The Egr-1 is an immediate early response gene encoding a transcription factor that functions in the regulation of cell growth, differentiation, and apoptosis. Estrogen has diverse physiological effects, including cellular proliferation and neuroprotection against brain injury. There are two types of estrogen receptors (ERs), ERα and ERβ. ERα-induced Egr-1 expression has been extensively studied; however, the role of ERβ is yet not known. In the present study, we investigated whether or not ERβ induces Egr-1 expression in C6 rat glioma cells, which express ERβ but not ERα. Our results show that ERβ promoted up-regulation of Egr-1 expression via a non-genomic mechanism involving the Raf/MEK1/Erk/Elk-1 signaling cascade. [BMB reports 2011; 44(7): 452-457]

INTRODUCTION

The steroid hormone 17β-estradiol (17β-E2) has a wide range of effects in many tissues, including those of the central nervous system (CNS). Specifically, 17β-E2 promotes growth of the hypothalamus, hippocampus, midbrain, and cortex in the developing brain (1), and it controls sexual behavior, neuroprotection, and cognition-related neuronal activity in the adult CNS, in addition to affecting one’s mood, mental state, memory, and ability to learn (2).

The estrogen receptors ERα and ERβ belong to a nuclear receptor superfamily of ligand-inducible transcription factors. Each ER is composed of several domains that are important for ligand binding, DNA binding, and transcriptional activation. The DNA-binding domains of ERα and ERβ are highly homologous; however, their ligand-binding and transactivation domains are less well-conserved (3). Upon binding to 17β-E2, the ERs interact with estrogen response elements (EREs) to stimulate the transcription of target genes in the nucleus. In addition to these genomic effects, several lines of evidence suggest that ERα functions via a non-genomic pathway (i.e., it functions outside the nucleus) through Shc, Src, and p85 of phosphatidylinositol 3-kinase (4).

Early growth response-1 (Egr-1) is an immediate early response gene whose expression is rapidly induced by diverse growth factors in many cell systems (5). Previous studies have demonstrated that 17β-E2-bound ERα activates Egr-1 transcription via a non-genomic pathway in MCF-7 breast cancer cells (6-9). However, very little is known about the role of ERβ in the regulation of Egr-1 expression.

In the present study, we investigated whether or not ERβ induces Egr-1 expression in C6 glial cells, which express ERβ but not ERα (10). Here, we demonstrate that ERβ induced Egr-1 expression via a non-genomic pathway involving the Raf/MEK1/Erk/Elk-1 signaling cascade.

RESULTS

Activation of estrogen receptor induces Egr-1 expression in C6 cells

To investigate whether or not ERβ stimulates Egr-1 expression, we used rat C6 glial cells, which express ERβ but not ERα (10). Serum-starved C6 cells were treated with increasing concentrations of 17β-E2 for 1 h, after which the amount of Egr-1 protein was measured by Western blotting. Treatment with 17β-E2 increased the abundance of Egr-1 protein in a concentration-dependent manner (Fig. 1A). Time-course analysis showed that 17β-E2 induced Egr-1 expression within 1 h, reaching a peak at 2 h, and then declined (Fig. 1B). Typical anti-estrogen ICI 182,780 exhibits antagonistic activity toward ERβ (11). Treatment with ICI 182,780 dose-dependently attenuated the accumulation of Egr-1 by 17β-E2 (Fig. 1C), suggesting that Egr-1 induction by 17β-E2 is mediated by ER. Since C6 cells express ERβ only, it was concluded that ERβ may play a role in the activation of the Egr-1 gene.

ERβ activates Egr-1 transcription

To determine whether or not the accumulation of Egr-1 protein by 17β-E2 was due to mRNA expression, we performed
Fig. 1. Effect of ERβ on expression of Egr-1 in C6 cells. (A and B) C6 cells were starved with 0.5% serum for 24 h and then treated or untreated with different concentrations of 17β-E2 for 1 h (A) or 10 μM 17β-E2 for the indicated time periods (B). (C) Serum-starved C6 cells were treated or untreated with ICI 182,780 for 30 min, followed by stimulation with 17β-E2 as indicated for 2 h. Total cell lysates were then prepared and probed with anti-Egr-1 antibodies. The same blot was reprobed with anti-GAPDH antibodies as an internal control. Each blot shown is representative of three separate experiments.

Fig. 2. Effect of ERβ on transcriptional activation of the Egr-1 gene. (A) Serum-starved C6 cells were treated with 10 μM 17β-E2 for the indicated time periods. Total RNA was then isolated from the cells, electrophoresed on a 1% agarose gel (10 μg/lane), and capillary-transferred to a nylon filter. The blot was hybridized with a 32P-labeled Egr-1 cDNA probe. The amount of Gapdh mRNA was used as an internal control for the amount of RNA in each lane. Each blot is representative of at least three separate experiments. (B) C6 cells were co-transfected with 0.5 μg of Pegr1-Luc(-780/+1) and 0.5 μg of an expression plasmid for ERα (pCMV/ERα) or ERβ (pCMV/ERβ), along with 50 ng of pRL-null vector. At 24 h post-transfection, firefly luciferase activity was normalized to Renilla activity. The data shown represent the mean ± S.D. of three independent experiments performed in triplicate. The statistical significance of the results was evaluated using Student’s t-test (*P < 0.05 compared with untreated cells; n = 9).

Erk MAPK is involved in ERβ-mediated Egr-1 promoter activation
It has been demonstrated that Egr-1 expression by ERα is mediated by a non-genomic pathway involving Erk MAPK in MCF-7 breast cancer cells (6-9). To determine whether or not ERβ also stimulates MAPK signaling, serum-starved C6 cells were treated with 17β-E2, and the activation status of three major MAPK pathways was measured using phospho-specific antibodies. Phosphorylation of Raf1 and Erk1/2 increased in a time-dependent manner in response to 17β-E2 treatment, whereas little changes in the phosphorylation of JNK and p38 MAPK were observed (Fig. 3A), suggesting that the Erk MAPK pathway is activated by 17β-E2 in C6 cells.

To determine the role of the Erk MAPK pathway in ERβ-induced Egr-1 expression, we examined the effect of U0126, a selective MEK1 inhibitor, on the accumulation of Egr-1 using Western blot analysis. As shown in Fig. 3B, pretreatment of C6 cells with U0126 completely blocked 17β-E2-induced accumulation of Egr-1. Furthermore, U0126 strongly reduced the Egr-1 promoter activity induced by ectopic expression of ERβ (Fig. 3C). These data suggest that the Erk MAPK pathway is involved in ERβ-mediated Egr-1 expression.

Elk-1 functions as an Erk downstream mediator in ERβ-mediated Egr-1 expression
Elk-1 is a member of the Ets family of transcription factors (12). It has been demonstrated that phosphorylation of Elk-1 by Erk
MAPK results in activation of Egr-1 promoter activity (13, 14). We next tested whether or not Elk-1 is involved in the transcriptional activation of Egr-1 by ERβ. The Egr-1 promoter reporter Pegr1-Luc(-780/+1) was co-transfected with expression plasmids for ERβ with or without Elk-1. As shown in Fig. 4A, ectopic expression of Elk-1 enhanced ERβ-mediated activation of the Egr-1 promoter. To assess whether or not Elk-1 functions as a downstream mediator of ERβ MAPK in ERβ-mediated Egr-1 expression, we examined the transcriptional activity of Elk-1 using a trans-activating reporter assay system. A Gal4-Elk1 trans-acting plasmid was co-transfected into C6 cells together with a pFR-Luc reporter construct containing Gal4-binding sequences. As shown in Fig. 4B, ectopic expression of ERβ increased the trans-acting activity of Gal4-Elk1, which was prevented by transfection of dominant negative (dn)-Raf, dn-MEK1, and dn-Erk2. These data suggest that ERβ stimulates Elk-1 trans-acting activity via the Raf/MEK1/Erk MAPK pathway in C6 cells.

To determine whether or not the Raf/MEK1/Erk/Elk-1 signaling cascade is functionally linked to ERβ-mediated Egr-1 expression, an Egr-1 promoter reporter, Pegr1-Luc(-780/+1), was co-transfected into C6 cells along with expression plasmids for dominant negative constructs of the MAPK pathway. As shown...
in Fig. 4C, the ability of ERβ to activate the Egr-1 promoter was strongly prevented by the expression of dn-Raf-1, dn-MEK1, and dn-Erk2. Thus, it was concluded that ERβ activates Egr-1 transcription via the Raf/MEK1/Erk/Elk-1 signaling pathway in C6 cells.

DISCUSSION

In this study, we investigated the role of ERβ in the regulation of transcription factor Egr-1 gene expression. The major finding of our study is that ERβ controls Egr-1 gene expression via a non-genomic pathway, which activates the Raf/MEK1/Erk/Elk-1 signaling cascade.

Estrogen receptors, ERα and ERβ, are traditionally considered to act as transcription factors that bind to EREs in promoter regions, leading to the regulation of gene expression. However, a non-transcriptional mechanism of signal transduction through ERα, a so-called non-genomic pathway, has been identified. This non-genomic pathway is associated with ERα, which is localized to the cell membrane and interacts with c-Src or growth factor receptor tyrosine kinase (RTK), thereby activating multiple signal transduction pathways such as the Ras/Raf/MEK1/Erk and PI-3K pathways. Similar to ERα, ERβ at the cell membrane could activate the non-genomic signaling pathway. Indeed, ERβ stimulates Erk MAPK signaling in ERβ-transfected CHO cells (13), and it interacts with Src in LNCaP prostate cancer cells (16). In this study, we found that Erk1/2, but not JNK1/2 or p38 MAPK, was rapidly activated following 17β-E2 treatment in C6 cells, which express ERβ only. Moreover, pretreatment with the MEK1 inhibitor U0126 or expression of dn mutants of Raf1, MEK1, or Erk2 blocked Egr-1 expression by ERβ. Thus, similar to ERα, ERβ activates the Raf/MEK1/Erk/Elk-1 signaling pathway to induce Egr-1 gene expression.

Egr-1 is a prototypical immediate early response gene whose expression is rapidly induced by stress, injury, mitogens, and differentiation factors (17-19). Transcriptional regulation of Egr-1 is regulated by multiple cis-acting elements in the Egr-1 gene promoter, including cAMP response element, NF-κB-like element, and serum response element (20). Two half palindromic estrogen response elements (EREs) separated by 20 bp exist in the promoter regions, leading to the regulation of gene expression.

Moreover, pretreatment with the MEK1 inhibitor U0126 or expression of dn mutants of Raf1, MEK1, or Erk2 strongly inhibited ERβ activation. Moreover, transient expression of dn mutants of Raf1, MEK1, or Erk2 strongly inhibited ERβ-mediated Elk-1 trans-activity. Based on these findings, we concluded that ERβ stimulates SRE in the Egr-1 promoter via Elk-1, which is activated by the Raf/MEK1/Erk MAPK signaling pathway.

MATERIALS AND METHODS

Cell culture and reagents
C6 rat glioma cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT). 17β-E2 and the MEK1 inhibitor U0126 were purchased from Calbiochem (La Jolla, CA). ICI 182,780 was obtained from Tocris (Ballwin, MO). Rabbit anti-Egr-1 and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-c-Raf (Ser338), anti-phospho-Erk1/2 (Thr202/Tyr204), anti-phospho-JNK (Thr183/Tyr185), and anti-phospho-p38 MAPK (Thr180/Tyr182) antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Plasmids
The full-length Egr-1 promoter-reporter plasmid expressing firefly luciferase [pEgr1(-780/+1)] is described elsewhere (20). Plasmid pFA2-Elk1 encoding a fusion protein consisting of the yeast Gal4 DNA-binding domain (residues 1-147) and the activation domain of Elk-1 (residues 307-427), along with plasmid pFR-Luc containing five Gal4-binding-element repeats upstream of the luciferase gene, were purchased from Stratagene (La Jolla, CA). The pRL-null plasmid encoding Renilla luciferase was purchased from Promega (Madison, WI). pCMV/ERα and pCMV/ERβ were gifts from Dr. I.W. Kim (Department of Life Science, Pohang University of Science and Technology, Korea). Expression plasmids for dn-Raf1 (pSV-SPOT/c-Raf1DN), dn-MEK1 (pCGN1/DNMEK1), and dn-Erk2 (pHA-Erk2 K52R) were kindly provided by Dr. P.-G. Suh (Department of Life Science, Pohang University of Science and Technology, Korea). pCMV/flagElk1 was a gift from Dr. A.D. Sharrocks (Faculty of Life Science, University of Manchester, United Kingdom).

Northern blot analysis
Total RNA (10 μg) from each sample was separated by electrophoresis on a formaldehyde/agarose gel and then transferred to a Hybond N+ nylon membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Northern blotting was performed using a [α-32P]dCTP-labeled cDNA probe, followed by hybridization with a glyceraldehyde-3-phosphate dehydrogenase (Gapdh) cDNA probe, as described previously (25).

Western blot analysis
Cells were lysed in 20 mM HEPES (pH 7.2) containing 1%
Triton X-100, 10% glycerol, 150 mM NaCl, 10 μg/ml of leupeptin, and 1 mM PMSF. The protein samples (20 μg each) were then separated by 10% SDS-PAGE and transferred to nitrocellulose filters. The blots were incubated with the appropriate primary antibodies and developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech Inc.).

**Egr-1 promoter reporter assay**

C6 cells were seeded into 12-well plates and transfected with 0.5 μg of the Egr-1 promoter. Where indicated, mammalian expression vectors encoding dn-Raf, dn-MEK1, and dn-Erk2 were also included. At 24 h post-transfection, the levels of firefly and Renilla luciferase activity were measured as described previously (26).

**Trans-acting reporter assay**

Elk-1 transacting activity was measured using a luciferase reporter assay system. C6 cells were co-transfected with 50 ng of trans-activator plasmid (pFA2-Elk1) and 0.5 μg of reporter plasmid (pFR-Luc). pRL-null plasmid (50 ng) was included in all of the samples in order to monitor transfection efficiency. After transfection, the levels of firefly and Renilla luciferase activity were assayed.

**Statistical analysis**

Each experiment was repeated at least three times. All data are plotted as the mean ± S.D. The statistical significance of the data was evaluated using Student’s t-test. A P-value < 0.05 was considered to be significant.

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