Identification of Protein Arginine Methyltransferase 2 as a Coactivator for Estrogen Receptor α*

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In an attempt to isolate cofactors capable of influencing estrogen receptor α (ERα) transcriptional activity, we used yeast two-hybrid screening and identified protein arginine methyltransferase 2 (PRMT2) as a new ERα-binding protein. PRMT2 interacted directly with three ERα regions including AF-1, DNA binding domain, and hormone binding domain in a ligand-independent fashion. The ERα-interacting region on PRMT2 has been mapped to a region encompassing amino acids 133–275. PRMT2 also binds to ERβ, PR, TRβ, RARα, PPARγ, and RXRα in a ligand-independent manner. PRMT2 enhanced both ERα AF-1 and AF-2 transcriptional activity, and the potential methyltransferase activity of PRMT2 appeared pivotal for its coactivator function. In addition, PRMT2 enhanced PR, PPARγ, and RARα-mediated transactivation. Although PRMT2 was found to interact with two other coactivators, the steroid receptor coactivator-1 (SRC-1) and the peroxisome proliferator-activated receptor-interacting protein (PRIP), no synergistic enhancement of ERα transcriptional activity was observed when PRMT2 was coexpressed with either PRIP or SRC-1. In this respect PRMT2 differs from coactivators PRMT1 and CARM1 (coactivator-associated arginine methyltransferase). These results suggest that PRMT2 is a novel ERα coactivator.

The estrogen receptor (ER)† is a transcription factor that belongs to the nuclear receptor superfamily (1, 2). Upon estrogen binding, ER regulates the transcription of specific target genes by binding to specific DNA response elements referred to as estrogen response elements (EREs) in their promoters or by interacting with other transcription factors such as Jun and Fos (1, 2). In addition to hormone-mediated activation, ER is also activated by growth factors including epidermal growth factor and insulin-like growth factor-1 probably through phosphorylation (3, 4). ER contains two transcriptional activation function (AF) domains: AF-1 located in the N terminus and the ligand-dependent AF-2 located in the ligand binding domain (5). The ability of AF-1 and AF-2 to activate transcription varies according to the promoter context and the cell type (6).

There are two isoforms of estrogen receptors, namely ERα and ERβ (7). ERα and ERβ recognize identical DNA elements and have similar affinity for a certain estrogen, but exhibit distinct tissue distribution (7). Evidence provided by gene knock experiments indicates that ERα is the receptor responsible for the estrogen-induced growth of mammary gland and the reproductive tract (8).

The precise mechanism by which ER modulates cell- and gene-specific transcription is not fully understood. Recent evidence suggests that ER activates transcription by recruiting coactivators that appear to act by modifying chromatin structure or facilitating the formation of transcriptional initiation complexes (9, 10). Among a growing list of cofactors that regulate nuclear receptors, including ER, are the well studied coactivators of the SRC-1 family (9), CREB-binding protein (CBP/p300) (11, 12), and PBP (13). PBP is a component of the thyroid hormone receptor-associated protein (TRAP)/vitamin D3 receptor-interacting protein (DRIP) complexes (14–16). Both SRC-1 and CBP/p300 have intrinsic histone acetyltransferase activity and recruit other acetyltransferases (13–16). The acetylation of histone results in the modification of chromatin and increases the access of the DNA to other components of transcription apparatus. The multiprotein TRAP/DRIP complexes exhibit no intrinsic histone acetyltransferase activity and appear to function through the direct interaction with general transcriptional machinery (15, 16). The observation that certain coactivators such as SRC-3 (AIB1; ACTR, p/CIP, RAC3) (21–24), AIB3 (PRIP, ASC2, RAP250, NRC, TRBP) (25–29), and PBP (30) are amplified and overexpressed in some breast cancers underscores the importance of nuclear receptor coactivators in transcriptional activation and also points to their possible role in neoplastic conversion.

Post-translational modification of proteins by arginine methylation has recently been implicated in a variety of cellular processes including nuclear receptor transcriptional regulation (31). Among the five members of protein arginine methyltransferases (PRMTs) identified thus far based on protein sequences, PRMT1 is the first identified and the predominant PMRT in mammalian cells (32). PRMT1 has been shown to interact with SRC-2 (GRIP1) and enhance the nuclear receptor transactivation function (33). Coactivator-associated arginine methyltransferase 1 (CARM1/PRMT4) was identified by its interaction with nuclear receptor coactivator SRC-2 (GRIP1) (34). PRMT1 and CARM1 are able to methylate the histones H4 and H3, respectively, suggesting their role in modulating...
Fig. 1. The estrogen-independent interaction of PRMT2 with ERα in yeast. pACT2 expressing GAL4 activation domain alone or pACT2-PRMT2 expressing the fusion protein between the GAL4 activation domain and PRMT2 was cotransformed into yeast H7C with pGBKTK7 expressing GAL4 DNA binding domain alone or pGBKTK7-ERα expressing fusion protein between GAL4 DNA binding domain and ERα into yeast H7C. The β-galactosidase activities from equal number of cells were measured as an indication of the relative strength of interaction in the presence or absence of ligand 17β-estradiol.

Fig. 2. In vitro interaction of PRMT2 with ERα, ERβ, PR, TRβ, RARα, PPARγ, and RXRα. GST-Sepharose beads bound with purified E. coli expressed GST-PRMT2 or with GST were incubated with [35S]methionine-labeled ERα, ERβ, PR, TRβ, RARα, PPARγ, and RXRα in the presence (+) or absence (−) of ligand. The ligands used were: 17β-estradiol for ERα and ERβ, progesterone for PR, T3 for TRβ, 9-cis-retinoic acid for RARα and RXRα, and Invitrogen 49653 for PPARγ. Following four times washing with NETN solution, the bound proteins were eluted and separated using 10% SDS-polyacrylamide gel electrophoresis and autoradiographed.

EXPERIMENTAL PROCEDURES

Plasmids—pCDNA3.1-ERα, ERβ-TK-LUC, PCMX-RARα, RARE-TK-LUC, PCMV-PPARγ, and PPRE-TK-LUC and the vectors for in vitro translation of PR, RXRα, TRβ1 have been described elsewhere (26). ERβ is a gift from Dr. Laird D. Madison (Northwestern University). pGBKTK7-ERα was constructed by inserting the full-length coding region of ERα cDNA into Neo/Slall site of pGBKTK7 (CLONTECH). PCMV-PRMT2 was an IMAGE clone purchased from Invitrogen and confirmed by sequencing. GST-PRMT2 was produced in Escherichia coli BL21 and bound to glutathione-Sepharose beads according to the manufacturer’s instructions (Amersham Biosciences). In vitro translation was performed using rabbit reticulocyte lysate (Promega) and labeled with [35S]methionine. In GST pull-down assays, a 25-μl aliquot of GST fusion protein loaded on glutathione-Sepharose beads was incubated with 5 μl of [35S]methionine-labeled in vitro translated proteins for 2 h in 500 μl of NETN (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.7 mM EDTA, 0.05% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride). The binding was assayed in the presence or absence of specific ligands: 17β-estradiol (10−8 M) for ER; BRL49653 (10−6 M) for PPARγ; 9-cis-retinoic acid (10−6 M) for RXRα and RARα; Wy-14,643 (10−5 M) for PPARγ; and T3 (10−6 M) for TRβ1. Bound proteins were washed five times with binding buffer, eluted by boiling for 2 min in 20 μl of SDS sample buffer, analyzed by SDS-PAGE, and autoradiographed.

Immunoprecipitation—COS-7 cells were transfected with 5 μg of pCDNA3.1-ERα and 5 μg of pCMV-FLAG-PRMT2 using LipofectAMINE 2000 (Invitrogen). 24 h after transfection, the cells were harvested. The lysate was immunoprecipitated with anti-FLAG (Sigma) or control serum. The precipitates were resolved by SDS-PAGE and subjected to Western blot analysis using anti-ERα and anti-PRMT2 antibodies.

S-Adenosyl-l-methionine Binding Assay—Purified GST, GST-PRMT2, GST-PRMT2M, or GST-PRMT1 (10 μg) was incubated with 20 μCi of S-adenosyl-l-[methyl-3H]methionine (Amersham Biosciences) in the buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2 mM MgCl2) at 37°C for 10 min. The protein was trapped on HAWP 02500 filter (Millipore). The filter was washed with the buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2 mM MgCl2) to remove unbound S-adenosyl-l-
methyl-3H]methionine. The filters were dried, and the amount of bound S-adenosyl-L-[methyl-3H]methionine was quantified by liquid scintillation counting.

Cell Culture and Transfection—CV-1 cells (1 × 10^5) were plated in 6-well plates and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum for 24 h before transfection. Cells were transfected for 5 h with 1.25 μg of luciferase reporter DNA, 20 ng of plasmid expressing the receptor, and 1.25 μg of appropriate expression plasmid DNA or as indicated in the figure legends using LipofectAMINE 2000 (Invitrogen); 0.1 μg of β-galactosidase expression vector pCMVβ (CLONTECH) DNA was always included as an internal control. Cell extracts were prepared 24 h after transfection and assayed for luciferase and β-galactosidase activities (Tropix). Three independent transfections were performed for each assay.

RESULTS

Isolation of PRMT2 as an ERα-binding Protein by Two-hybrid Screening—Using full-length ERα as a bait in yeast two-hybrid system, we isolated from human mammary gland cDNA library a partial cDNA encoding PRMT2 (amino acids 10-433). To examine the influence of estrogen on the interaction, pACT2-PRMT2, which was isolated by yeast two-hybrid screening and expressed as fusion protein between GAL4 activation domain and PRMT2 (amino acids 10-433), or pACT2 was cotransformed with PGBKT7-ERα expressing fusion protein between GAL4 DNA binding domain and ERα or PG-BTKT7 into yeast HF7C. The β-galactosidase activity was measured as an indication of the relative strength of interaction in the presence or absence of ligand. In the absence of ligand, we observed an interaction between ERα and PRMT2 that resulted in a 40-fold increase in the β-galactosidase activity (Fig. 1). The presence of the ligand estrogen did not significantly affect the interaction between PRMT2 and ERα (Fig. 1).

Interaction of PRMT2 with ERα and Other Nuclear Receptors in Vitro—The direct interaction between PRMT2 and ERα was further tested by in vitro GST binding assay. The immobilized GST-PRMT2, but not GST alone, retained [35S]methionine-labeled ERα fusion protein for luciferase and β-galactosidase activities (Tropix). Three independent transfections were performed for each assay.

FIG. 3. Mapping the regions for ERα and PRMT2 interaction. A, GST pull-down assay was performed using [35S]methionine-labeled PRMT2 and fusion proteins between GST and four different ERα fragment. Three ERα regions including AF-1 (amino acids 1-184), DNA binding domain (amino acids 185-250), and hormone binding domain (amino acids 302-595) interact with PRMT2. The binding of PRMT2 to the hormone binding domain is estrogen-independent. B, the SH3 domain on PRMT2 is dispensable for PRMT2 and ERα interaction. [35S]methionine-labeled PRMT2 with SH3 domain deletion is able to bind to the GST-ERα fusion protein but not GST alone. C, the interaction region of PRMT2 to ERα was mapped to the fragment from amino acid 133 to 275 by GST pull-down assay using [35S]methionine-labeled truncated PRMT2 and GST-ERα fusion protein.

FIG. 4. PRMT2 interacts with ERα in vivo. Plasmids expressing FLAG-tagged PRMT2 and ERα were cotransfected into COS-7 cells in the presence or absence of 17β-estradiol. The cell extracts were immunoprecipitated with either anti-FLAG or control serum. The precipitates were then analyzed by Western blot using anti-ERα antibody.

FIG. 5. PRMT2 interacts with SRC-1 and PRIP. GST or GST-PRMT2 fusion protein was incubated with [35S]methionine-labeled SRC-1, PRIP, PBP, or CBP. Bound proteins were resolved by SDS-PAGE and detected by autoradiography.

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Interaction of PRMT2 with PRIP, SRC-1, and with PRMT2 Itself—The potential interaction between PRMT2 and other known nuclear receptor coactivators was investigated using the GST pull-down assay. We detected the interaction of PRMT2 with PRIP and SRC-1 (Fig. 5). No interaction was observed between PRMT2 and PPB or PRMT2 and CBP (Fig. 5).

The methyltransferase PRMT1 is able to form homodimer or homooligomers (39, 40). A GST pull-down assay was performed to see if PRMT2 exhibits this property. GST and PRMT2 fusion protein but not GST alone retained [35S]-methionine-labeled PRMT2 suggesting that PRMT2 is capable of forming homodimer or homooligomers (Fig. 6).

PRMT2 Binds S-Adenosylmethionine—PRMT2 was initially isolated by its protein sequence similarity to other PRMTs and so far no methyltransferase activity has been revealed. Using bacterially expressed GST-PRMT2 fusion protein, we did not demonstrate that PRMT2 was capable of methylating histone and ERα (data not shown). We then tested the ability of PRMT2 to bind the methyl donor S-adenosylmethionine by a filter binding assay. Just like PRMT1, PRMT2 was found to be able to bind S-adenosylmethionine, whereas PRMT2 with point mutation in the S-adenosylmethionine binding motif (41) failed to bind S-adenosylmethionine (Fig. 7).

PRMT2 Potentiates ERα Transcriptional Activity and Its Potential Methyltransferase Activity Is Pivotal While Its SH3 Domain Is Dispensable for This Function—Having established that PRMT2 is an ERα-binding protein, we investigated the effect of increased levels of PRMT2 upon ERα transcriptional activity in CV-1 cells. The luciferase activity expressed from ERE-TK-LUC that contains one copy of ERE serves as the indicator of the ERα transcriptional activity. Expression of PRMT2 increased the estrogen-dependent transcription of luciferase gene by about 8-fold with minimal effort on basal transcription, which provided evidence that PRMT2 acts as a coactivator for ERα (Fig. 8). However, the mutated PRMT2 that was incapable of binding S-adenosylmethionine enhanced the ERα transcriptional activity by about 2.5-fold, which is much less than the 8-fold obtained with wild-type PRMT2 indicating the importance of the potential methyltransferase activity for the role of PRMT2 as a coactivator (Fig. 8). On the other hand, PRMT2 with deletion of the SH3 domain increased ERα activity to the same extent as that for wild-type PRMT2, indicating that the SH3 domain is dispensable for its coactivator function (Fig. 8).

PRMT2 Increases Both AF-1 and AF-2 Transcriptional Activity of ERα—As ERα contains the autonomous activation domain AF-1 and ligand-dependent activation domain AF-2, we further examined the effect of increased expression of PRMT2 on their individual activities. The AF-1 (1–184) and AF-2 (251–595) were fused to GAL4 DNA binding domain, respectively, and then cotransfected with GAL4 responsive element-directed luciferase as the reporter gene. In comparison with the GAL4 DNA binding domain alone, AF-1 increased the luciferase activity by about 3-fold. The expression of PRMT2 further increased AF-1-mediated luciferase expression by about 4.5-fold (Fig. 9A). Therefore, PRMT2 is able to enhance the ERα AF-1 activity. Just as other nuclear receptors show transcription repression in the absence of their corresponding interaction with ERβ, PR, TRβ, RARα, PPARγ, and RXRα (Fig. 2).

To determine which region of ERα binds to PRMT2, a GST pull-down assay was performed using fusion proteins between GST and different regions of ERα. As shown in Fig. 3A, PRMT2 bound to the AF1 region, DNA binding domain, and hormone binding domain but not to the hinge region. The binding to ERα hormone binding domain was ligand-independent. The interaction between PRMT2 and ERα AF-1 region or DNA binding domain is stronger than that between PRMT2 and ERα hormone binding domain.

PRMT2 contains a Src homology 3 (SH3) domain that binds to proteins with a proline-rich motif and plays a pivotal role in a wide variety of biological processes (38). A GST pull-down assay revealed that PRMT2 with a SH3 domain deletion was still able to bind to the GST-ERα fusion protein but not GST alone. Therefore, this domain is not considered necessary for PRMT2 and ERα interaction (Fig. 3B). The region of PRMT2 that interacts with ERα was further defined by GST pull-down assay using different truncated PRMT2 fragments. A fragment from amino acid 133 to 275 was found to interact with ERα (Fig. 3C).

PRMT2 Interacts with ERα in Vivo—The potential interaction between PRMT2 and ERα in the intact cell was examined by coexpressing ERα and FLAG-tagged PRMT2 in COS-7 cells followed by immunoprecipitation and Western blot analysis. As shown in Fig. 4, PRMT2 interacts with ERα both in the presence and absence of estrogen.

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Fig. 9. The effects of PRMT2 on AF-1 and AF-2 transcriptional activities of ERα. Gal4-DBD-ERα (1–184) and Gal4-DBD-ERα (251–595) were generated by inserting the corresponding ERα cDNA fragment downstream of the Gal4 DNA binding domain (Gal4-DBD). The Gal4 responsive element-directed luciferase vector (Gal4-TK-LUC) was used as the reporter of activities. A, PRMT2 increases AF-1 activity. CV-1 cells were cotransfected with 1.5 μg of GAL-TK-LUC, 25 ng of Gal4-DBD or Gal4-DBD-ERα (1–184), and 1.5 μg of PRMT2 expression vector or control PCDNA3.1. The activity obtained on transfection of GAL-TK-LUC and Gal4-DBD without exogenous PRMT2 was taken as 1. B, PRMT2 also increases AF-2 activity. CV-1 cells were cotransfected with 1.5 μg of GAL-TK-LUC, 25 ng of Gal4-DBD or Gal4-DBD-ERα (251–595), and 1.5 μg of PRMT2 expression vector or control PCDNA3.1. The activity from transfection of ER/H9251 and GAL-TK-LUC in the absence of exogenous PRMT2 was taken as 1.

On the other hand, the hormone-dependent AF-2 slightly decreased the luciferase activity without estrogen over the control. The addition of estrogen increased the AF-2-mediated luciferase expression by about 3-fold, which is further enhanced by coexpression of PRMT2 by about 5-fold, demonstrating that PRMT2 also potentiates the AF-2 activity (Fig. 9B).

No Synergistic Enhancement of ERα Activity by Coexpression of PRMT2 and SRC-1 or PRIP—Given that PRMT2 binds to SRC-1 and PRIP, we sought to determine whether there was synergistic enhancement of ERα activity by PRMT2 and SRC-1 or PRIP. In transient transfection assay with ERα and its reporter gene, PRMT2, SRC-1, and PRIP all enhanced the expression of reporter gene to different levels (Fig. 10). When PRMT2 was cotransfected with either SRC-1 or PRIP, the expression of the reporter gene luciferase was modestly de-

creased in comparison with PRMT2 alone (Fig. 10). Therefore, there appears no synergistic activation when PRMT2 are coex-

PRMT2 Contains No Intrinsic Transcriptional Activity—In an attempt to define the mechanism by which PRMT2 acts as a coactivator, we tested if PRMT2 contains intrinsic transcriptional activity similar to that reported with other coactivators such as SRC-1 (42). PRMT2 was linked to the GAL4 DNA binding domain and transfected into CV-1 cells along with GAL4 responsive element-directed reporter gene luciferase. PRMT2 enhanced the PR ligand-dependent transcriptional activity by about 8-fold. In comparison with ERα and PR, PRMT2, which also increased the ligand-dependent PPARγ and RARα transactivation by about 5- and 4.5-fold, respectively, showed less effect on PPARγ and RARα transactivation (Fig. 11).

Fig. 10. No synergistic activation was observed for coexpression of PRMT2 and SRC-1 or PRIP. CV-1 cells were transfected with 1.5 μg of reporter construct ERE-TK-LUC, 35 ng of PCDNA3.1-ERα, and 0.8 μg of expression vector as indicated in the absence (−) or presence (+) of 10−7 M 17β-estradiol. Transfection without indicated expression vector was compensated by adding the same amount of PCDNA3.1. The activity obtained on transfection of ERE-TK-LUC without exogenous PRMT2 in the absence of ligand was taken as 1.

In DISCUSSION

Using a yeast two-hybrid system with ERα as bait to screen a human mammary gland cDNA library, we isolated PRMT2 as a new ERα-interacting protein. The interaction between PRMT2 and ERα was confirmed by in vitro binding and in vivo immunoprecipitation assay. A transient transfection assay demonstrated that PRMT2 increased the ERα transcriptional activity. In agreement with the finding that PRMT2 bound to both ERα AF-1 domain and the hormone binding domain, PRMT2 enhanced both ERα AF-1 and AF-2-mediated transcription. These results established that PRMT2 is a coactivator of ERα. However, unlike other coactivators such as SRC-1 family and CBP that show ligand-dependent binding to the nuclear receptors, PRMT2 binds to ERα both in the presence and absence of estrogen but enhances the ERα activity only
with the estrogen. It appears that the interaction between ERα and PRMT2 is not enough for ERα transcriptional activation, which occurs only after the binding of estrogen resulting in most probably the recruitment of other ligand-dependent coactivators.

Two types of PRMT activities have been identified in mammalian cells (31). Type 1 PRMT enzymes including PRMT1, PRMT3, and CARM1 catalyze the formation of monomethylarginine and asymmetric dimethylarginine. Type 2 PRMT enzymes catalyze the formation of monomethylarginine and symmetric dimethylarginine. PRMT5/JBP1 is the only type II enzyme identified so far (43). Based on the protein sequence, PRMT2 was identified as a methyltransferase most probably belonging to type I enzyme, but so far its methyltransferase activity has not been identified (37). Although we demonstrated PRMT2 is capable of binding S-adenosylmethionine, we failed to detect any methyltransferase activity using bacterially expressed GST-PRMT2 fusion protein with substrates including histone and ERα (data not shown). A systematic approach to identify the substrates for PRMT2 will be required, and it is also possible that some modification such as phosphorylation or some cofactor may be required for its activity. Nevertheless, the mutation in the conserved PRMT2 binding site for S-adenosylmethionine, which would abolish the potential methyltransferase activity, substantially diminished the PRMT2 coactivator function. The finding that PRMT2 does not have any intrinsic transcriptional activity favors the hypothesis that PRMT2 acts by modifying chromatin structure or the transcriptional apparatus through methylation. The elucidation of the substrates will be crucial for the understanding of PRMT2 coactivator function.

PRMT1 and CARM1 are two arginine methyltransferases that have been found to participate in the nuclear receptor transcriptional activation. Both PRMT1 and CARM1 interact with the carboxyl-terminal activation domain of coactivator GRIP1 and are able to methylate histones H4 and H3, respectively. PRMT1 or CARM1 enhance the nuclear receptor activity mildly by itself but substantially when coexpressed with GRIP1, suggesting that PRMT1 and CARM1 act as the secondary coactivators that are recruited by the first coactivator to modify the chromatin structure. Instead, PRMT2 strongly enhances the ERα transcriptional activity by direct interaction with ERα. Although PRMT2 was found to interact with other coactivators SRC-1 and PRIP, no synergistic activation was found with coexpression of PRMT2 and PRIP or SRC-1. The coexpression of SRC-1 or PRIP even modestly decreased PRMT2 coactivation. Therefore, although PRMT2 is a protein arginine methyltransferase highly homologous to PRMT1 and CARM1, PRMT2 may have a very different mechanism by which it acts as a coactivator, possibly because it has different substrates involved in transcriptional activation.

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**Fig. 11.** PRMT2 also enhances PR, PPARγ, and RARα-mediated transactivation. CV-1 cells were transfected with 1.5 μg of reporter vector (PRE-TK-LUC for PR, PPRE-TK-LUC for PPARγ, RARE-TK-LUC for RARα) cotransfected with 1.5 ng of control pCDNA3.1 or PRMT2 expression vector in the presence or absence of ligand (10 μM progesterone for PR, 10 μM BRL49653 for PPARγ, and 10 μM 9-cis-retinoic acid for RARα). The activity obtained on transfection of the reporter vector without exogenous PRMT2 in the absence of ligand was taken as 1.

**Fig. 12.** PRMT2 does not have intrinsic transcriptional activity. 1 μg of Ga4-DDB, Ga4-DDB-PRMT2, or Ga4-DDB-SRC-1 was cotransfected with 2 μg of GAL-TK-LUC into CV-1 cells. The activity of luciferase from transfection of GAL-TK-LUC into CV-1 cells was taken as 1.
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