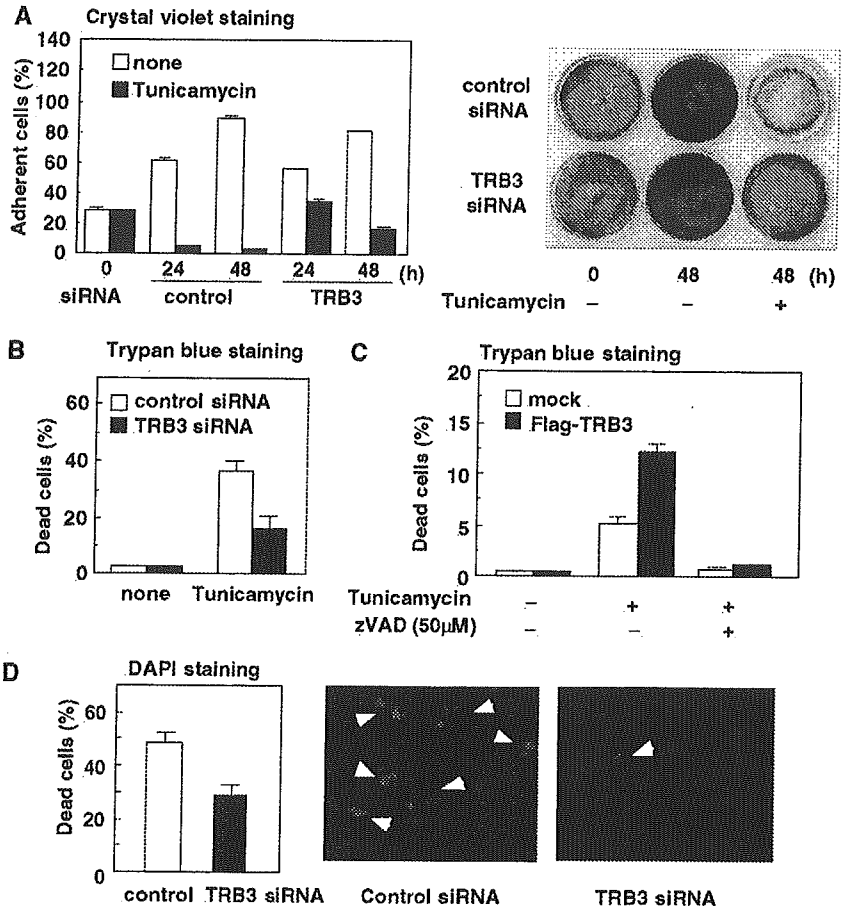

Figure 8 TRB3 induces ER stress-dependent cell death. (**A**) 293 cells were transiently transfected with control siRNA or TRB3 siRNA. After 48 h, cells were treated with 2 μg/ml of tunicamycin for the indicated periods. The percentage of adherent cells was measured by crystal violet staining. The right panel shows the stained cells treated with tunicamycin for 48 h. (**B, C**) HeLa cells (B) were transiently transfected with control siRNA or TRB3 siRNA. 293 cells (C) were transiently transfected with control vector or Flag-TRB3. After 48 h, cells were treated with 2 μg/ml of tunicamycin for 24 h or left untreated in the absence or presence of 50 μM of zVAD. The percentage of dead cells was measured by Trypan blue staining. (**D**) HeLa cells were transiently transfected with control siRNA or TRB3 siRNA. After 48 h, cells were treated with 2 μg/ml of tunicamycin for 24 h. Apoptotic cells were measured by staining with DAPI. Arrowheads in the right panel indicate apoptotic cells. Similar results were obtained in three independent experiments.

(Figures 2D and 3F, and Supplementary Figure S9). These results suggest that the regulation of CHOP activity by TRB3 requires the association not only with CHOP but also with other molecule(s), for example, coactivator(s), corepressor(s) or other members of TRB family. Alternatively, TRB3/SKIP3 and its family contain the classic substrate-binding domains (C-terminal region) of a protein kinase, but lack the ATP-binding and kinase-activation domains (N-terminal region) (Bowers *et al*, 2003), so original kinase(s) or other modifying enzyme(s) cannot recruit to CHOP when CHOP binds to TRB3. TRB3 could be a novel type of endogenous kinase inhibitor, acting as a decoy kinase-like protein for CHOP, Akt or other substrates.

TRB3 functions as a negative regulator of ATF4, another bZIP protein that is critical for the induction of CHOP, as well. As TRB3/SKIP3 promotes the degradation of ATF4 (Bowers *et al*, 2003), this function may be explained by the ATF4 protein degradation.

Several genes are activated by ER or mitochondrial stress in a CHOP-dependent manner; however, the activation of these target genes does not account for CHOP-dependent cell growth inhibition. High levels of sustained ER stress can result in apoptosis, and some of the factors controlling this response, the activation of c-Jun N-terminal kinase (JNK) (Nishitoh *et al*, 2002), the activation of caspase-12 (Nakagawa *et al*, 2000) and transcriptional induction of CHOP (Zinszner *et al*, 1998), have recently been identified. During ER stress, CHOP is induced by a PERK–ATF4 pathway and/or IRE1/ATF6 pathway. The reports from the studies of overexpression or knockout of CHOP indicate that CHOP is related to ER stress-induced apoptosis; however, there is a missing link in the CHOP-mediated apoptosis-signaling pathway. We have indicated that *TRB3* is a CHOP target gene and induces apoptosis during ER stress. Very recently, TRB3 has been shown to be a crucial factor for insulin resistance, and binds to Akt/PKB to inhibit its kinase activity (Du *et al*, 2003). Akt is known to maintain cell survival, by inhibiting apoptosis, and promote cell cycling (Brazil and Hemmings, 2001). On the other hand, it has recently been shown that CHOP-induced apoptosis is mediated by translocation of Bax

from the cytosol to mitochondria (Gotoh *et al*, 2004). In addition, it has been described that the dephosphorylation of Akt is essential to the Bax conformational change and translocation to mitochondria of Bax (Pervin *et al*, 2003; Rathmell *et al*, 2003). Taken together, our observations and reports suggest that TRB3 could be the link between ER stress-induced CHOP and Akt dephosphorylation in inducing translocation of Bax to induce cell death.

Recently, there have been several reports that CHOP acts as an antiapoptotic factor (Southwood *et al*, 2002; Mayerhofer and Kodym, 2003). Southwood *et al* (2002) showed that oligodendrocytes but not kidney cells undergo apoptosis with greater frequency in Chop/rsh null mice than in controls. These data reveal a major divergence in the importance of CHOP as a crucial mediator of cell death for different cell types that conceivably could be related to the cell type-specific target genes of this transcription factor. A likely possibility is the pattering of CHOP with oligodendrocyte-specific bZIP transcription factor. Further studies should be carried out to resolve this question.

In a study using CHOP or NF-IL6 knockout MEFs, Wang *et al* have indicated that several CHOP target genes, *DOC1* (stress-induced form of carbonic anhydrase VI), *DOC4* and *DOC6*, are expressed dependent on the presence of NF-IL6 as well as CHOP. In this paper, by knockout/knockdown analysis, we have revealed that TRB3 expression during ER stress is independent of the presence of NF-IL6, but that the ATF4–CHOP pathway is crucial for this expression. From the promoter analysis, unlike CHOP-binding site, NF-IL6 alone negatively regulated the activation of the *TRB3* promoter (Figures 3C and 6B). Overexpressed NF-IL6 supported the ectopic CHOP-dependent activation of *TRB3* promoter probably because it worked as a dimerization partner of CHOP as shown in Figure 4B. However, tunicamycin-induced endogenous CHOP, whose expression level might be quite low, may choose ATF4 as a dimerization partner for *TRB3* promoter activation, probably because the DNA-binding affinity of ATF4 to *TRB3* promoter is higher than that of NF-IL6. ATF4 expression alone activated the *TRB3* promoter; however, the promoter mutagenesis study revealed that this activation is not correlated with tunicamycin-induced activation. CHOP-induced *TRB3* promoter activation is usually correlated with tunicamycin-induced activation, and TRB3 induction was late compared to CHOP induction. These findings suggest that ATF4 expression alone is not sufficient for TRB3 induction, and CHOP is essential for this as well. Indeed, CHOP and ATF4 cooperated to activate the *TRB3* promoter, and the knockdown of CHOP significantly repressed the TRB3 expression. Here, we show that ATF4 is a novel partner of CHOP in the PERK–eIF2α pathway during ER stress.

CHOP is also induced by other stress signals such as oxidative stress, amino-acid deprivation and hypoxia. Therefore, it is possible that the CHOP–TRB3 pathway operates in response to these stresses as well. *TRB3* is one of the targets of CHOP, and acts as a regulator of CHOP as well, suggesting that CHOP signaling is strictly regulated by TRB3 via a negative feedback mechanism. In terms of the physiological significance, TRB3 could be a sensor for ER stress-induced apoptosis. If the ER stress is transient and mild, the induced TRB3 blocks the CHOP and ATF4 function by binding to them. However, when potent and prolonged ER stress occurs, excess TRB3 will be produced and lead to apoptosis. TRB3 may be a potential therapeutic target for diseases with stress-dependent cell death, such as neurodegenerative diseases or type I diabetes.

Materials and methods

Reagents

RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), anti-β-actin monoclonal antibody (AC-15), anti-Flag monoclonal antibody (M2), tunicamycin and MMS were purchased from Sigma (St Louis, MO). Fetal bovine serum (FBS) was from HyClone (Logan, UT). Anti-Myc monoclonal antibody (9E10) was from Roche (Indianapolis, IN). Anti-GST monoclonal antibody (DG 122-2A7) was from Upstate Inc. (Lake Placid, NY). Anti-GADD153 monoclonal antibody (B-3) was from Santa Cruz (Santa Cruz, CA). A23187 was obtained from Calbiochem (La Jolla, CA); carbobenzoxyl-L-leucyl-L-leucyl-L-leucinal (MG132) and benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone (zVAD) were obtained from Peptide Institute (Osaka, Japan). Cycloheximide was obtained from Nacalai Tesque (Kyoto, Japan).

Cell culture

The human melanoma cell line A375, the human embryonic kidney cell line 293 and the human hepatocellular carcinoma cell line HepG2 were cultured as described previously (Hattori *et al*, 2001, 2003a).

Construction of expression plasmids

TRB3 (GenBank accession #AK026945) was kindly provided by the human cDNA sequencing project of the New Energy and Industrial Technology Development Organization (NEDO) in Japan (Ota *et al*, 2004). pCMV5-Flag-TRB3, pCMV5-Flag-TRB3ΔN lacking aa 1–127, pCMV5-Flag-TRB3ΔC lacking aa 283–358, pCMV5-Flag-TRB3N179 lacking aa 180–358, pCMV5-Flag-TRB3C179 lacking aa 1–179 or pCMV5-Flag-TRB3Δ239–265 lacking aa 239–265, the region essential for the binding with Akt1 of human *TRB3* were generated by PCR. pGEX-TRB3(1–50) was constructed by ligating pGEX-6P-1 (Amersham Bioscience, Little Chalfont, UK) with the *TRB3* cDNA fragment (corresponding to aa 1–50). The plasmids pcDNA3.1-Myc-CHOP, pcDNA3.1-Myc-CHOPSer^{79,82}Ala, pcDNA3.1-Myc-CHOPΔBR, pcDNA3.1-Myc-CHOPΔLZ and pCMV-Flag-NF-IL6 were constructed as described previously (Hattori *et al*, 2003a,b). pcDNA3.1-Myc-CHOPΔN9, ΔN18, Δ19–26 and ΔN70 lacking the amino-terminus of human CHOP were generated by PCR. Gal4-CHOP(WT) was kindly provided by Dr D Ron (New York Univ.). pCMV5-Gal4-CHOPΔN9, ΔN18 and ΔN70, pcDNA3.1-NF-IL6 and pcDNA3.1-CHOP were generated by PCR. To obtain p(CHOP)$_4$-Luc, four tandem repeats of the CHOP-binding element were amplified by PCR and ligated with pGL3 promoter (Promega, Madison, WI). pTRB3-Luc was generated by ligating the human TRB3 promoter region (−1265 to +609) with pGL3-basic. pcDNA3.1-Flag-ATF4, pcDNA3.1-Flag-XBP1, pcDNA3.1-Flag-NF-YA and pcDNA3.1-Flag-NF-YA DN, replacing Arg^{283}, Gly^{284} and Glu^{285} with Ala, were generated by PCR. To obtain p(ERSE)$_2$-Luc or p(UPRE)$_2$-Luc, two tandem repeats of ERSE or two tandem repeats of the UPR response element, respectively, were amplified by PCR and ligated with pGL3 promoter. All constructs were verified by sequencing.

Reporter gene assays

Cells were transfected with luciferase reporter plasmids, and at 24 h post-transfection, treated for specific periods with ER stressors. Lysates were prepared and luciferase assays were performed according to the manufacturer's instructions (Promega). All experiments were performed a minimum of three times before calculating means and standard deviations.

RNA extraction, RT–PCR and Northern blot analysis

Total RNA was extracted from cells seeded in 60 mm plates. The RT-PCR and Northern blot analysis used were described previously (Matsumura *et al*, 2000). Human *TRB3*, *CHOP*, *GAPDH*, and mouse *TRB3*, *CHOP-10*, carbonic anhydrase VI, *GAPDH*-radiolabeled hybridization probes were generated using the individual cDNA fragments. Primers used for human *TRB3* were 5′-ATGCGAGCCA CCCCTCTAGC and 3′-CTAGCCATACAGAACCACTTC; for human *CHOP* were 5′-GCGTCTAGAATGGCAGCTGAGTCATTGCC and 3′-GC

GTCTAGATCATGCTTGGTGCAGATTC; and for human *GAPDH* were 5′-TGAAGGTCGGAGTCAACGGATTTGGT and 3′-CATGTGGGCCATG AGGTCCACCAC.

Preparation of antiserum against human TRB3

TRB3 (1–50) protein fused with GST was prepared by transformation of JM109 with pGEX-TRB3 (1–50). This region of TRB3 (aa 1–50) has little homology with other human orthologs of *tribbles*, TRB1 or TRB2. Two female Japanese White rabbits (2–3 kg) were immunized with recombinant TRB3 (1–50) protein four times and bled to prepare the antiserum against human TRB3.

Immunoprecipitation and Western blot analysis

Cells were transiently transfected and treated as described in the figure legends. The cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate and 1% Triton X-100) supplemented with protease inhibitors. The lysates were subjected to immunoprecipitation, and 1–2% of the lysate or co-immunoprecipitate was subjected to SDS–PAGE (12.5%), transferred onto PVDF membrane and probed with antibodies indicated in the figure legends. The immunoreactive proteins were visualized using ECL Western blotting detection reagents (Amersham Bioscience), and light emission was quantified with a LAS1000 lumino image analyzer (FUJI, Japan).

RNA interference

Double-stranded RNA duplexes corresponding to human TRB3 (5′-CGAGCUCGAAGUGGGCCCC-3′), human ATF4 (5′-GCCUAGGUCUC UUAGAUGA-3′) and human CHOP (5′-GCCUGGUAUGAGGACCU GC-3′) were designed. Control siRNA was used for the scramble II duplex (5′-GCGCGCUUUGUAGGAUUCG-3′). They were purchased from Dharmacon Inc. (Chicago, IL).

Transfection

A375 cells were transfected by a lipofection method using Effectene (Qiagen, Hilden, Germany) according to the manufacturer's instructions. 293 and HepG2 cells were transfected by the Chen-Okayama method (Chen and Okayama, 1987)

Cell proliferation assay and apoptosis assay

Cells were washed with PBS. Crystal violet was added to stain cells and the stained cells were lysed in 1% SDS and measured at an OD of 595. Cells were pelleted and washed with PBS. Trypan blue was added to cell pellets and stained cells were counted as dead.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Dr Sumio Sugano, Dr Toshio Ota, Dr David Ron and Dr Shizuo Akira for generously providing expression plasmids and MEFs deficient in NF-IL6 and Dr Ryuichiro Sato for discussion. This work was supported in part by Grant-in-Aids for Scientific Research (B) from Japan Society for the Promotion of Science and Grant-in-Aids for Scientific Research on Priority Areas (C) from the Ministry of Education, Science, Sports and Culture.

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日本臨牀　63巻 増刊号8（2005年8月28日発行）別刷

広範囲 血液・尿化学検査 免疫学的検査

—その数値をどう読むか—
［第6版］

(4)

IX. プロスタノイド，サイトカイン，増殖因子，ケモカイン

インターロイキン-1(IL-1) α，β，IL-1受容体，IL-1受容体アンタゴニスト(IL-1ra)

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インターロイキン-1(IL-1) α，β，IL-1受容体，IL-1受容体アンタゴニスト(IL-1ra)

Interleukin-1(IL-1) α， β， IL-1 receptor,
IL-1 receptor antagonist(IL-1ra)

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Key words: IL-1α, IL-1β, IL-1 receptor(IL-1R), IL-1 receptor antagonist(IL-1ra), soluble IL-1R(sIL-1R)

1. 概　　説

インターロイキン-1(IL-1)は主に単球・マクロファージから産生される分子量17.5kDaのサイトカインであり，免疫，炎症，造血，内分泌など種々の生体反応に重要な役割を果たしている[1,2]．IL-1には等電点の異なるα，β型の2種類存在する．いずれのIL-1も同じ受容体に結合し，ほぼ同様の活性を示す．いずれも約30kDaの前駆体として産生され，IL-1αはカルパイン，IL-1βはIL-1β変換酵素(ICE，caspase 1)によって切断，成熟化され，分泌される．いずれも分泌蛋白特有のシグナルペプチドを欠いている．IL-1αは前駆体でも活性を示し，細胞膜結合型としても存在するが，IL-1βは成熟体のみが活性を示す．

受容体(IL-1R)にはI型とII型の2種類が存在するが，現在のところ，II型は全くシグナルを細胞内に伝達できず，IL-1のI型受容体への結合を調節していると考えられている．また，同じIL-1Rスーパーファミリーの一つで，IL-1の結合能はないが，IL-1R(I)にIL-1が結合後，複合体を形成し，その後のシグナル伝達に必須であるIL-1RAcP(IL-1R accessory protein)も同定されている．いずれの受容体とも可溶型のものが存在しており，IL-1の作用を制御していると考えられている．

IL-1ファミリーには，ほかにIL-1受容体アンタゴニスト(IL-1ra)が知られている[2,3]．IL-1raには現在，分泌型(sIL-1ra)のほか，非分泌型(icIL-1ra)のアイソフォームも報告されているが機能ははっきりしていない．IL-1raはIL-1に比べ親和性は低いが，両IL-1Rに結合し，自らはシグナルを伝達できず，IL-1α，βの結合を競合的に阻害することにより，IL-1の作用を抑制する．その親和性はII型よりもI型の受容体の方が高い．

更に，IL-1ファミリーに属する分子としてIL-18のほか，最近，次々に新しい分子(IL-1F5～IL-1F10)が見いだされている．IL-1F9，IL-1F7のように活性が報告されているものもあるが，多くのものはまだ機能が明らかにされていない．

2. 検査の目的

IL-1は種々の活性を示すが，特に炎症時には内因性発熱因子として働き，肝臓からの急性期蛋白の誘導，白血球増加，PGE_2，IL-6，IL-8などの産生を誘導する．また，エンドトキシンショックの重要なメディエーターである．何らかの理由で局所的，恒常的にIL-1が産生されると，**表1**に示すような慢性炎症性疾患がもたらされる．また，疾患により，IL-1ばかりではなく，IL-1raおよび可溶型のIL-1R(sIL-1R)が産生され，IL-1の作用を制御していると考えられている．したがって，体液中，あるいは単球・マクロファージから産生されるIL-1やIL-1ra，sIL-1Rの活性や量を測定することは病態を知るうえで重要である．

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0047-1852/05/¥40/頁/JCLS

表1 IL-1の関与が示唆される疾患

疾患名	IL-1の作用
関節リウマチ，ライム病	発熱，滑膜細胞の増殖，軟骨の破壊，骨破壊
骨粗鬆症	骨吸収の促進
川崎病	血管の損傷
エンドトキシンショック	血管の損傷，低血圧
toxic shock syndrome	血管の損傷
虚血再灌流に伴う組織障害	血管の損傷
痛　風	尿素結晶による局所で産生されるIL-1による炎症
I型糖尿病	β-ランゲルハンス細胞の障害
糸球体腎炎	メサンギウム細胞の増殖，PGE_2産生誘導
拡張性心筋症	β-受容体作動薬に対する心筋収縮の抑制
coxsackieウイルス感染症の自己免疫性心筋炎	心臓浸潤炎症性細胞によるIL-1産生
子宮内膜炎	受精卵の発育阻止
早　産	PGE_2，$PGF_{2\alpha}$産生誘導による子宮収縮
肉芽腫	肉芽腫形成促進，線維芽細胞増殖
強皮症	線維芽細胞の活性化
B細胞慢性リンパ白血病に伴う骨髄の線維化	線維芽細胞増殖
急性骨髄性白血病	オートクリンな増殖促進
Kaposi肉腫	オートクリンな増殖促進
Alzheimer症候群，Down症候群	ミクログリアによるIL-1産生とアストログリアの増殖，APPの分解促進
実験的アレルギー性脳脊髄炎	炎症の惹起・促進

3. 試料の採取方法，保存条件

細胞の培養上清の場合には，遠心後上清を−20℃以下で保存すれば問題はない．血漿，血清，その他の体液の場合には，なるべくEDTAあるいはヘパリン添加の状態で採取し，早急に細胞，沈殿物を取り除いたのち，0.45 μmのフィルターを通し微量の不溶物(主に，脂肪)を取り除いたものを用いるとよい．やはり，測定開始までは冷却しておいた方がよい．

4. 測　定　法

原理的には，IL-1に特異的に反応する細胞を用いたバイオアッセイ法と特異的抗体を用いた免疫学的アッセイ法(ELISA，EIA，RIA)がある．最近ではその簡便さや特異性の観点から免疫学的アッセイ法が主流になっている．

a. バイオアッセイ法

歴史的にはマウス胸腺細胞をコンカナバリンA(ConA)存在下，IL-1で刺激し，増殖を調べるLAF(lymphocyte activating factor)活性測定法が知られている．その他，IL-1特異的に増殖するマウスTh2型ヘルパーT細胞株D10(N4)Mを用いる方法[4]，IL-1刺激によりT細胞から誘導されるIL-2を測定する方法，線維芽細胞からのPGE_2産生誘導能，ヒトメラノーマ細胞株(A375)に対する増殖阻害活性を指標に調べる方法などが知られている．

b. 免疫学的アッセイ法

抗IL-1α，IL-1β抗体を用い，IL-1α，βをそれぞれ交差なく測定できるキットが各社から市販されている．また，IL-1raおよびsIL-1R(II)の測定キットも入手可能である．多くのキットはELISA法によるものであるが，EIAやRIAによる測定キットも市販されている．

ELISAなどの場合，測定値が必ずしもIL-1活性を表しているとは限らない．前駆体と成熟体をともに認識する抗体と，個々に認識する抗体を用いる場合とでは値が変わってくる．先述したように，IL-1βの場合，その前駆体に活性

はみられないため，重要なポイントである．最近では，IL-1β成熟体だけを特異的に検出するもの，あるいは，前駆体とは10%ほどの交差しか示さず，IL-1β成熟体を選択的に測定できるキットなどが市販されている．また，前駆体だけを検出するキットも入手可能になり，いろいろと組み合わせれば，より正確な測定が可能になってきている．

また，最近，サスペンションアレイ法(Bio-Rad社など)，あるいは蛍光マイクロビーズアレイ法(Luminex社，BD Pharmingen社など)と呼ばれる，蛍光ビーズとフローサイトメーターとを組み合わせた手法が開発され，一度に多種類のサイトカインを定量することが可能となっている．複数のサイトカインに対する特異的抗体を各スポットにコートしたものに試料を加え，ELISAを行うプロテインアレイ法(TAKARAバイオ社，Novagen社など)という方法が開発され，多種のサイトカインのハイスループットスクリーニングが可能になってきている．更に，IL-1産生細胞の定量法として，フローサイトメーターを用いる細胞内サイトカイン測定キットやメンブランを用いるELISpotキットが各社から販売されている．これらはいずれも特異的抗体を用いた免疫学的定量法である．

5. 基 準 値

ヒト血漿中のIL-1は，正常の状態では極めて低く検出が難しいが，報告によればIL-1βとして44.7±4.4 pg/ml(n=21，26-54歳，平均38歳)である[5]．また，IL-1raは681 pg/ml(n=200)[6]，sIL-1R(I)，sIL-1R(II)はそれぞれ，2.0±1.5 ng/ml(n=114)，4.76±0.16 ng/ml(n=134)という報告がある[7]．

6. 生理的変動(測定に影響を及ぼす因子)

激しい運動をした後のヒトの血漿中のIL-1β量は上昇していると報告されている．また，女性の場合，血中IL-1β量は月経周期に連関した挙動を示すことが知られている．測定に影響を及ぼす因子については様々なものがある．バイオアッセイに関しては，IL-1特異的細胞を用いているとはいえ，未知の因子の影響が考えられる．また，αとβ型IL-1の区別がつかない．そこで，必要であれば，それぞれのIL-1に対する中和抗体や，IL-1raなどを添加し，活性が抑制されることを確認する．バイオアッセイ，免疫学的アッセイにかかわらず，血清中にIL-1raのほか，sIL-1R，あるいはIL-1αに対する自然抗体が存在している場合，あるいは可溶性受容体やα2-マクログロブリンなどの結合蛋白によって干渉され，測定値が変化することもある．

血清，血漿中には未知の活性阻害物質が存在する．そこで，ゲル濾過カラムで分画するか，血漿をクロロホルムで抽出したり，ポリエチレングリコールで処理し，阻害因子を除く方法も試みられている．最近のELISAなどのキットでは，血漿や血清などの検体でも測定可能なものもみられる．

7. 臨床的意義

一般的に，多くの炎症時には，IL-1特に，IL-1βの産生が亢進し，続いて，IL-1raやsIL-1Rの産生が誘導され，IL-1の作用を制御すると考えられる．しかし，多くの炎症性疾患などでは，恒常的なIL-1αやIL-1βの産生が起こっている．また，ある種の癌では，IL-1raがその増殖の間接的な促進因子として作用していることも報告されている．'検査の目的'の項や，**表2**に示すように，敗血症や関節リウマチなど，種々の炎症性疾患の病態を知るうえで意義がある．また，活性期サルコイドーシス患者のLPS刺激肺胞マクロファージ，慢性疲労症候群患者のLPS刺激血液単核球，強皮症患者の無刺激単球からのIL-1やIL-1raの産生は正常人に比べ，亢進している．以上のように，単球からのIL-1，IL-1ra産生能も病態を示す一つの指標となる．

表2 体液中のIL-1，IL-1ra，sIL-1Rレベルの上昇が認められる疾患

サイトカイン	疾患	体液
IL-1α	細菌感染嚢胞性線維症	喀痰中
IL-1β	潰瘍性大腸炎，Crohn病	血中
	細菌感染嚢胞性線維症	喀痰中
	人工透析	血中
	敗血症	血中
	DIC	血中
	関節リウマチ	血中，関節液中
	熱傷	血中
	統合失調症	血中
	panic disorder	血中
	前立腺炎	精液
	慢性肝炎	血中
	細菌性髄膜炎	血中，髄液中
	慢性骨髄性白血病	血中
	散在性汎気管支炎	気管支洗浄液中
	癒着性腹膜炎(手術後)	血中
IL-1ra	手術	血中
	IL-2，IL-4，IL-6投与癌患者	血中
	子癇前症	血中
	高IgD症候群	血中
	エンドトキシン，IFNα投与健常人	血中
	敗血症	血中
	潰瘍性大腸炎，Crohn病	血中
	細菌感染嚢胞性線維症	血中，喀痰中
	熱傷	血中
	紫外線皮膚炎	角質層中
	成人性呼吸窮迫症候群	気管支肺洗浄液中
	無精子症，精子奇形	精液中
	老化	血中
	尿路感染症	血中
	慢性肝炎	血中
	人工透析	血中
	細菌性髄膜炎	血中，髄液中
	散在性汎気管支炎	気管支洗浄液中
	慢性関節炎(破壊性)	血中
	統合失調症	血中
	気管支喘息	血中
sIL-1R(I)	*手術	血中
sIL-1R(II)	(大きな)手術	血中
	敗血症	血中
	細菌性髄膜炎	血中，髄液中
	慢性関節炎(非破壊性)	血中
	Alzheimer症候群	脳脊髄液中

*濃度が低下する.

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International Immunopharmacology 6 (2006) 170–181

International Immunopharmacology

www.elsevier.com/locate/intimp

Estrogen stimulates transcription of human immunodeficiency virus type 1 (HIV-1)

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Received 14 June 2005; received in revised form 14 July 2005; accepted 27 July 2005

Abstract

Gene expression from human immunodeficiency virus (HIV) provirus is a crucial step for the viral replication. Here we examined a potential role of 17β-estradiol (E2) in HIV-1 transcription. Transient luciferase expression studies revealed that E2 activated HIV-LTR reporter gene in HEK293 cells when the cells were co-transfected with estrogen receptor α (ERα) but not ERβ expression plasmid. This E2 effect was abrogated by a specific antagonist to ER, ICI 182,780, indicating that it was mediated by ERα. Mutation analysis revealed that Sp1 binding site but not nuclear factor-kappa B (NF-κB) binding site of HIV-1 LTR is critical to the E2 effect. In addition, whereas E2 could not induce DNA-binding activity of NF-κB, E2 could augment both Sp1 DNA-binding and transcriptional activity. These findings suggest a contribution of estrogen for HIV-1 replication through ERα by augmenting Sp1 DNA-binding and transcriptional activity.

Keywords: HIV; Estrogen; Estrogen receptor; Sp1; NF-κB

1. Introduction

It is well documented that women are more vulnerable than men in acquiring HIV infection in heterosexual encounter, which has been ascribed to biological factors, such as the greater area of mucous membrane exposure during sexual intercourse in women than men, the greater quantity of fluids transferred from men to women, the higher viral content of male sexual fluids, and the micro mechanical tears that can occur in vaginal (or rectal) tissue from sexual penetration [1–3]. Social factors are also expected to contribute to the gender difference [4]. It is also reported that the rate of disease progression is greater

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doi:10.1016/j.intimp.2005.07.017

in women than men as shown by the rapid declines in $CD4^+$ cells [5] and higher rate of HIV-1 replication over time in women than in men [6].

Estrogen is a female sex hormone that has been recognized as a risk factor for breast and endometrial cancer, and autoimmune diseases [7]. There are two types of estrogen receptors, namely estrogen receptor α (ERα) and estrogen receptor β (ERβ) [8,9]. Following ligand binding, ERα and ERβ form homo- or heterodimmers that bind to specific DNA sequences (estrogen responsive element, ERE) of target genes through its DNA-binding domain (DBD), and activate the transcription of vitellogenin [10], progesterone receptor [11] and c-Myc [12] genes. Recent studies have revealed that ERα physically interacts with many transcription factors, including Sp1 [13–15], AP-1 [16], NF-κB [17], C/EBPβ and STAT5 [18], and modulates their transcriptional activity. Therefore, estrogen can modulate target gene activation through ER in both ERE-dependent and ERE-independent manners.

On the other hand, it is well known that nuclear factor-κB (NF-κB) and Sp1 are potent cellular activators of HIV-1 gene expression [19–21], and activation of NF-κB together with constitutively active Sp1 could trigger the transcription of viral genes in cells chronically infected with HIV-1 [22–25]. Therefore, it is possible that estrogen activates HIV-1 transcription through interacting with NF-κB or Sp1. In this study we show that 17β-estradiol (E2) stimulates HIV-1 transcription via ERα but not ERβ by augmenting Sp1 DNA-binding and transcriptional activity.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) without phenol red, 17β-estradiol (E2) and anti-β-actin monoclonal antibody (AC-15) were purchased from SIGMA (St. Louis, MO). ICI 182,780 was from Torcris Cookson, Inc. (Ballwin, MO). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT) and was used after treatment with charcoal to remove serum-derived estrogen. Anti-human ERα rabbit antibody (HC-20), anti-human ERβ rabbit antibody (HC-150), anti-human p65 rabbit antibody (SC-372), anti-human Sp1 rabbit antibody (SC-59) and anti-human Sp3 rabbit antibody (SC-644) were purchased from Santa Cruz Biotech, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) was from Jackson ImmunoResearch Lab. Inc. (West Grobe, PA). Horseradish peroxidase-conjugated sheep anti-mouse IgG was purchased from Amersham Bioscience.

2.2. Cell cultures

A human embryonic kidney cell line 293 was obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and a human mammary tumor cell line MCF-7 was kindly provided by Dr. H. Utsumi (Kyushu Univ., Fukuoka, Japan). These two cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin [26].

2.3. Plasmids

The luciferase reporter plasmids containing the full length LTR (LTR-FF-Luc (κBw)) and with mutated two κB sites (MJW-37-Luc (κBm)) were generous gifts from Dr. J. Kam [27]. HIV-1 LTR-based luciferase expression plasmids including CD12-Luc (containing the full-size LTR U3 and R), CD23-Luc (containing positions −117 to +80 of HIV-1 LTR), CD52-Luc (containing positions −65 to +80 of HIV-1 LTR) and CD54-Luc (containing positions −48 to +80 of HIV-1 LTR) were constructed as described previously [24]. Expression vector pCIneo-Sp1 and GAL4-Sp1 were generous gifts from Dr. S. Kojima (RIKEN, Wako, Japan) [28]. Expression vectors pcDNA3-ERα and pcDNA3-ERβ, and a reporter plasmid $pERE_3tk$-Luc (containing three tandem repeats of ERE) were generous gifts from Dr. S. Kato (Univ. Tokyo, Japan) [29]. pFR-Luc (5× GAL4-Luc) and pFC2-dbd (GAL4-dbd) were purchased from Stratagene (La Jolla, CA).

2.4. Transfection conditions and luciferase assay

Transient transfection of 293 cells was performed by the Chen–Okayama method [30]. After 16 h of transfection, cells were incubated with the indicated concentrations of E2 for additional 24 h and then harvested. Gene expression was measured by luciferase activity as previously described [31]. Transfection efficiency was monitored by β-galactosidase activity with pCMV-β-gal plasmid as an internal control. All luciferase activities shown in transient transfection assays were corrected by the internal control activity of β-galactosidase activity by pCMV-β-gal.

2.5. Electrophoretic mobility shift assay (EMSA)

293 cells were transfected with or without pcDNA3-ERα plasmid. After 16 h of transfection, cells were treated

with or without 10 nM E2 or TNFα (1 ng/ml) for 24 h. Preparation of nuclear extracts and EMSA was carried out as previously described [19,32,33]. The double stranded oligonucleotide probe for NF-κB or GC rich sequence was synthesized and end-labeled by γ-^{32}P-ATP. Both sequences were taken from the HIV-1 LTR. The κB sequence used was forward (5′-TTTCTAGGGACTTTCC-GCCTGGGGACTTTCCAG-3′) and complement (5′-TTT-CTGGAAAGTCCCCAGGCGGAAAGTCCCTAG-3′). The GC rich sequence used was forward (5′-CAGGGA-GGCGTGGCCTGGGCGGGACTGGGGAGTGGCGTC-3′) and complement (5′-G ACGCCACTCCCCAGTCCCGC-CCAGGCCACGCCTCCCTG-3′). Nuclear extracts were incubated in 10 μl EMSA buffer containing the radio-labeled oligonucleotide probe.

2.6. RNA preparation and reverse transcriptase (RT) reaction

Total RNA from cells was extracted according to the method of Chomczynski and Sacchi [34]. The amount of RNA was determined spectrophotometrically. The RT reaction was performed by incubating 2 μg of total RNA in 20 μl reaction volume containing 50 mM Tris–HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTP, 40 ng Random Primer $p(dN)_6$, 6 U ribonuclease inhibitor and 40 U M-MLV Reverse Transcriptase. The reaction mixtures were incubated at 37 °C for 60 min, then followed by 70 °C for 10 min in Gene Amp PCR System 2400 (Applied Biosystems). The reaction mixtures were diluted to five fold of volume with sterilized distilled H_2O and used as cDNA solutions. cDNA samples were stored at −20 °C until PCR analysis.

2.7. PCR analysis

Primers used for human ERα were 5′-GTCTGA-GGCTGCGGCGTTCGGCTCC-3′ and 5′-ATTCCATAGC-CATACTTCCCTTGTC-3′, for human ERβ were 5′-GGCAACTACTTCAAGGTTTCGAGAG-3′ and 5′-ACTC-GCATGCCTGACGTGGGACAGG-3′, and for human GAPDH were 5′-TGAAGGTCGGAGTCAACGGATTT-GGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′. PCR reactions contained 1× PCR buffer, 0.4 mM dNTPs, 2.5 ng forward and reverse primers, 0.005 U Ampli Taq Gold™ DNA Polymerase (Applied Biosystems), and 5 μl cDNA solution in a 10 μl volume. ERα and ERβ cDNAs were amplified for 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. GAPDH cDNA was amplified for 24 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. PCR products were analyzed on 1.5% agarose gels in the presence of ethidium bromide. Fragments of 281 bps (ERα), 265 bps (ERβ) and 983 bps (GAPDH) were generated and their identities were confirmed by restriction enzyme digestion and sequencing. Experiments were conducted three times.

2.8. Preparation of cell extracts and Western blot analysis

Preparation of cell extracts from cells seeded in 60-mm dishes and Western blot analysis were performed as described previously [35]. After the cells were treated for indicated times, the medium was removed. The cells were washed three times with ice-cold PBS, and 0.4 ml of ice-cold lysis buffer (20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 120 mM NaCl, 0.5% Triton X-100, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml antipain, 100 μg/ml benzamidine hydrochloride, 50 μg/ml aprotinin, 100 μg/ml soybean trypsin inhibitor, 10 μg/ml pepstatin A and 1 mM PMSF) was added into the plates. The cell lysates were collected and the cell debris was pelleted to collect the supernatants. For Western blots, equal amounts of proteins were suspended in SDS-sample buffer. The proteins were separated by 8% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to an Immobilon™-P PVDF membrane (Millipore, Bedford, MA). After blocking with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST), membranes were incubated with appropriately diluted rabbit antiserum against human ERα, ERβ or anti-human β-actin mouse monoclonal antibody, and then with horseradish peroxidase-conjugated anti-rabbit IgG (1:10000) or anti-mouse Ig, horseradish peroxidase linked whole antibody (1:5000), respectively. The reactive proteins were detected with enhanced chemiluminescence reagents, ECL (Amersham Bioscience) and analyzed by a chemiluminescence image analyzer, LAS-1000 (Fuji Film, Japan).

3. Results

3.1. 293 cells express ERβ but not ERα

To examine the effect of E2 on HIV-LTR activation we used 293 cells because of their high transfection efficiency. First, we examined whether 293 cells express ERα or ERβ by determining mRNA and protein levels of ERα and ERβ by RT-PCR and Western blotting. As shown in Fig. 1, 293 cells express ERβ mRNA and low level of ERα mRNA. Western blot analysis confirmed the expression of ERβ but not ERα. In MCF-7 cells used as a positive control, ERα and ERβ were expressed at both mRNA and protein levels.

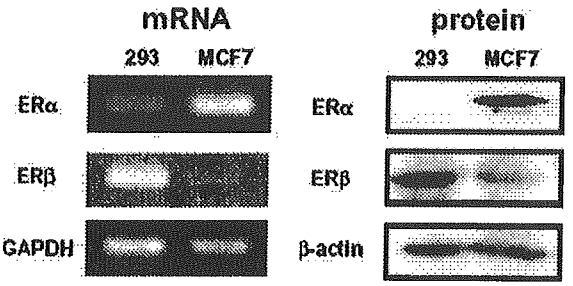

Fig. 1. Expression of ERα and ERβ in HEK293 cells and MCF-7 cells. Total RNA was extracted from each cell lines and then mRNA expression levels of ERα, ERβ and GAPDH were determined by RT-PCR. Cell lysates from each cell lines were separated by SDS-PAGE. Protein expression levels of ERα, ERβ and β-actin were determined by Western blotting. Representative data of three experiments are shown.

3.2. E2 activates ERE reporter gene by expression with ERα or ERβ

Prior to examining the effect of E2 on HIV-1 LTR activation, we constructed E2 responding 293 cells. As 293 cells do not express ERα, the cells were transiently transfected with expression plasmid of ERα or ERβ, together with ERE_3tk-Luc, a reporter plasmid containing three tandem repeats of ERE, and stimulated with varying concentrations of E2. Up to 100 nM are physiological levels of E2 in circulation during the early and late pregnancy [36]. As shown in Fig. 2, E2-induced luciferase activity in a dose-dependent manner in the presence of ERα or ERβ, at up to 100 and 1000 pM, respectively. Without expression of either ERα or ERβ, E2 did not affect the level of luciferase activity, suggesting that endogenous ERβ is not functioning in 293 cells.

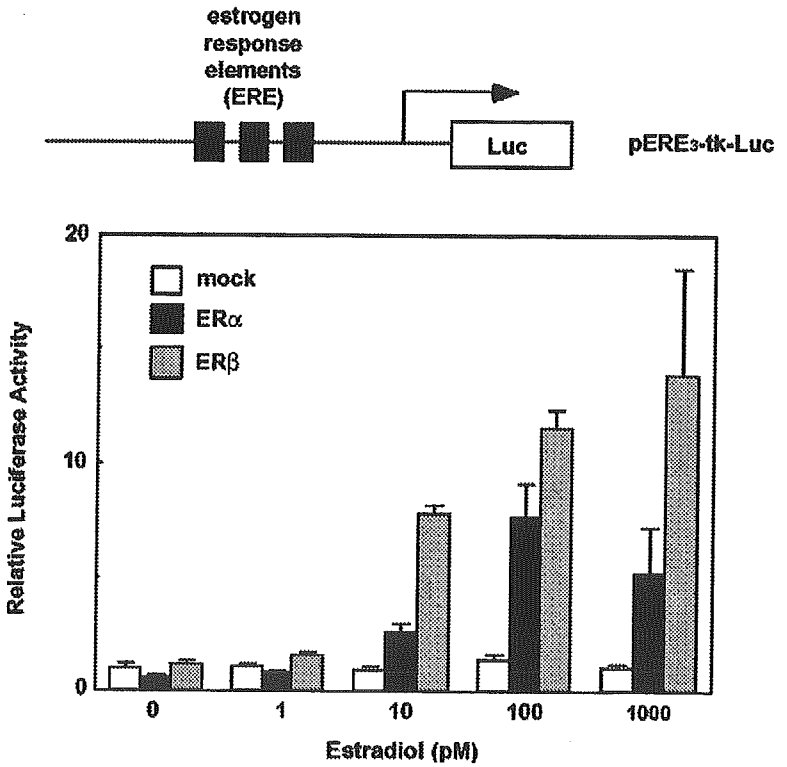

Fig. 2. The effect of 17β-estradiol (E2) on ER-dependent gene activation from estrogen response element (ERE) in HEK293 cell. 293 cells were co-transfected with ERE_3tk-Luc reporter gene with or without pcDNA3-ERα? or pcDNA3-ERβ plasmid. After 16 h of transfection, cells were incubated with varying concentrations of E2. Cells were harvested 24 h after transfection, and luciferase activity was measured. The luciferase activity was normalized by the β-galactosidase activity and mean ± SD based triplicated cultures are shown. Representative data of three experiments are shown.

3.3. E2 activates HIV-1 LTR transcription via ERα

Using E2 responding 293 cells, we determined the effect of E2 on HIV-1 LTR activation. 293 cells were transiently transfected with expression plasmid of ERα or ERβ, together with the CD12-Luc reporter plasmid containing the full-length HIV-1 LTR, and stimulated with E2. As shown in Fig. 3B, the basal transcription level of CD12-Luc was not significantly stimulated by E2. In contrast, E2 activated transcription of CD12-Luc in a dose-dependent manner at up to 10 nM in the presence of ERα but not ERβ. The stimulating effect of E2 was also observed by increasing the expression level of ERα (Fig. 3C). In order to determine whether the stimulating effect of E2 is mediated

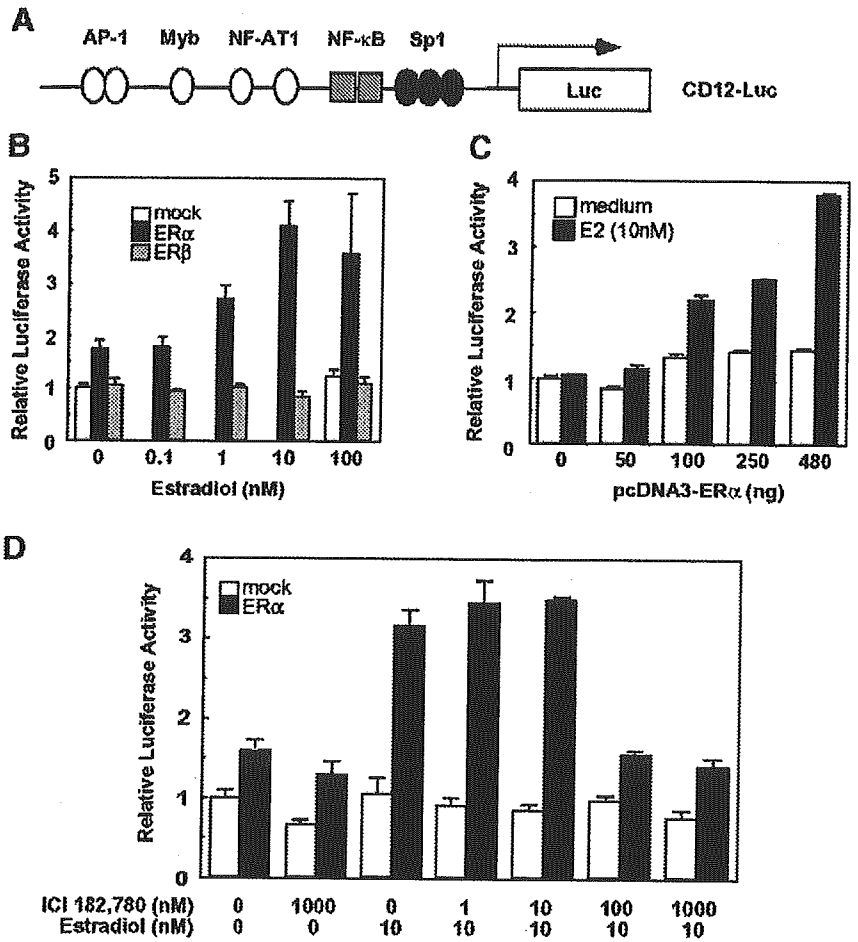

Fig. 3. The effect of E2 on gene expression from HIV-1 LTR. (A) Schematic diagram of HIV-1 LTR, position of various *cis*-elements for transcription factors, and reporter constructs. Binding sites for AP-1, Myb, NF-AT1, two tandem repeats of NF-κB binding sites, and three tandem repeats of Sp1 binding sites are indicated. CD12-Luc contains the full sequence of both U3 and R regions. (B) Dose-dependent effect of E2 on gene expression from HIV-1 LTR. 293 cells were co-transfected with CD12-Luc plasmid with or without pcDNA3-ERα or pcDNA3-ERβ plasmid.? After 16 h of transfection, cells were incubated with varying concentrations of E2. Cells were harvested 24 h after transfection, and luciferase activity was measured as described in Fig. 2. (C) Dose-dependent effect of ERα expression on the E2-induced activation of gene expression from HIV-1 LTR. 293 cells were co-transfected with CD12-Luc plasmid with pcDNA3 or without indicated amount of pcDNA3-ERα plasmid.? After 16 h of transfection, cells were incubated with 10 nM of E2. Cells were harvested 24 h after transfection, and luciferase activity was measured. (D) Effect of ER antagonist on the E2-dependent activation of gene expression from HIV-1 LTR. 293 cells were co-transfected with HIV-1 LTR-Luc reporter plasmid (CD12-Luc) in the presence or absence of ERα plasmid. After 16 h of transfection, cells were incubated for 24 h with indicated concentrations of ICI 182,780, in the presence or absence of 10 nM E2, and luciferase activity was measured. Representative data of more than three experiments are shown.

by ERα, ICI 182,780, a specific antagonist to ER, was added to the reporter gene assay. As shown in Fig. 3D, the basal transcription level of CD12-Luc was not affected by ICI 182,780. However, the E2-induced CD12-Luc transcription was completely abrogated by ICI 182,780. These findings indicate that E2 activates transcription of HIV-1 LTR via ERα.

3.4. Sp1 binding site is critical to the E2-induced gene expression from HIV-1 LTR

To identify the *cis*-regulatory region responsible for the E2-induced activation of HIV-1 LTR transcription, we compared the effect of E2 on gene expression of CD12-Luc and various 5′-deletion mutants: CD23-Luc (deleting the upstream sequences from position-117 of HIV-1 LTR, including AP1-binding site but still retaining NF-κB and Sp1 sites), CD52-Luc (deleting the upstream from position-65 but retaining the promoter-proximal two tandem repeats of Sp1 sites), and CD54-Luc (deleting the upstream from −48 and retaining no Sp1 site) (Fig. 4A). Similar to the results with CD12-Luc, gene expressions of CD23-Luc and CD52-Luc were significantly activated by E2 in the presence of ERα (Fig. 4B). However, CD54-Luc lost its responsiveness to E2. These results indicate that E2 can activate HIV-1 gene expression even in the absence of NF-κB and that the Sp1 site is indispensable for the effect of E2.

3.5. NF-κB is not involved in the E2-mediated activation of HIV-1 gene expression

To further examine a role of NF-κB in the E2-induced HIV-1 gene activation, we examined the effect of E2 on the gene expression from HIV-1 LTR and its mutant containing mutations at two κB sites (MJW-37-Luc (κBm)). As demonstrated in Fig. 5B, E2 could activate the gene expression

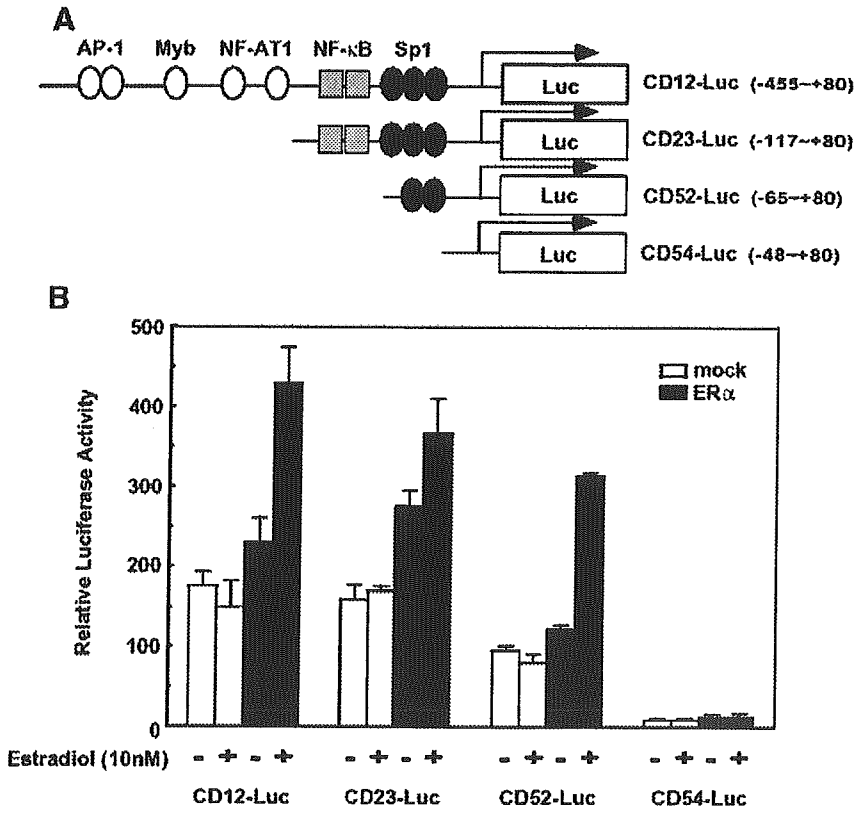

Fig. 4. Determination of crucial region for the E2-induced activation of gene expression from HIV-1 LTR. (A) Schematic diagram of HIV-1 LTR reporter genes, CD12-Luc (containing the full sequence of both U3 and R regions), CD23-Luc (containing both NF-κB and Sp1 sites), CD52-Luc (containing only the two Sp1 binding sites), and CD54-Luc (retaining no Sp1 site). (B) Each HIV-1 LTR reporter gene with or without pcDNA3-ERα plasmid was transfected into 293 cells. After 16 h of transfection, cells were incubated with or without 10 nM E2. Cells were harvested 24 h after transfection, and luciferase activity was measured as described in Fig. 2.

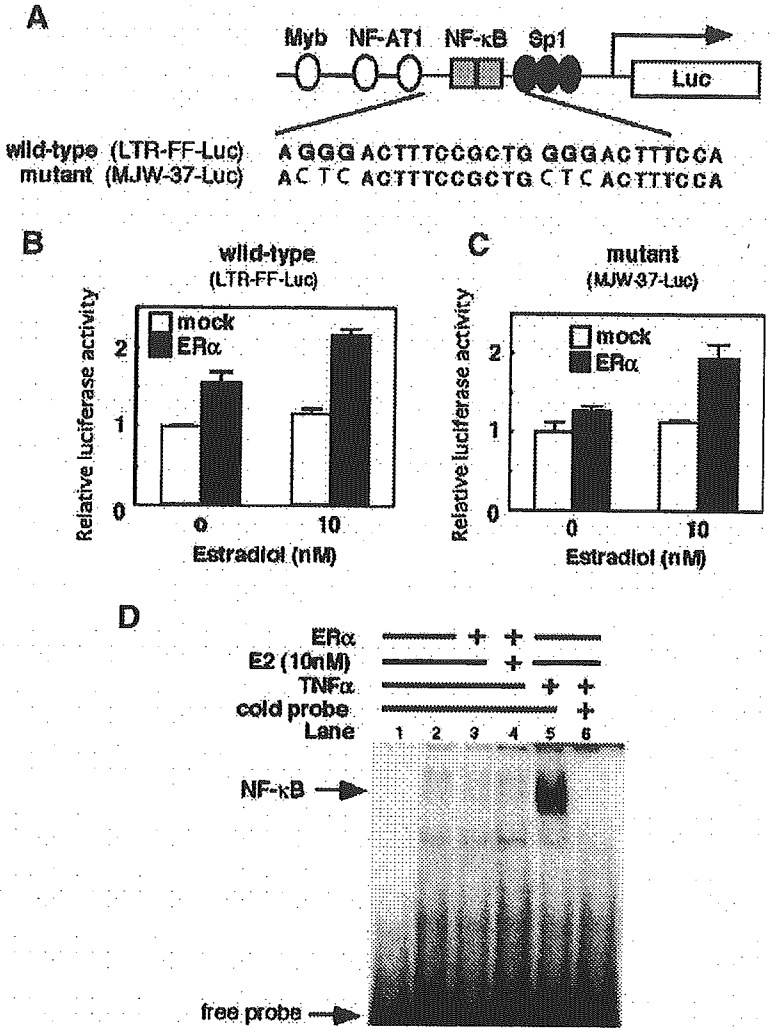

Fig. 5. NF-κB is not involved in the E2-induced activation of HIV-1 LTR. (A) Schematic diagram of LTR-FF-Luc (containing wild type NF-κB binding sites) and MJW-37-Luc (containing mutated NF-κB binding sites). (B, C) Mutation in the NF-κB site in HIV-1 LTR had no effect on the E2-induced activation of HIV-1 LTR-Luc in 293 cells. Cells were co-transfected with LTR-FF-Luc (wild type) (B) or MJW-37-Luc (mutant) (C) with pcDNA3-ERα plasmid. After 16 h of transfection, cells were incubated with or without 10 nM E2. Cells were harvested 24 h after transfection, and luciferase activity was measured as described in Fig. 2. (D) DNA binding activity of nuclear extracts from the E2 treated 293 cells to the NF-κB site in HIV-1 LTR. Cells were transfected with or without pcDNA3-ERα plasmid. After 16 h of transfection, cells were treated with or without 10 nM E2 or 100 U/ml TNFα. Twenty-four hours after cells were harvested and nuclear extracts were prepared and subjected to electrophoretic mobility shift assay (EMSA) analysis with ^{32}P-labeled NF-κB probe. Cells were treated with TNFα as a positive control (Lane 5). Lane 1; free labeled probe. Lane 6; 250-fold excess of cold NF-κB unlabeled was added to the labeled probe.

from the wild type HIV-1 LTR (LTR-FF-Luc (κBw)) in the presence but not in the absence of ERα, and the similar extents of stimulation by E2 were obtained even with the HIV-1 LTR promoter containing the mutated κB sites (Fig. 5C). We also assessed the effect of E2 on NF-κB DNA-binding by EMSA with κB DNA probe (Fig. 5D). Nuclear extracts obtained from 293 cells or from cells transfected with ERα stimulated with or without E2 or TNFα were allowed to bind with the end-labeled κB DNA probe. Although TNFα, used as a positive control, stimulated the NF-κB DNA-binding, E2 did not significantly augment the DNA-binding in the presence or absence of ERα. These observations indicate that NF-κB is not involved in the HIV-1 LTR activation induced by E2.

3.6. Sp1 activates transcription from HIV-1 LTR in a manner dependent on the GC box

As Sp1 site appeared to be important for the E2-induced transcription from HIV-1 LTR, EMSA was performed with the GC box DNA derived from HIV-1 LTR. As shown in Fig. 6A, specific band was observed in the absence of stimulation, and the level was not affected in the presence of ER. However, E2 augmented the binding level in the presence of ERα. Supershift assay using antibodies against Sp1 or Sp3 revealed that the major bands are mostly comprised of Sp1 (Fig. 6B). Sp3 also appeared to be a component of the major band. Antibody against p65 used as a control did not induce supershift of the band. Supershift was not observed with antibodies against either ERα or ERβ, suggesting that Sp1 or Sp3 is not bound to either ERα or ERβ or their interaction is weak.

3.7. E2 induces transcriptional activity of Sp1

We then examined whether E2 induces the transcriptional activity of Sp1 through ERα. 293 cells were transfected with expression plasmid encoding GAL4-DBD (DNA-binding domain) or Sp1 fused with GAL4-DBD (GAL4-Sp1), together with a reporter plasmid containing five tandem repeats of GAL4-binding sites (Fig. 7A). E2 treatment did not induce transcriptional activity of GAL4-DBD in the presence or absence of ERα. In the presence of ERα, however, E2 could induce transcriptional activity of GAL4-Sp1 (Fig. 7B). These results indicate that E2 induces transcriptional activity of Sp1 in a manner dependent on ERα. We further examined whether Sp1 contributes to the E2-induced stimulation of transcription from HIV-1 LTR. As shown in Fig. 7C, E2 induced the transcription from HIV-1 LTR in a dose-dependent manner, and this E2 effect was further augmented by Sp1 overexpression. These results indicate that Sp1 is involved in the E2-induced activation of transcription from HIV-1 LTR.

4. Discussion

In this study, we observed that E2 stimulates HIV-1 transcription via ERα-dependent activation of DNA-

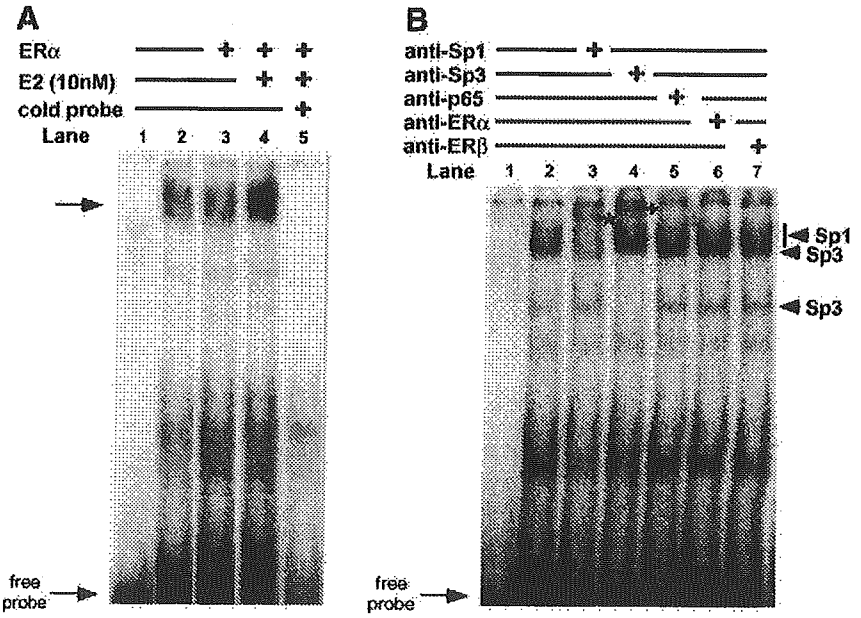

Fig. 6. E2 augments the binding of nuclear extracts from 293 cells to the GC rich region of HIV-1 LTR. (A) Effect of E2 on DNA-binding activity of nuclear extracts from 293 cells to the GC rich region of HIV-1 LTR. 293 cells were transfected with or without pcDNA3-ERα plasmid. After 16 h of transfection, cells were treated with or without 10 nM E2. Twenty-four hours after, cells were harvested and nuclear extracts were prepared and subjected to EMSA analysis with ^{32}P-labeled GC rich probe. Lane 1; free labeled probe. Lane 5; 250-fold excess of unlabeled GC rich probe was added to the labeled probe. (B) Binding of Sp1 and Sp3 to the GC rich region of HIV-1 LTR. 293 cells were transfected with pcDNA3-ERα plasmid. After 16 h of transfection, cells were treated with 10 nM E2. Twenty-four hours after that the cells were harvested and nuclear extracts were prepared and subjected to EMSA analysis with ^{32}P-labeled GC rich probe. Supershift analysis was performed using anti-Sp1 or anti-Sp3 antibodies. Supershifted bands are indicated with asterisk (*; Sp1) and plus (+; Sp3). Lane 1; free labeled probe. Representative data of more than three experiments are shown.

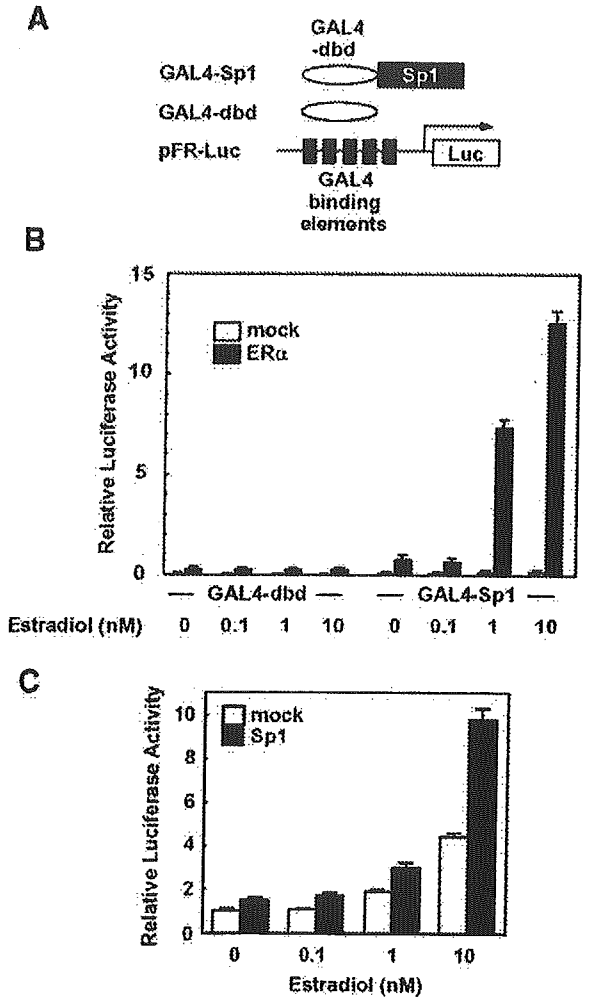

Fig. 7. Contribution of Sp1 to the E2-induced activation of HIV-1 LTR. (A) Schematic diagram of GAL4-Sp1 plasmids, which express Sp1 in fusion with GAL4-dbd and 5× GAL4-Luc, containing five tandem repeats of GAL4-binding sites. (B) Effect of E2 on transactivation activity of Sp1. 293 cells were cotransfected with 5× GAL4-Luc, GAL4-dbd or GAL4-Sp1 expression plasmids in the presence or absence of pcDNA3-ERα plasmid. After 16 h of transfection, cells were incubated with varying concentrations of E2. Cells were harvested 24 h after transfection, and luciferase activity was measured as described in Fig. 2. (C) Effect of E2 on transactivation activity of Sp1 for HIV-1 LTR. 293 cells were co-transfected with CD12-Luc and pcDNA3-ERα with or without pCIneo-Sp1. After 16 h of transfection, cells were incubated with indicated concentrations of E2 for 24 h and luciferase activity was measured. Representative data of three experiments are shown.

binding and transcriptional activity of Sp1. In an effort to analyze the effect of E2 on HIV-1 transcription, 293 cells, which do not express ERα but express ERβ, were transiently transfected with expression plasmid of ERα or ERβ together with CD12-Luc containing the full-length HIV-1 LTR, and examined the effect of E2. E2 activated the transcription from HIV-1 LTR in a manner dependent on ERα but not ERβ. Furthermore, the ERα-mediated E2 effect was verified by a specific antagonist to ER. E2 binds to both ERα and ERβ with a similar high affinity [37] and activates their functions. Indeed, in 293 cells E2 induced the transcriptional activity of both ERα and ERβ when it was assessed with ERE_3tk-Luc, a reporter plasmid containing three tandem repeats of ERE. Although 293 cells express ERβ, it did not appear to be functional. The endogenous ERβ may, therefore, be mutated. ERα and ERβ consist of three functional domains: an N-terminal domain involved in ligand-independent transcription (A/B), a central domain highly conserved in these two ERs and responsible for specific DNA-binding, and a C-terminal domain involved in ligand binding and ligand-dependent transcription. The N-terminal A/B domain of ERβ is short in its length as compared to ERα, consequently their functions are different depending on ligands, cell types and tissues [7,38,39].

As there is no ERE in the promoter region of HIV-1 LTR, we presumed that E2/ERα indirectly activates HIV-1 transcription through binding to other transcription factors. It is reported that ERα physically interacts with many transcription factors, including Sp1 [13–15], AP-1 [16], NF-κB [17], C/EBPβ and STAT5 [18], and modulates their transcriptional activity. By the experiments using deletion mutant reporter genes of HIV-1 LTR, E2 did not appear to require the transcription binding sites, including AP-1 (−247 to −222), NF-AT1 (−254 to −216), c-Myb (−304 to −299) and NF-κB for its augmenting effect. Reporter gene assay using the mutant containing mutations at two κB sites and EMSA also supported that NF-κB is not involved in the effect of E2. In contrast, the GC rich region corresponding to Sp1 sites appeared to be important for the effect of E2. Similar to these findings, we recently found that 3-methylcholanthrene activates HIV-1 replication via aryl hydrocarbon receptor and that Sp1, but not NF-κB, is involved [40].

We observed that Sp1 and Sp3 specifically bound to the nucleotide containing GC rich region in HIV-1 LTR, and that E2 in the presence of ERα augmented

Sp1 DNA-binding. The specific binding of Sp1 and Sp3 to the GC rich region was observed in the absence of ERα and E2 and the reporter gene assay showed that Sp1 site is critical to the basal transcription activity. These findings suggest that Sp1 and Sp3 binding to the GC rich region is important to the basal transcription activity of HIV-1 LTR. We also showed that in a luciferase reporter assay using GAL4-Sp1 fusion protein E2 could augment the transcriptional activity of Sp1 in a manner dependent on ERα. However, as far as we examined, ERα was not associated with the Sp1–DNA complex. Similar to our findings, it was previously reported that although ERα and Sp1 could physically interact and enhance Sp1 DNA-binding, ERα was not detected in the Sp1–DNA complex [13,14,41]. Therefore, Sp1/ERα complex may not be stable or ERα is released upon Sp1 binding to DNA. The function of Sp1 is regulated by posttranslational modifications and interaction with other protein molecules. The former type regulation includes phosphorylation [42], acetylation [15] and glycosylation [43]. The DNA-binding ability of Sp1 is regulated by phosphorylation of Ser located within the N terminal Glu-rich region, and Sp1 is phosphorylated by ERK1/2 or JNK upon stimulation with extracellular stimuli such as FGF, HGF and peroxide [44–46]. Therefore, it is possible that E2-activated ERα may induce such posttranslational modification of Sp1 to stimulate its transcriptional activity.

The reports that tamoxifen, an antiestrogenic reagent, inhibits the replication of HIV-1 in lymphocytes [47,48] are in agreement with our findings. We also observed that E2 augmented HIV-1 replication in OM10.1 cells (promyelocytic cell line latently infected with HIV-1) in a dose-dependent manner (data not shown). However, the E2 effect was not inhibited by ICI 182,780, rather ICI 182,780 augmented HIV-1 replication. However, it is reported that the effect of ICI 182,780 depends on cell types. In breast tumor cells MCF-7 and MDA-MB231 and prostate tumor cells LNcaP, ICI 182,780 works as an agonist for ERα-dependent gene activation [49]. Therefore, the E2 effect on OM10.1 cells does not rule out the involvement of ERα. Peripheral T cells, B cells and monocytes express ERα and ERβ mRNA [50]. Therefore, it is feasible that E2 augments HIV-1 replication in these cells.

Acknowledgments

This work was supported by grants-in-aid for Scientific Research on Priority Areas from the Ministry of Health and Welfare, the Ministry of Education, Science, Culture and Sports of Japan and the Human Science Foundation of Japan. We thank Dr. J. Karn (Medical Research Council Laboratory of Molecular Biology, UK) for generous gifts of luciferase reporter plasmids (LTR-FF-Luc and MJW-37-Luc), Dr. S. Kojima (Wako Institute, RIKEN, Japan) for expression vector pCIneo-Sp1, and Dr. S. Kato (The Institute of Molecular and Cellular Biosciences, the University of Tokyo, Japan) for expression vectors pcDNA3-ERα and pcDNA3-ERβ, and a reporter plasmid pERE$_3$tk-Luc.

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