300 and the cAMP response element binding protein (CREB) binding protein (CBP) are potent HATs possibly related with the histone acetylation within c-fos promoter (Usenko et al., 2003), while the potential interaction between βarr1 and HAT protein Tip60 has been suggested (Salwinski et al., 2004). Our data showed that reducing nuclear βarr1 by its siRNA decreased, while overexpression of βarr1 increased the accumulation of p300 at $p27$ and c-fos promoters (Figure 4A), but the accumulation of CBP and Tip60 at these regions was not affected. In addition, the level of Tip60 at the tested regions was very low, and the accumulation of these HAT proteins in the promoter regions of c-jun (Figure 4A), $cyclin A$, and $cyclin D1$ (data not shown) was not affected by βarr1. Coincidently, DPDPE stimulation also increased p300 accumulation at $p27$ and c-fos promoters, which was temporally parallel to the enrichment of βarr1 and the increase of H4 acetylation in these two promoter regions (Figures 3B and 4B, data not shown for c-fos). Coimmunoprecipitation using HeLa nuclear extracts showed the presence of endogenous βarr1 in the p300 immunocomplex and the endogenous p300 in the βarr immunocomplex, indicating that βarr1 may interact with p300 in the nucleus (Figure 4C). Collectively, these results suggest that βarr1 may promote gene-specific H4 hyperacetylation through recruiting p300 to the target genomic regions.

p300 Plays a Role in βarr1-Mediated Histone H4 Hyperacetylation and Gene Transcription

The potential role of p300 in βarr1-mediated gene-specific H4 acetylation was then investigated. H4 acetylation at $p27$ and c-fos promoter regions was strongly increased by overexpression of p300 in HeLa and $βarrs^{-/-}$ MEF cells, and this effect of p300 was augmented by coexpression of
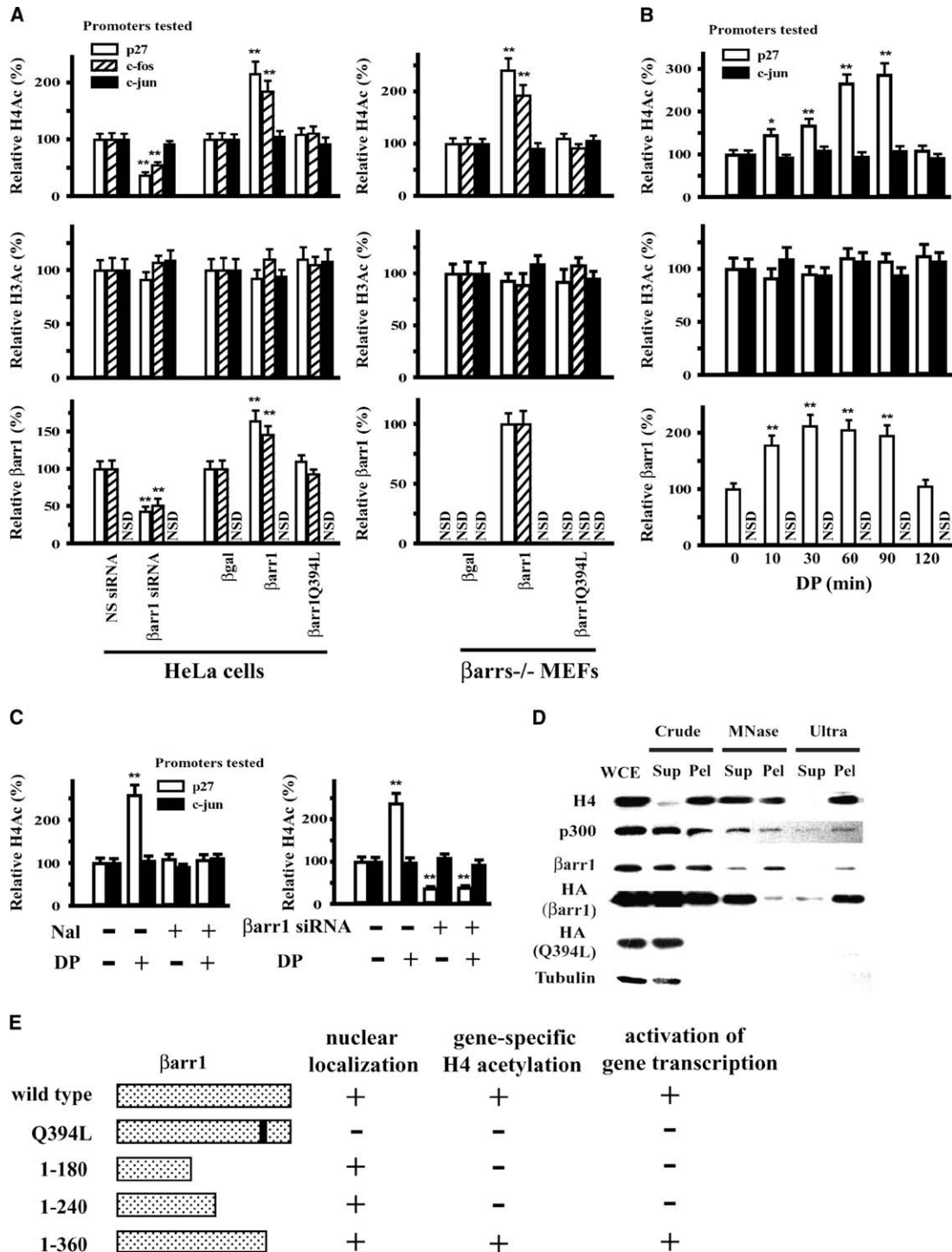
Figure 3. Nuclear Accumulation of βarr1 Promotes Acetylation of Histone H4 at *p27* and *c-fos* Promoters

(A–C) ChIP experiments were carried out using antibodies against acetylated H4 (H4Ac), H3 (H3Ac), βarr1, and human or mouse IgG (as a negative control) and the presence of the *p27*, *c-fos*, and *c-jun* promoter sequences in the input DNA and that recovered from antibody-bound chromatin segments were analyzed by qPCR. The data were normalized to the corresponding input controls. Primer sets covering different regions of the same promoter were used and produced similar results. The data shown are the means ± SD of three independent experiments of one set of primers. (A) HeLa (left) and *βarrs*^{−/−} MEF (right) cells expressing the indicated plasmids. NSD, no signal detected. (B) HeLa cells expressing DOR alone were incubated with 1 μM DP for the time indicated. (C) HeLa cells expressing DOR or DOR and βarr1 siRNA as indicated were treated with or without 100 nM Nal for 5 min before incubated with 1 μM DP for 60 min.
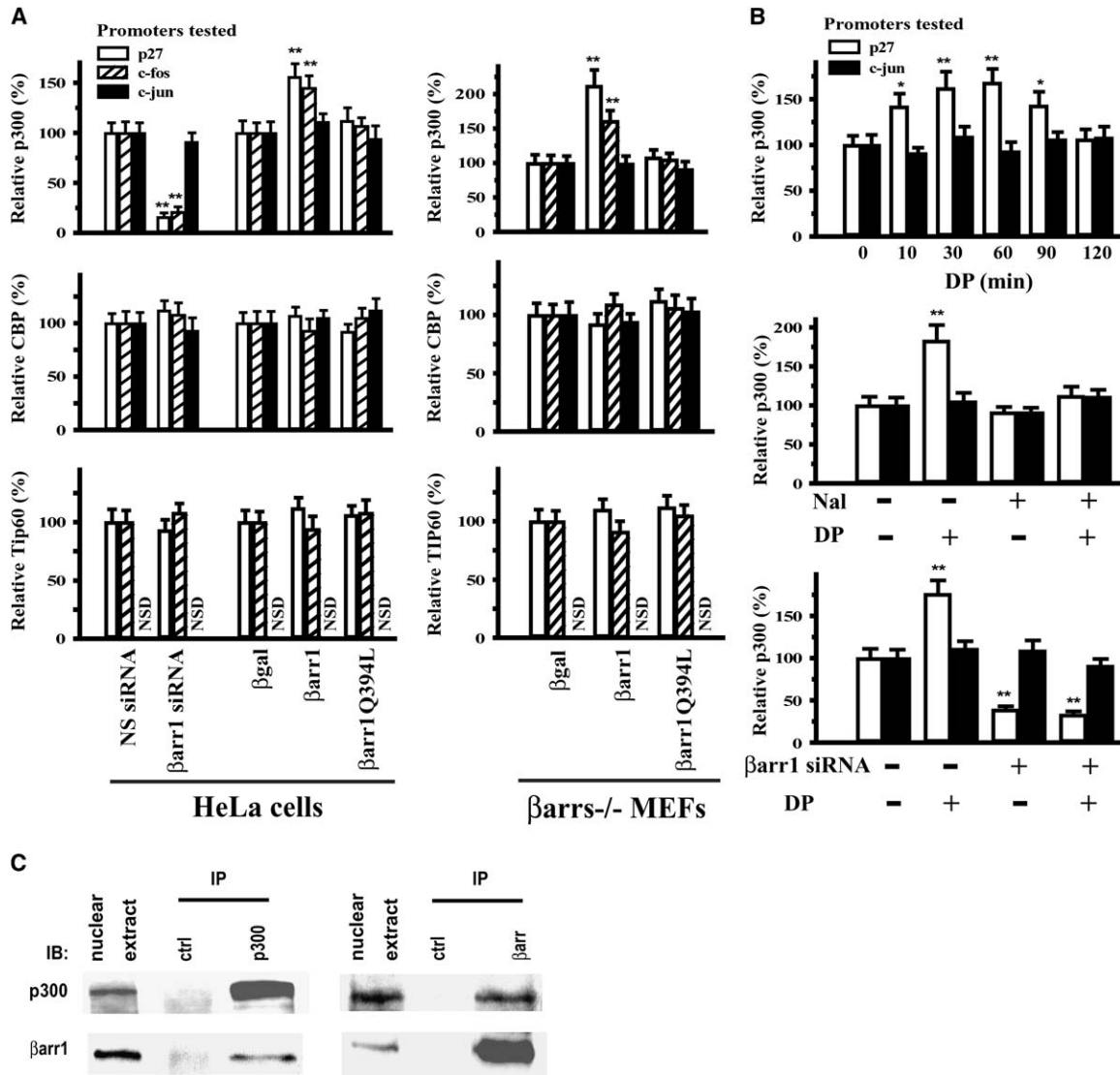
Figure 4. βarr1 Recruits p300 to *p27* and *c-fos* Promoter Regions

(A and B) ChIP experiments were done using antibodies against p300, CBP, Tip60, or human or mouse IgG (as a negative control). The presence of the *p27*, *c-fos*, and *c-jun* promoter sequences in the input DNA and antibody bound chromatin segments were analyzed by qPCR. The data were normalized to the corresponding input. The data shown are the means ± SD of three independent experiments of one set of primers. *p < 0.05, **p < 0.01 versus the corresponding control. (A) HeLa (left) and *βarrs*$^{-/-}$ MEF (right) cells expressing the indicated plasmids. (B) HeLa cells expressing DOR were incubated in 1 μM DP for the time indicated (upper). HeLa cells expressing DOR or DOR and βarr1 siRNA as indicated were treated with or without 100 nM Nal for 5 min before incubated with 1 μM DP for 60 min (middle and lower).

(C) HeLa nuclear extracts were immunoprecipitated with p300 or βarr antibody and the immunocomplexes were analyzed in Western using antibodies against p300 or βarr. 5% of total nuclear extracts was loaded as a control.

(D) Chromatin-protein binding assay. The whole-cell lysates (WCE) were centrifuged and the pellet (Crude Pel) was treated with micrococcal nuclease (MNase) and centrifuged again. The supernatant obtained (MNase Sup) was subjected to ultracentrifugation to obtain pellet (Ultra Pel) and supernatant (Ultra Sup). Samples were analyzed in Western using the antibodies indicated.

(E) Nuclear distribution of the wild-type and mutant βarr1 transiently expressed in HeLa cells and their effects on H4 acetylation and gene transcription. Nuclear localization of βarr1-HA was observed under confocal microscope. H4 acetylation in *p27* promoter regions was determined in ChIP assay and the transcription of *p27* was analyzed by RT-qPCR. Data shown are the means ± SD from three independent experiments, *p < 0.05, **p < 0.01 versus the corresponding control.
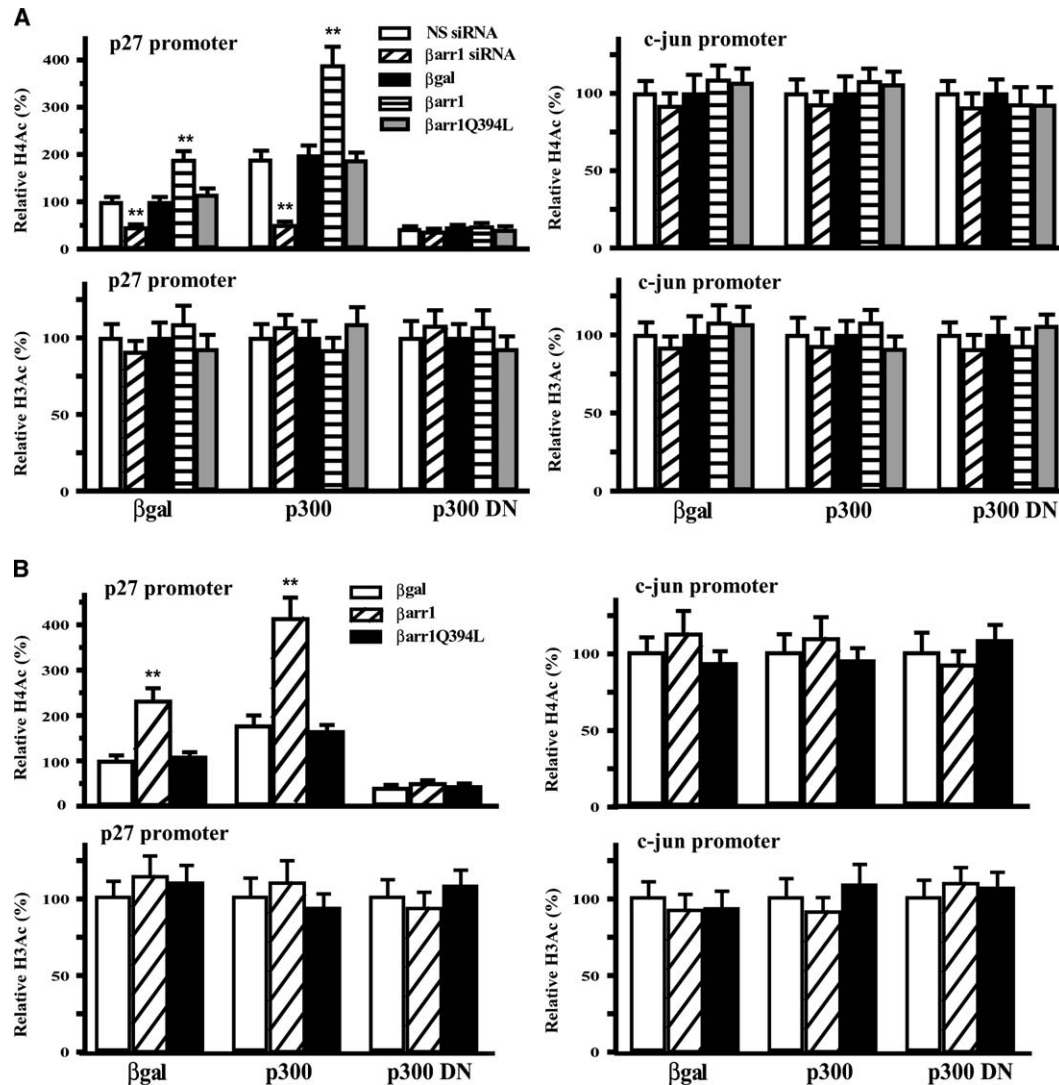
Figure 5. p300 Plays a Role in βarr1-Mediated Gene-Specific H4 Acetylation

ChIP experiments were carried out in HeLa (A) or *βarrs*$^{-/-}$ MEF (B) cells expressing the indicated plasmids using antibodies against H4Ac and H3Ac. The presence of the *p27* and *c-jun* promoter sequences in the input DNA and antibody-bound chromatin segments was analyzed by qPCR. The data obtained were normalized on the basis of the corresponding input control, and means ± SD from three independent experiments were plotted. **p < 0.01 versus the cells transfected with NS siRNA or βgal in the corresponding βgal and p300 group, respectively.

βarr1 and blocked by coexpression of βarr1 siRNA. Moreover, p300 DN, a dominant-negative mutant of p300, strongly attenuated the effect of βarr1 on H4 acetylation at *p27* and *c-fos* promoters (Figure 5 for *p27* and data not shown for *c-fos*). These data indicate that p300 plays a critical role in βarr1-mediated H4 hyperacetylation in these promoter regions. Similar to its effect on βarr1-mediated H4 acetylation, overexpression of p300 DN also diminished the effect of βarr1 on *p27* and *c-fos* transcription (Figures 6A and 6B), suggesting that gene-specific H4 hyperacetylation promoted by βarr1 and p300 contributes to transcriptional activation of these genes.

CREB is the known transcription factor that recruits the coactivators CBP and p300 (Mayr and Montminy, 2001) and regulates transcription of *p27* and *c-fos* (Garcia et al., 2004; Mayr and Montminy, 2001). Recent studies indicate the importance of stimulus-induced histone acetylation on CREB-dependent transcription of genes such as *c-fos* (Johannessen et al., 2004). Our coimmunoprecipitation data showed that endogenous βarr1 was present in the CREB immunocomplex and vice versa (Figure 6C). Further studies showed that the expression of CREB siRNA significantly attenuated the βarr1 accumulation at *p27* and *c-fos* promoters (Figure 6D), although expression of βarr1 and its siRNA had no effect on the binding of CREB (Figure 6D) or phosphorylated CREB (data not shown) at these regions. In addition, overexpression of βarr1 showed no significant effect on CREB-mediated transcriptional activity in both GAL4-CREB
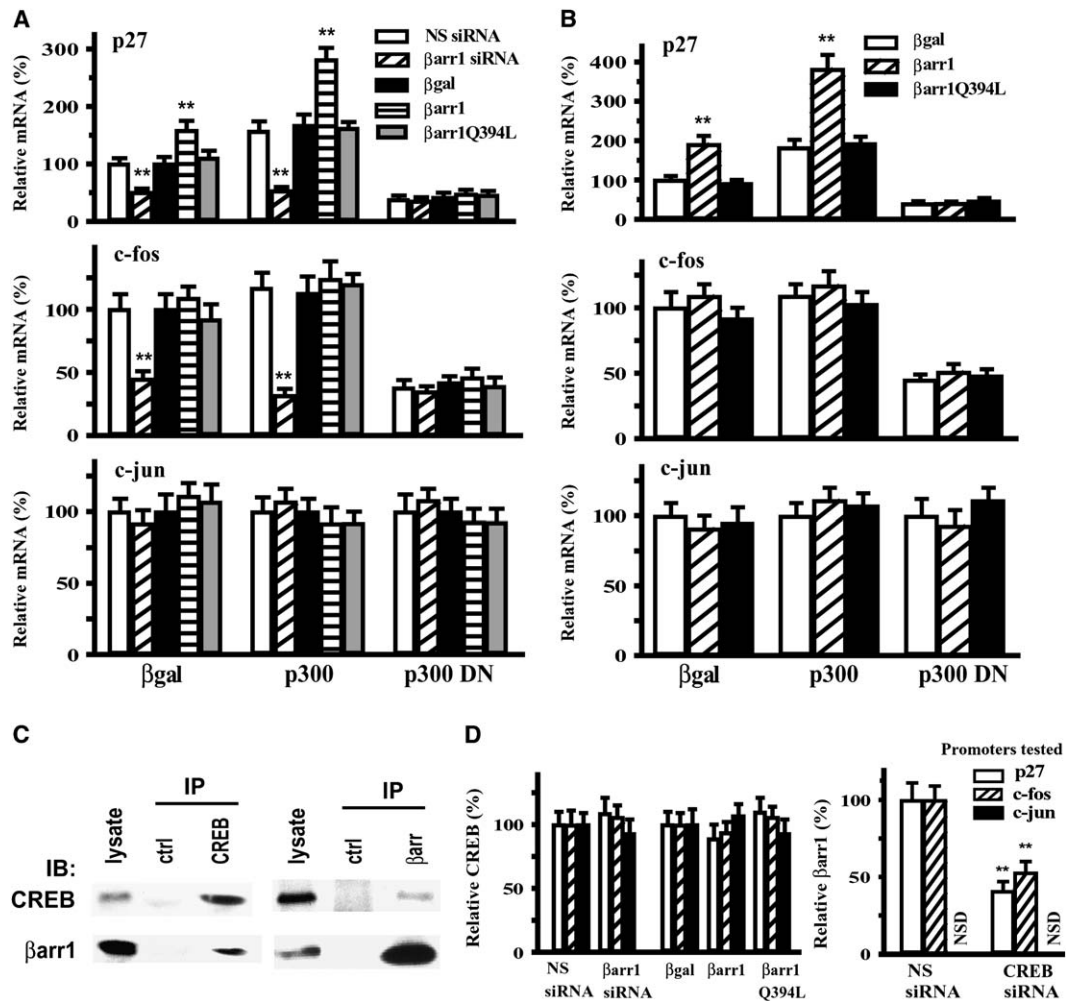
Figure 6. p300 Plays a Role in βarr1-Mediated Gene Transcription

(A and B) HeLa (A) and βarrs$^{-/-}$ MEF (B) cells expressing the indicated plasmids were used and RT-qPCR was performed to evaluate the transcription levels of *p27*, *c-fos* and *c-jun*. *HPRT* was used to normalize the amounts of cDNA template. The data shown are the means ± SD from three independent experiments. **p < 0.01 versus the cells transfected with NS siRNA or βgal in the corresponding βgal and p300 group, respectively.

(C) Immunoprecipitation assay for the presence of CREB or βarr in βarr- or CREB-immunoprecipitation complexes. 5% of cell lysate was loaded as a control.

(D) ChIP experiments were carried out to detect the binding of CREB or βarr1 at the *p27*, *c-fos*, and *c-jun* promoter regions. The data were normalized on the basis of the corresponding input control, and means ± SD from three independent experiments are plotted. **p < 0.01 versus the cells transfected with NS siRNA.

reporter system and the system with reporter plasmid carrying a CREB binding site (Figure S4E). These results suggest that βarr1 unlikely activates transcription of these genes through promoting CREB phosphorylation or in the absence of the intact chromatin structure, while the interaction of βarr1 and CREB may be involved in GPCR signal-stimulated accumulation of βarr1 and histone acetylation in specific chromosomal regions and the transcription of specific genes.

Activation of Endogenous DOR in Neural Cells Promotes βarr1-Dependent Histone H4 Hyperacetylation, *p27* Transcription, and Growth Inhibition

DOR is an important neurotransmitter receptor widely expressed in the central nerve system. To confirm whether the DOR activation-stimulated H4 acetylation and *p27* transcription occur under more physiological conditions, human brain neuroblastoma SK cells, which express DOR endogenously (Yu et al., 1986), were challenged with DPDPE. As shown in Figures 7A and 7B, DPDPE treatment time dependently increased the nuclear concentration of endogenous βarr1, the H4 acetylation at *p27* promoter, and the transcription of *p27*. These effects of DPDPE could be blocked by naltridole and βarr1 siRNA. Similarly, intracerebroventricular injections of DPDPE also DOR specifically increased the H4 acetylation at *p27* promoter and the transcription of *p27* in mouse hippocampus (Figure 7C) and cerebral cortex (data not shown), demonstrating the regulation of DOR activation on histone acetylation and *p27* transcription occurs in vivo. Since p27 is known to be involved in suppression of cell
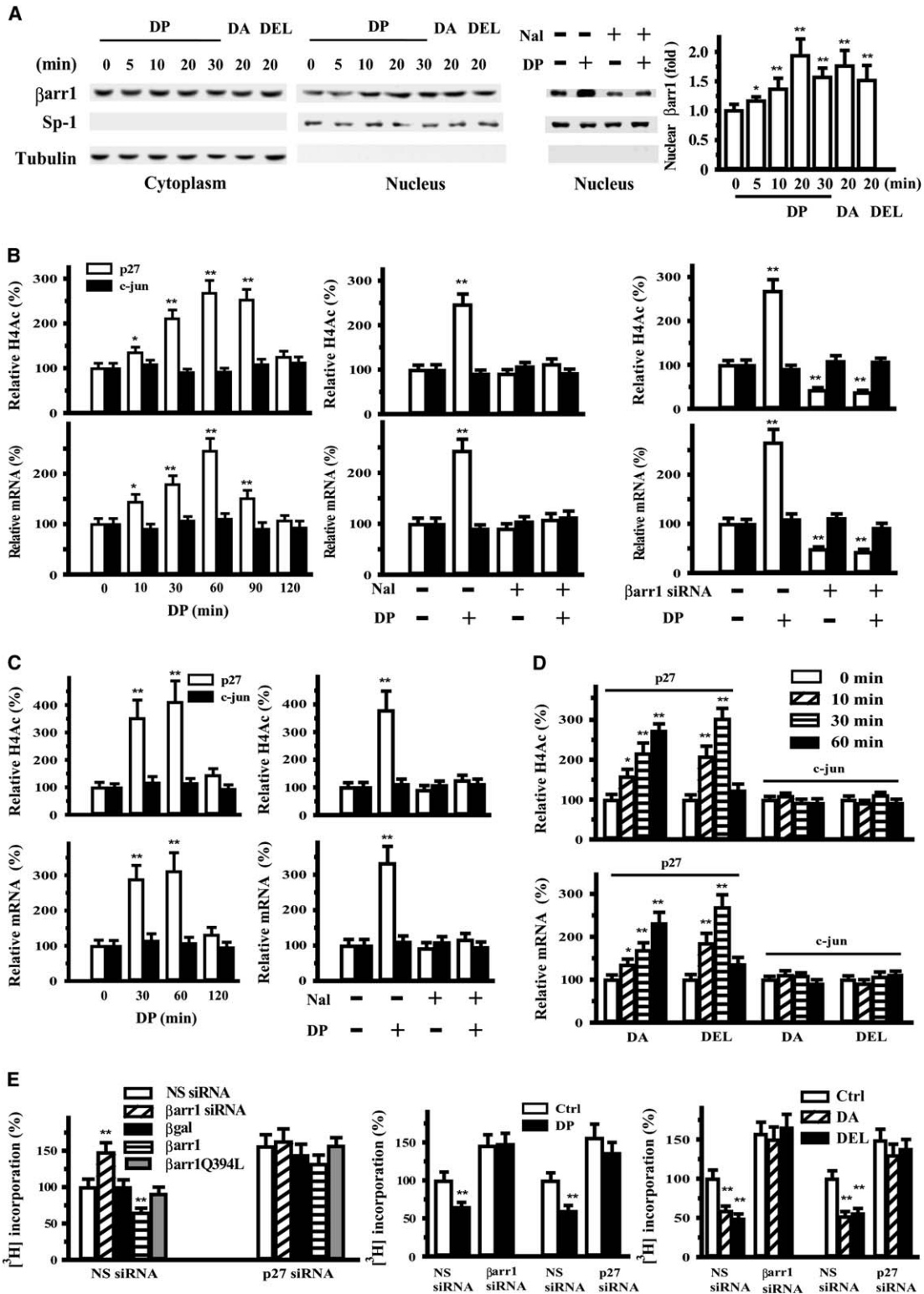
Figure 7. βarr1-Mediated Epigenetic Regulation of Gene Transcription in Neural Cells

(A) SK cells expressing DOR and βarr1 endogenously were incubated with 1 μM DP, 1 μM DADLE (DA), or 1 μM deltorphin-I (DEL) for the time indicated. In the right panel, cells were pretreated with or without 100 nM Nal for 5 min before incubated with 1 μM DP for 20 min. βarr1 content in nuclear extracts was analyzed by Western and quantified as described in the legend for Figure 1.

growth (Kiyokawa et al., 1996; Nakayama et al., 1996), whether βarr1 and DOR activation could p27-dependently inhibit cell growth was estimated by [³H]thymidine incorporation in neuroblastoma cells. As shown in Figure 7E, overexpression of βarr1 inhibited, while its siRNA promoted the growth of SK cells. The effect of βarr1 on cell growth was p27 dependent since it could be blocked by *p27* siRNA. Coincidently, DOR activation by DPDPE obviously inhibited the growth of SK cells in a βarr1- and *p27*-dependent manner. Moreover, treatment with other agonists of DOR including DADLE (structure similar to DPDPE) and deltorphin-I (structure different from DPDPE), capable of stimulating nuclear translocation of βarr1 (Figure 7A), *p27* promoter H4 hyperacetylation, and *p27* transcription (Figure 7D) in SK cells, also inhibited growth of these cells (Figure 7E). These data implicate that activation of endogenous DOR in neural cells may exert impact on physiological functions of these cells through a βarr1-mediated epigenetic mechanism.

DISCUSSION

βarr1 and βarr2 are previously known as cytosolic signaling regulatory and scaffold proteins. Recent studies revealed that βarr1 is distributed in both the cytoplasm and the nucleus, but the potential function of βarr1 in the nucleus is unknown. The present study showed that activation of DOR could induce βarr1 translocation to the nucleus and stimulate βarr-dependent *p27* and *c-fos* transcription, thus revealing a novel function of βarr as a messenger carrying receptor signals to the nucleus. Overexpression of βarr1 and nuclear translocation of βarr1 promoted histone H4 hyperacetylation at the *p27* and *c-fos* promoters and hence activated their transcription, indicating that epigenetic regulation of gene expression is one function of βarr in the nucleus. Collectively, this study demonstrates that chromatin is a direct target of GPCR-mediated signal transduction and reveals an epigenetic mechanism for GPCR signaling from cell membrane to the nucleus. It also provides the first evidence for a function of the arrestin family in the nucleus as a GPCR messenger.

Previous studies demonstrated that activation of GPCR recruits both βarr1 and βarr2 to the cell membrane and interactions of the phosphorylated GPCR and βarrs induce receptor endocytosis and signal inhibition. However, accumulating evidence also revealed potential functional differences between the two βarr subtypes as well as their receptor

specificity. For example, βarr1 binds some GPCRs such as β₂ AR, MOR, and endothelin type receptor with lower affinity than βarr2 and is less efficient in membrane translocation upon agonist stimulation, while βarr1 and βarr2 bind other receptors, including angiotensin II type 1A receptor, neurotensin 1 receptor, and substance P receptor with similar high affinities, and translocate to the membrane with similar efficiencies (Oakley et al., 2000). Furthermore, sequestration of the β₂AR was compromised in the βarr2—but not βarr1—knockout cells (Kohout et al., 2001). Our current study showed that nuclear accumulation of βarr1 occurred following stimulation of DOR and KOR, but not after activation of β₂AR or MOR, suggesting that this βarr1-mediated epigenetic pathway may be preferentially used by certain receptors. Although the underlying mechanisms are not understood, differential interactions of βarr1 with these receptors may contribute to the receptor specificity observed. Moreover, our data also showed that DOR activation stimulated βarr1, but not βarr2 accumulation in the nucleus. The distinct nuclear trafficking responses of βarr1 and βarr2 to DOR activation may be partially attributed to the difference in their structure. Previous studies showed that both βarrs are able to shuttle between cytoplasm and nucleus. But different from βarr1, βarr2 possesses a strong nuclear export signal in its C terminus, which hinders its retention in the nucleus (Scott et al., 2002; Wang et al., 2003b). Our data showing that agonist stimulation failed to induce nuclear accumulation of Q394L, a mutant βarr1 with βarr2 nuclear export signal, demonstrated the importance of the C-terminal domain in regulating nuclear concentration of different βarrs. These data also suggest that βarr1 subtype may play a more important role in GPCR-mediated nuclear signaling.

The classical GPCR pathways involve the activation of G proteins and the hydrolysis of GTP, the regulation of cAMP formation as well as various signaling molecules such as PKA and MAPKs, and the alteration of transcription of the target genes (Neves et al., 2002; Shaywitz and Greenberg, 1999). Signal transduction initiated by DOR stimulation activates Gi/Go proteins and ERK1/2, JNK, P38, and PI3K cascades (Eisinger and Schulz, 2004; Persson et al., 2003; Shahabi et al., 2003; Zhang et al., 1999). Our results suggest that DOR stimulation also triggers a direct signaling pathway from the cell surface to the nucleus mediated by βarr1 nuclear translocation. The precise molecular mechanism by which DOR stimulation leads to βarr1 nuclear translocation remains

(B) SK cells transfected with or without βarr1siRNA as indicated were incubated in 1 μM DP for the time indicated (left) or 60 min (middle and right) after pretreated with or without 100 nM Nal for 5 min. The level of H4Ac at *p27* and *c-jun* promoters and the transcription levels of *p27* and *c-fos* were analyzed by ChIP and RT-qPCR. *HPRT* was used in RT-qPCR to normalize the input cDNA. Values are expressed as the mean ± SD, *p < 0.05, **p < 0.01 versus the corresponding control.
(C) DP was intracerebroventricularly injected in mice and hippocampus samples were analyzed for H4Ac at *p27* and *c-jun* promoters and the transcription of *p27* and *c-jun* by ChIP and RT-qPCR. Left, samples were taken after different intervals of DP injection. Right, Nal was injected before DP injection and the hippocampi were taken 60 min after DP injection. Data are from three independent experiments. TATA box binding protein (*TBP*) was used in RT-qPCR to normalize the input cDNA. Values are expressed as the mean ± SD, **p < 0.01 versus mice treated without DP.
(D) SK cells were incubated with 1 μM DA or 1 μM DEL for the indicated times and then H4Ac at *p27* and *c-jun* promoters (left) and the transcription of *p27* and *c-jun* (right) were determined by ChIP or qPCR. *p < 0.05, **p < 0.01 versus 0 min control in the corresponding group.
(E) SK cells were transfected with βgal, βarr1, βarr1Q394L, NS siRNA, βarr1 siRNA, *p27* siRNA, or indicated combinations and incubated in the presence or absence of 1 μM of DP and DA for 60 min or 1 μM of DEL for 30 min. The [³H]thymidine incorporation was determined, and data shown are the means ± SD from three independent experiments, **p < 0.01 versus the corresponding control.

to be elucidated, but the process appears to be independent of the activation of Gi/Go, PI3K, P38, JNK, and ERK. In the JAK-STAT signaling pathway, receptor phosphorylation triggered by cytokine and growth factors induces phosphorylation of STATs, which subsequently translocate to the nucleus to regulate transcription (Levy and Darnell, 2002). The phosphorylation and nuclear translocation of Smad proteins also play a critical role in the propagation of TGF-β signaling from cell membrane to the nucleus (Shi and Massague, 2003). Both DOR and βarr1 could be phosphorylated at their C termini and the changes in DOR and βarr1 phosphorylation in response to agonist treatment have been observed (Lin et al., 1997; Zhao et al., 1997). Thus, an intriguing possibility is that, similar to the JAK-STAT and TGF-β signaling pathways, GPCR phosphorylation and posttranslational modification of βarr1 might play a role in the receptor signal-induced βarr1 nuclear translocation. Interestingly, a recent study has demonstrated that upon extracellular stimulation, endocytic protein APPL1 translocates into the nucleus and interacts with the nucleosome remodeling and histone deacetylase multiprotein complex NuRD/MeCP1 (Miaczynska et al., 2004). βarr1 is also a well known important regulator of receptor endocytosis. The potential roles of βarr1 membrane trafficking, receptor endocytosis, and the endocytosis machinery in GPCR activation-induced βarr1 nuclear translocation await future investigations.

Two possible mechanisms could be involved in βarr1-mediated histone hyperacetylation. βarr1 may function as a HAT or a HAT activator/recruiter to increase HAT activity in the targeted chromatin regions. Alternatively, βarr1 may inhibit HDAC activity or the binding of HDAC proteins to the chromatin. The data herein showed that HDAC activity was not required for βarr1-promoted histone acetylation and βarr1 itself did not have any HAT activity. Moreover, βarr1 did not affect the activity of HAT or HDAC proteins. Therefore we believe that the increased levels of histone H4 acetylation reflect the enhanced recruitment of HAT to chromatin mediated by βarr1. Consistent with that, our results showed that the accumulation of βarr1 and p300, a HAT protein, in the p27 and c-fos promoter regions was detected, and the level of p300 in these regions was regulated by βarr1. Furthermore, p300 DN inhibited βarr1-dependent regulation of H4 acetylation and gene activation and an interaction between p300 and βarr1 was detected in immunoprecipitation. These data support the hypothesis that in response to DOR activation, βarr1 associates with the target gene promoter regions and recruits p300 to these locations to induce the H4 hyperacetylation and the transcription of these genes. Since p300 is a common transcription cofactor, βarr1 thus could potentially influence multiple transcriptional pathways via its interaction with p300.

Among the five genes tested, DOR activation increased transcription of p27 and c-fos, but not c-jun, cyclin A, and cyclin D1 genes, suggesting that GPCR stimulation-induced, βarr1-mediated histone modification and transcriptional activation occur at a defined set(s) of genes. It has been shown that certain chromatin-remodeling enzymes, such as HATs, could directly interact with gene-specific transcription factors to ensure that chromatin remodeling is

targeted to the correct gene (Fry and Peterson, 2002). Furthermore, growing studies indicate that in addition to transcription factors, the preexisting nucleoprotein architecture of specific promoters also plays critical roles in chromatin remodeling of specific gene loci (Urnov and Wolffe, 2001). Consistent with this view, our results showed that agonist-stimulated accumulation of βarr1 and p300 and H4 acetylation occurred specifically at p27 and c-fos, but not c-jun promoter regions, and that expression of CREB siRNA strongly inhibited βarr1 accumulation at p27 and c-fos promoter regions. It is generally believed that CREB-dependent transcription is stimulated by signal-induced CREB phosphorylation at Ser-133 and the subsequent recruitment of the coactivators p300 and CBP. Interestingly, our data suggest that while both CREB and p300 are required in βarr1-mediated transcription, CBP appears to be dispensable and there does not appear to be a change in CREB phosphorylation at target promoter regions upon DOR stimulation (unpublished data). Thus, analogous to what happens in the cytoplasm and on the cell membrane, βarr1 may also function as a scaffold molecule in the nucleus by interacting with transcription factors and other nuclear proteins to recruit p300 to the target chromatin regions. Our preliminary mass spectrum analysis of Flag-βarr1 immunoprecipitation complex from the nuclear or total cell extracts of HEK293 cells suggests the presence of other nuclear protein components in addition to p300 and CREB. Thus, in addition to CREB, other sequence-specific factor(s) may also contribute to the recruitment of βarr1 to target promoter regions such as that of p27 and c-fos, which could provide a molecular basis for gene-specific transcriptional regulation by βarr1.

Epigenetic regulation is an important pathway to induce a coordinated transcriptional response to environmental signals and the balance of epigenetic networks contributes to the normal processes of human development, while the disruption of this balance can cause aberrant disease states such as cancer and mental retardation (Levenson and Sweatt, 2005; Sutherland and Costa, 2003). GPCRs transduce a vast number of extracellular stimuli to the interior of cell and play vital roles in regulation of various cellular functions. This study shows that activation of DOR induces trafficking of βarr1 to the nucleus and results in histone modification and gene activation, revealing that epigenetic events such as histone modification are subjected to direct regulation by GPCRs, and βarr is again an important player in this pathway. The physiological significance of this βarr-mediated epigenetic regulatory pathway is implicated by the results that activation of DOR led to βarr1- and p27-dependent growth inhibition in human neuroblastoma cells. Further research will be needed to elucidate mechanistic details of this βarr-mediated epigenetic signaling pathway and to provide further insight into the physiology regulated by this novel pathway.

EXPERIMENTAL PROCEDURES

Antibodies, Reagents, Plasmids, and siRNAs
Antibodies and reagents are shown in the Supplemental Data. Plasmid cDNA encoding βgal, HA-βarr1 (long form as previously described (Parruti

et al., 1993), HA-βarr2, βarr1Q394L, βarr1-GFP, βarr2-GFP, βarr1Q394L-GFP, and HA-βarr1 truncation mutants were generated as described (Wang et al., 2003a, 2003b). Construction of βarr2 siRNA, pBS/U6/βarr1 siRNA, and pBS/U6/nonspecific siRNA plasmids were as described previously (Sun et al., 2002; Wang et al., 2003b). The nucleotide sequences carried in the plasmids are 5′-GGAAGCTCAAGCACGAAGACAA-3′ (siRNA against both splicing isoforms of βarr1 (Parruti et al., 1993)). The wild-type and dominant negative (C/H1 deletion) p300 pCMVβ plasmids were purchased from Upstate Biotechnology. siRNAs for p27, CREB, and non-silencing were synthesized by Shanghai GeneChem Inc (Shanghai, China). The target sequences of siRNA oligonucleotides are: for p27, 5′-ACCGACGATTCTTCTACTCAA-3′ and for CREB, 5′-GCTCGAGAGTG TCGTAGAA3′.

Cell Culture and Transfection
HEK293, HeLa, and SK-N-SH (SK) cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in MEM medium (Gibco-BRL, Gaithersburg, MD). The wild-type and βarr1/2 double knockout (βarrs$^{-/-}$) murine embryonic fibroblast (MEF) cells, provided by Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC), were maintained in DMEM medium (Gibco-BRL). HEK293 cells were transfected using calcium phosphate coprecipitation and HeLa, SK, and βarrs$^{-/-}$ MEF cells were transfected by Lipofectamine (Invitrogen, Carlsbad, CA).

Immunofluorescence Microscopy
After a 2 hr starvation with serum-free MEM, HEK293 cells transfected with the indicated plasmids were treated with or without the receptor agonist for 5 min, fixed, and incubated with 12CA5 and then Texas Red-conjugated anti-mouse IgG. The fluorescence signals were observed under a TCS NT laser confocal microscope (Leica Microsystems, Bensheim, Germany). For observation of distribution of βarr1-GFP in live cells in real time, the fluorescence of βarr1-GFP in HEK293 cells transfected with receptor and βarr1-GFP was observed at 37ºC using TCS NT equipped with a temperature controller. The cells were scanned in a time series. The fluorescence density of βarr1-GFP in the entire compartment of the nucleus, plasma membrane, or cytoplasm of the same cell was quantified using Image-Pro Plus 5.1 software (Media Cybernetic, Silver Spring, MD).

Nuclear Extract Preparation
Nuclear extracts were prepared as described previously (Dignam et al., 1983) with minor modifications. After a 12 hr serum starvation, cells were incubated with 1 μM DPDPE for different times, washed and resuspended in 400 μl of hypotonic buffer. After incubation on ice for 10 min, 3 μl of 1% NP-40 for HEK293, 4 μl of 10% NP-40 for SK, and 30 μl of 10% NP-40 for HeLa cells were added. After separation, the nuclei (pellet) were resuspended in hypertonic buffer and shaken for 1 hr at 4ºC. After centrifugation, the supernatant (nuclear extracts) were saved.

Western Blotting
In Western blotting analysis, the protein bands visualized by enhanced chemiluminescence method were quantified by Scion Image Beta 4.02 software (SynGene, Cambridge, Great Britain). For more quantitative measurement (as in Figures 1 and 7), the blots were incubated with IRDye 800CW-conjugated secondary antibody, the infrared fluorescence image was obtained using Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE), and the bands were quantified by Image-Pro Plus 5.1 software.

Intracerebroventricular Injection
2 pmol DPDPE (DP, 1.5 μl/mouse) were injected into the third cerebral ventricle of 3-week-old C57 mice. In some experiments, saline or 20 fmol naltridole (1.5 μl/mice) were injected 15 min before DP injection. Mice were sacrificed at different time points after DP injection and hippocampi were immediately separated, snap-frozen in liquid nitrogen, and stored at −80ºC. All animal treatments were carried out strictly in accor-

dance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Reverse Transcription Quantitative Real-Time PCR
Total RNAs were extracted from cultured cells or mouse hippocampi with TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription of purified RNA was performed using oligo(dT) priming and superscript II reverse transcriptase (Invitrogen). The quantification of all gene transcripts was done by qPCR, using Brilliant SYBR Green QPCR Master Mix and a Light Cycler apparatus (Stratagene). The primer pairs used are described in the Supplemental Data.

Chromatin Immunoprecipitation
Chromatin Immunoprecipitation (ChIP) assay was performed according to the protocol for the ChIP assay kit (Upstate Biology). The presence of the target gene promoter sequences in both the input DNA and the recovered DNA immunocomplexes was detected by qPCR. The primer pairs for specific promoter regions (within −1000 to +100 region, corresponding to the transcription start sites of genes) are shown in the Supplemental Data. The data obtained were normalized to the corresponding DNA input control.

Protein-Chromatin Binding
The assay was performed as described previously (Donovan et al., 1997; Liang and Stillman, 1997). Briefly, HeLa cells were lysed in extraction buffer containing protease inhibitor cocktail (Roche Molecular Biochemicals), and centrifuged. The pellet (Crude Pel) was digested for 20 s with 5 units of micrococcal nuclease (MNase, Takara Biotechnology). The supernatant after MNase digestion (MNase Sup) was centrifuged at 500,000 ×g for 1 hr again to yield ultracentrifugation pellet (Ultra Pel) and supernatant (Ultra Sup). All pellet fractions were resuspended in extraction buffer, and the volumes of all fractions were adjusted to reflect the same cell equivalent before Western analysis.

Immunoprecipitation
Cells and the nuclear extracts were lysed in buffer containing 1% Triton X-100, 10% glycerol, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 20 mM NaF, and 1 mM phenylmethylsulfonyl fluoride for 4 hr at 4ºC. After centrifugation at 15,000 ×g for 10 min, the supernatant was incubated at 4ºC with indicated antibodies for 12 hr. The immunocomplexes were captured by rotating for 1 hr with protein G Sepharose.

[^{3}H]Thymidine Incorporation
SK cells were transfected with βarrs for 42 hr or βarr1 siRNA for 90 hr. For estimation of DOR activation effect, SK cells were transfected with βarr1 siRNA or p27 siRNA for 90 hr, starved with serum-free MEM for 2 hr, and treated with 1 μM of DP and DA for 60 min, or 1 μM of DEL for 30 min. Then differently treated cells were incubated with fresh MEM containing 1 μCi/ml of [^{3}H]thymidine (24 Ci/mmol; Amersham) for 6 hr, and the [^{3}H]thymidine incorporation in DNA was determined using a Beckman scintillation S6500 counter.

Statistical Analysis
Quantitative data are expressed as the means ± standard deviation (SD). The statistical significance was determined by ANOVA followed by Bonferroni post-hoc test for multiple comparisons or Student's t test.

Supplemental Data
Supplemental Data include Supplemental Experimental Procedures, four figures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/123/5/833/DC1/.

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