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MDM2 Enhances the Function of Estrogen Receptor α in Human Breast Cancer Cells

Shigehira Saji,*,†,[1] Naoki Okumura,*,† Hidetaka Eguchi,* Shigeru Nakashima,‡ Akio Suzuki,§ Masakazu Toi,§ Yoshinori Nozawa,‡ Shigetoyo Saji,† and Shin-ichi Hayashi*,[2]

**Division of Endocrinology, Laboratory of Cancer Diagnosis and Therapy, Saitama Cancer Center Research Institute, 818 Komuro, Ina-machi, Kitaadachi-gun, Saitama 362-0806, Japan; †Department of Surgery II and ‡Department of Biochemistry, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500-8705, Japan; and §Department of Surgery, Tokyo Metropolitan Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan*

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Overexpression of the oncoprotein MDM2, a negative feedback regulator of p53, is often observed in breast cancer tissue and cell lines, particularly in those which express estrogen receptor α (ERα). In this study, we report a novel function of MDM2, i.e., as a positive regulator of ERα. This function does not involve p53. MDM2 overexpressing clones derived from the breast cancer cell line, MCF-7 cells, showed a remarkable growth advantage only in estradiol supplemented conditions, and this profile coincided with increased transcriptional activity of ERα in these cells. Though p53 has been reported to be an inhibitor of ERα function, p53 protein in MDM2 overexpressing clones was more abundant than in the parental cells. When ERα was exogenously expressed in p53-null cells, its activity was enhanced by coexpression of MDM2. Mammalian two-hybrid assays and GST pull-down assays indicated that MDM2 could interact with ERα. These results indicate that MDM2 is a direct activator of ERα function, and suggest such a role for MDM2 in ERα-positive breast cancer.

Key Words: **mdm2; breast cancer; estrogen receptor; MCF-7; p53.**

Since mdm2 gene is frequently amplified in some types of cancers, including osteosarcoma (1–3), and shows oncogenic activity in NIH3T3 cells when exogenously introduced (4), it is considered to be an oncogene. The human mdm2 gene encodes a polypeptide consisting of 491 amino acids that contains a binding domain for the tumor suppressor p53, an acidic region, zinc finger motifs and a ring finger domain (4–7). MDM2 is involved in down regulation of p53 functions. It binds to the N-terminal region of p53, inhibiting its transcriptional activity by concealing its transactivation domain (1, 5). In addition, MDM2 promotes the rapid degradation of p53 via a ubiquitin-proteasome pathway (8–10). On the other hand, MDM2 is transcriptionally upregulated by p53, and this system is thought to be important in the negative feedback regulation of p53 (5, 11).

Besides p53, MDM2 has been shown to interact with other molecules such as retinoblastoma protein pRB (12), transcriptional factor E2F (13), ribosomal protein L5 (14), RNA (15), cell fate regulator Numb (16), and cell cycle inhibitor p19Arf (17, 18). Interaction of MDM2 with pRB or E2F1 promotes G1/S cell cycle progression through both disruptions of pRB function and stimulation of transcriptional activity of E2F1. Since MDM2 binds to the ribosomal protein L5, it may enhance the translation process. Based on these findings, MDM2 may play important roles in cell growth and differentiation. In some circumstances these actions are independent of p53 action.

Estrogen receptors, ERα and ERβ, belong to a superfamily of nuclear receptors that regulates the transcription of various target genes upon binding to estrogen response elements (ERE) present within the regulatory region of the genes (19–21). Expression of ERα in breast tumors is closely associated with cancer biology, since the presence of ERα indicates a less aggressive phenotype and a more favorable prognosis in endocrine therapy (22, 23). In most ERα-positive breast cancers, especially in early stages, the expression level of ERα is considerably higher than that in normal breast epithelium. We have previously shown that transcriptional regulation from a specific distal promoter is responsible for this enhanced expression of ERα protein (24). In addition to ERα, the expression level of MDM2 protein is up-regulated in approxi-

[1] Current address: Department of Medical Nutrition, Karolinska Institute, Novum, S-141 86 Huddinge, Sweden.
[2] To whom correspondence should be addressed. Fax: +81-48-722-1739. E-mail: h.shin@nifty.com.

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mately 40% of breast cancer specimens, though gene amplification of MDM2 in breast cancer is uncommon (2, 3). Interestingly, there is a significant correlation between the levels of MDM2 and ERα in breast cancer specimens and breast cancer cell lines (25–28). In contrast to ERα-negative breast cancer cell lines, all ERα-positive cell lines are found to express an elevated level of *mdm2* mRNA (26), suggesting some functional relationship between MDM2 and ERα. These observations prompted us to look for this functional interaction of MDM2 and ERα in breast cancer cells.

In this study, we discovered a novel role of MDM2 that is independent of p53: enhancement of the transcriptional activity of ERα. This finding indicates that MDM2 is a positive regulator for ERα and may play an important role in breast carcinogenesis.

MATERIAL AND METHODS

Cells and cell culture. Human breast cancer cell line MCF-7 was obtained from the National Cancer Institute (Bethesda, MD) and maintained routinely in RPMI1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco BRL), 1 nM of 17β-estradiol (E2, Wako Pure Chemical Industries, Osaka, Japan), 100 units/ml penicillin G and 100 μg/ml streptomycin. For experiments evaluating the effect of E2, cells were cultured in phenol red-free RPMI1640 (PRF-RPMI, Gibco BRL) containing 10% FBS stripped of steroids by absorption to dextran-coated charcoal (DCC-FBS). MCF-7/pCmdm2 clones S1 and S3 were established by transfection of mammalian expression vector containing human *mdm2* cDNA (pCmdm2) into MCF-7 cells as described previously (29, 30). Saos-2, a p53-deficient osteosarcoma cell line, was from American type culture collection (Manassas, VA) and grown in McCoy's 5A medium (Gibco BRL) containing 15% FBS and antibiotics.

Colony formation assay in soft agar. Three thousand cells were suspended in 300 μl of 0.3% Difco's noble agar in phenol-red free Dulbecco's modified Eagle medium (PRF-DMEM, Gibco BRL) with 10% DCC-FBS and layered over 300 μl of 0.6% agar-medium basal layer in 24-well tissue culture plates. Cells were then fed with PRF-DMEM/10% DCC-FBS, supplemented with or without 1 nM E2, and incubated at 37°C for 16 days. Culture medium was replaced every 3 days. On day 16, the number of colonies >30 μm in diameter was then counted under microscope.

Western blotting of p53. Cells were grown in PRF-RPMI/10% DCC-FBS up to 60% confluency in 100 mm dishes. For E2 treatment, cells were incubated in the medium containing 1 nM E2 for 24 h. Cells were washed twice with ice-cold PBS and harvested in lysis buffer (1% Triton X-100, 50 mM NaCl, 25 mM Hepes (pH 7.4), 2 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin). Lysates were scraped and transferred into microtubes and centrifuged at 12,000*g* for 10 min. The supernatants were used as cell extracts. Protein concentration of the extracts was determined using Bio-Rad DC protein assay kit (Bio Rad, Hercules, CA). Same amount of cell extracts were applied on to 9% SDS–PAGE and subjected to Western blot analysis using 0.1 μg/ml mouse monoclonal p53 antibody (Ab-6, Oncogene Research Products, Cambridge, MA) as described before (30).

Plasmids. Estrogen-responsive reporter plasmid ptk-ERE-Luc was constructed by insertion of estrogen response element (ERE) (5′-AGC TAG GTC AGG ATG ACC TAG CTA-3′) into *Hin*dIII site of tk-luciferase plasmid (31). MDM2 expression plasmid pCmdm2 (32) was kindly supplied by Dr. K. Roemer (University of California, San Diego, CA). The human ERα expression plasmid pCER (33) was a gift from Dr. B. S. Katzenellenbogen (University of Illinois, Urbana, IL). Plasmids pCMX-GAL4 and pCMX-VP16 for mammalian two-hybrid experiments were kindly supplied by Dr. K. Umesono (Kyoto University, Kyoto, Japan) together with the reporter tk-GALpx3-Luc possessing GAL4 binding sequence (31). An internal control plasmid for luciferase assay, pRL-TK was purchased from Promega (Madison, WI). For construction of expression plasmids of fusion protein with GAL4 DNA binding domain or VP16 activation domain, *Eco*RI and *Bam*HI sites were designed adjacent to the initiation and termination codons of inserted ERα cDNA and MDM2 cDNA, respectively. Glutathione *S*-transferase (GST) fusion protein expression vectors were constructed as following: full-length MDM2 cDNA fragments were ligated into pGEX-2T vector (Pharmacia). N-terminus portion of the p53 cDNA was amplified by PCR with Pyrobest DNA polymerase (Takara Shuzo, Japan), using specific primers P53N; TAG GAT CCA TGG AGG AGC CGC AGT and P53MR; AAA CTC GAG GCT CCC CTT TCT TGC GG, and plasmid containing full length p53 cDNA, pcNXR5 (kindly provided by Dr. Takashi Takahashi, Aichi Cancer Center Research Institute, Japan) as a template. The amplified fragment was digested with *Bam*HI and *Xho*I, then ligated with pGEX-4T-2 vector (Pharmacia). Coding region of ERα cDNA was prepared using PCR with primers ER-5′; TAT AGG GCG AAT TCG GCC ACG GAC CAT, and ER-3′; ATA CTC GAG CTC TCA GAC TGT GGC AGG GAA. The amplified fragment was digested with *Eco*RI and *Xho*I, and ligated with pGEX-4T-2 vector. The sequences of all constructed plasmids were confirmed using ABI Prism 310 automatic DNA sequencer.

Luciferase assay using estrogen-responsive reporter plasmid. The reporter plasmid ptk-ERE-Luc was transfected into MCF-7, MCF-7/pCmdm2 clones and Saos-2 cells. Briefly, cells were plated on culture dishes to 30–40% confluency, and medium was replaced with PRF-RPMI medium. Plasmid cocktail with or without mdm2 expression vector was mixed with TransIT reagent (Mirus, Madison, WI) and added to the culture. Total amount of the plasmid was kept constant by adding plasmid pCMX vector. After 4 h incubation, the medium was replaced with fresh PRF-RPMI/10% DCC-FBS containing 1 or 10 nM E2. After 24 h incubation, luciferase activity was measured by the standard method using Luciferase Assay System (Promega) with Luminescence Reader BLR-201 (Aloka, Tokyo, Japan). All the luciferase activities were normalized against the protein concentration determined by BCA protein assay kit (Pierce, Rockford, IL); in some experiments, luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega) to normalize transfection efficiency.

Protein–protein interaction assay using mammalian two-hybrid system. Saos-2 cells were cultured in 60 mm diameter dishes in McCoy's 5A medium/15% FBS. The reporter plasmid tk-GALpx3-Luc (1 μg) was cotransfected with pCMX-VP16-MDM2 (0.1 μg) and pCMX-GAL4-ER (0.1 μg) using TransIT reagent per manufacturer's instruction. After 4 h incubation, medium was changed to fresh McCoy's 5A medium/15% DCC-FBS supplemented with or without 10 nM E2. The luciferase activity was measured as described above.

In vitro translation of MDM2 and ERα. *In vitro* expression of radiolabeled ERα and MDM2 protein were performed by using *in vitro* transcription and translation (TNT)-coupled rabbit reticulocyte extracts in the presence of [^{35}S]methionine, according to manufacturer's recommended conditions (TNT T7 Quick Coupled Transcription-Translation kit; Promega).

Recombinant protein preparation. GST fusion proteins GST-MDM2 in pGEX-2T, GST-ER in pGEX-4T-2 were prepared essentially as described previously (24). Briefly, fusion proteins were expressed in *Escherichia coli* BL-21 that had been grown for 8 h at 37°C and induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside at 27°C for 3 h. Bacterial pellets were resuspended in ice-cold PBS containing 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT) and lysed by probe sonication. Triton X-100 to a final concentration of 1% (w/v), was added to the sonicates. They were incubated at 4°C for 30 min with gently

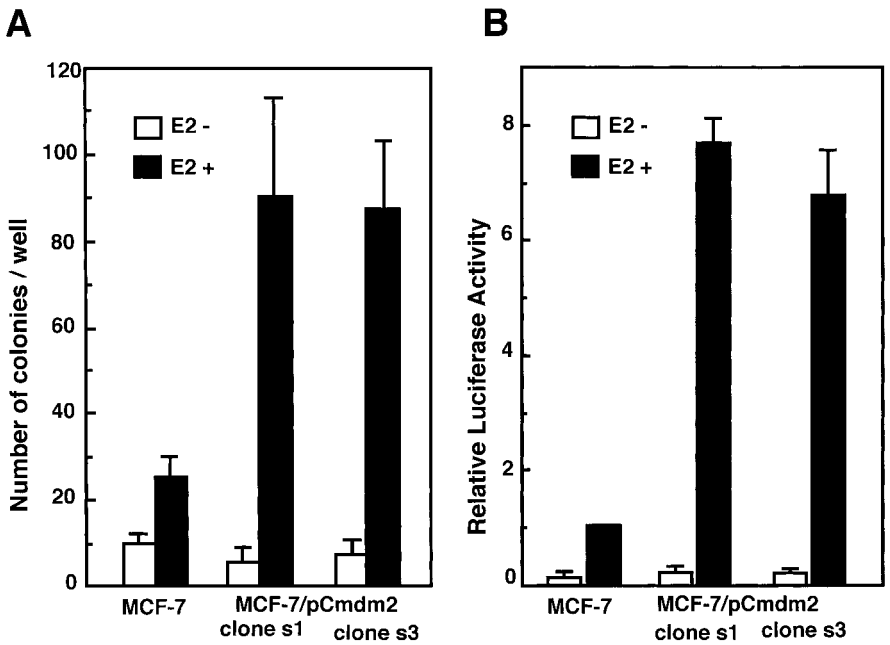

FIG. 1. Overexpression of MDM2 in MCF-7 cells potentiate a cell growth as well as ERα-mediated transcription activity in the presence of E2. (A) MDM2 overexpression clones of MCF-7, MCF-7/pCmdm2 s1 and s3, were established as described under Materials and Methods. Cells were fed in 0.3% agar medium with or without 1 nM E2 for 16 days in 24-well tissue culture plate. The numbers of colonies greater than 30 μm in a diameter were counted. The results shown are means ± SD of six experiments. (B) ERα-mediated transcription activity was assessed by transfection of 1 μg of ptk-ERE-Luc into MCF-7 and MCF-7/pCmdm2 s1 and s3 cells. The luciferase activities were measured after 24 h incubation in the presence or absence of 1 nM E2. Transfection efficiency was normalized against the *Renilla* luciferase activity of internal control plasmid pRL-TK. These assays were triplicated, and the mean values relative to the activity of MCF-7 in the presence of E2 are shown. Bars indicate SD.

shaking, and clarified by centrifugation at 13,000 rpm for 10 min. The supernatant was incubated with glutathione-Sepharose 4B (GS-4B) beads (Amersham Pharmacia Biotech). The beads were washed three times with ice-cold PBS containing 0.1% (w/v) NP-40. Levels of expression of GST fusion protein were estimated by SDS-PAGE followed by staining with Coomassie Brilliant Blue G-250. The known amounts of bovine serum albumin were used as standards.

GST pull-down assay. Twenty micrograms of fusion proteins bound to GS-4B beads were incubated with 5 μl of *in vitro* translated protein in 500 μl of binding buffer (50 mM KCl, 20 mM Hepes (pH 7.9), 2 mM EDTA, 0.1% NP-40, 5% glycerol (w/v), 0.5% nonfat dry milk and 5 mM DTT) at 4°C for 30 min. Before binding assays, GST fusion proteins were treated with ethanol (vehicle) and 100 nM estradiol at 4°C for 15 min. After washing three times with the binding buffer, bound proteins were recovered in 1× SDS–PAGE sample buffer and applied onto SDS–PAGE gel, and then gels were dried and visualized by autoradiography with a Fuji Bio-Image Analyzer BAS300 (Fuji, Tokyo).

RESULTS

Overexpression of MDM2 in MCF-7 Cells Accelerates ERα-Mediated Transcription

To study the biological role of MDM2 in ERα positive breast cancer cells, we have established two MDM2 overexpressing clones of MCF-7 cells, which have abundant amounts of ERα protein but very little of ERβ (34). We found that MCF-7/pCmdm2 clones s1 and s3 expressed 3.8- and 3.0-fold more MDM2 protein than the parental MCF-7 cells (30). In a soft agar colony formation assay, E2 supplementation resulted in remarkable growth advantages for the MDM2 overexpressing clones, compared with the parental MCF-7 cells, though no obvious difference was observed between the tested cells in E2 depleted culture medium (Fig. 1A). To clarify the mechanism of this growth acceleration, we examined the ERα function in these cells: the reporter plasmid ptk-ERE-Luc, which possesses a perfect palindromic estrogen response element (ERE) in front of the thymidine kinase (tk)-promoter, was transfected into MCF-7 and MCF-7/pCmdm2 clones s1 and s3. As shown in Fig. 1B, the reporter activity in s1 and s3 clones was 7.1- and 6.4-fold higher than that in MCF-7 cells in the presence of E2. In this experiment, expression levels of ERα protein in the tested cells, evaluated by Western blot, did not change (data not shown).

p53 Proteins Are Not Reduced in Stably Overexpressing Clones of MDM2

p53 has been reported to interact with ERα and inhibit the transactivation function of ERα in a dose-dependent manner (35, 36). To test the possibility that p53 reduction in MDM2 overexpressing clones was the cause of the enhancement of ERα function, we mea-

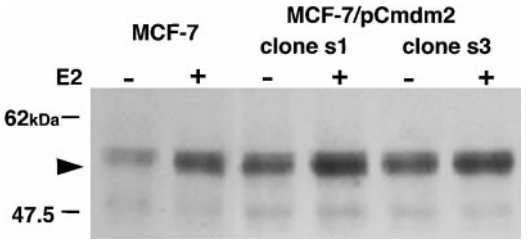

FIG. 2. Comparison of p53 protein expression in between MDM2 overexpression clones and parental MCF-7 cells. Cells were maintained without E2, and indicated cells were incubate with 1 nM E2 for 24 h. Equal amount of cell extracts were separated by SDS–PAGE and transferred onto PVDF membranes. Detection of p53 proteins were performed using Ab-6 mouse monoclonal p53 antibody (arrow head).

sured the expression level of p53 proteins in these clones under either E2 depleted or supplemented culture condition. As shown in Fig. 2, the expression levels of p53 in these cells were not less than that in parental MCF-7 cells, indicating that the enhancement of ERα function in MDM2 overexpressing clones is not due to the reduction of p53.

MDM2 Enhances ERα Function in the Absence of p53

To determine whether modulation of ERα function by MDM2 is independent of p53, we next carried out a transient transfection experiment using p53-null osteosarcoma Saos-2 cells. The reporter plasmid ptk-ERE-Luc and the ERα expression plasmid pCER were cotransfected into Saos-2 cells with or without various amounts of mdm2 expression plasmid. Transfection of MDM2 expression plasmid dose-dependently enhanced ERE-luciferase activity over that of control in the presence of E2 (Fig. 3), strongly indicating that MDM2 can potentiate the ERα function even in the absence of p53.

MDM2 Interacts with ERα in Vivo and in Vitro

The action of ER is regulated not only by ligands, but also by interaction with other proteins, including an array of co-regulatory ones, such as SRC-1 family members and CBP/p300 (37). To examine whether MDM2 physically interacts with ERα, we evaluated the interaction *in vivo*, using a mammalian two-hybrid assay. Cotransfection of a reporter plasmid, tk-GALpx3-Luc, along with expression vectors for GAL-ER and VP16-MDM2 chimeras, each alone or together, was performed in Saos-2 cells, which lacked detectable levels of both ERα and MDM2 proteins by Western blotting (data not shown). As shown in Fig. 4, GAL-ER transfected cells showed an enhanced reporter activity in a ligand dependent manner, probably due to its transactivating nature. Importantly, coexpression of VP-MDM2 with GAL-ER remarkably increased luciferase activity only in the presence of E2; it had no effect in the absence of E2. These results indicate a possibility that MDM2 physically interacts with ERα in a ligand-dependent manner *in vivo*.

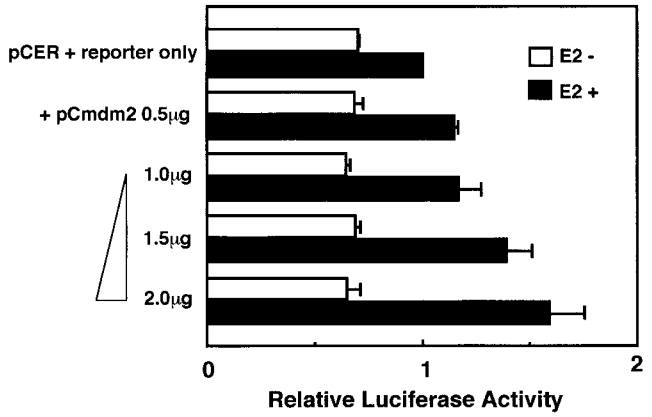

FIG. 3. MDM2 enhances ERα-mediated transcriptional activity in the absence of p53. One μg of reporter plasmid ptk-ERE-Luc and 0.1 μg of ERα expression plasmid pCER were cotransfected into p53-null Saos-2 cells with or without an indicated amount of MDM2 expression plasmids pCmdm2. After 24 h incubation in the presence or absence of 10 nM E2, luciferase activity was measured. Total amount of transfected plasmids was kept constant by addition of pCMX vector. These assays were triplicated, and the mean activities relative to that of reporter plasmid in the presence of E2 are shown. Bars indicate SD.

To confirm this, interaction of ERα and MDM2 protein *in vitro* using GST pull-down assay was performed. As shown in Fig. 5, *in vitro* translated MDM2 protein interacted specifically with ERα both in the absence or presence of E2. Addition of E2 could enhance the binding of GST-ERα to MDM2, however, this interaction was much weaker than that between GST-p53NM and MDM2 protein (Fig. 5). This results indi-

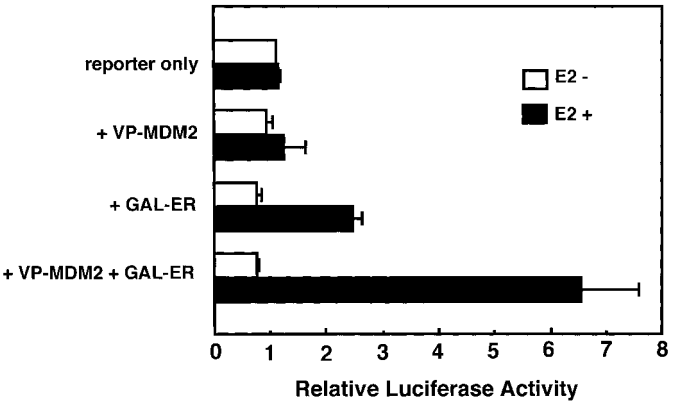

FIG. 4. MDM2 directly interacts with ERα in the presence of ligand *in vivo*. The reporter plasmid tk-GALpx3-Luc (1 μg) was cotransfected with 0.1 μg of pCMX-VP16-MDM2 and/or pCMX-GAL4-ER into Saos-2 cells. Luciferase activity was measured after 24 h incubation in the presence or absence of 10 nM E2. These assays were triplicated, and luciferase activity was normalized against protein concentration. Results shown are mean activities (±SD) relative to that of reporter plasmid in the absence of E2.

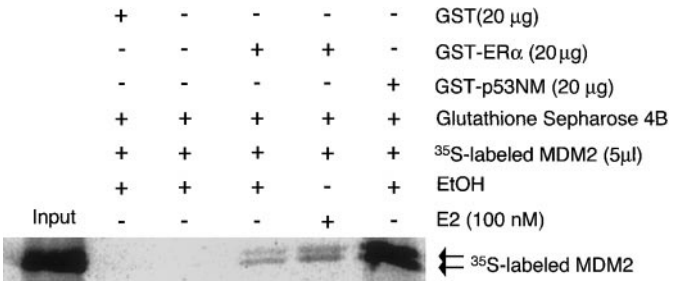

FIG. 5. MDM2 directly interacts with ERα *in vitro*. *In vitro* translated and [^{35}S]Met-labeled MDM2 protein was incubated with recombinant GST, GST-ER, or GST-MDM2 proteins in the presence or absence of estradiol. Proteins which bound to GST fusion protein were recovered with Glutathione Sepharose 4B and resolved by SDS–PAGE after eluted in SDS sample buffer. The gels were dried and visualized by autoradiography. Ethanol was used as a control of estradiol supplement.

cates that ERα and MDM2 can interact directly each other also *in vitro* assay preferably in the presence of E2.

DISCUSSION

We showed here that the oncogene product MDM2 physically interacts with ERα, leading to increased levels of estrogen dependent transcription. As for the biological significance of this finding, MDM2 overexpression in ERα positive breast cancer cell line MCF-7 was found to afford a remarkable growth advantage in response to E2. At first, from this accelerated cell growth in MDM2 overexpressing clones, it was expected that there would be down-regulation of p53 and a resultant loss of inhibition of ERα function since p53 can inhibit transactivation of ERα. Generally, overexpression of MDM2 promotes rapid degradation of p53 through an ubiquitin-proteasome system (5, 8–10). However, our MDM2 overexpressing clones, which were established by stable transfection of mdm2 cDNA, contained higher levels of p53 protein than the parental MCF-7 cells (Fig. 2). Though the mechanism of enhanced p53 expression in MDM2 overexpressing clones is still unclear, MDM2 and p53 may balance each other under stable condition. This notion is supported by other reports which showed overexpression of both wild type p53 and MDM2 in clinical samples (38, 39).

Since p53 modification by MDM2 was not the source of the E2 stimulated growth advantage, we tested the possibility that it was due to the function of MDM2 itself. Many studies have revealed that MDM2 binds to various proteins other than p53, altering their functions independently of p53 (12–17). ERE-reporter studies in p53-null Saos-2 cells clearly indicated that MDM2 by itself can modulate the ERα function in a p53 independent manner (Fig. 3). Although MDM2 increased ERα function only up to 1.5 fold of control in our experiment using Saos-2 cells, this might have been due to a lack of some regulatory molecules required for maximal response in this cell line.

It has been revealed that coregulatory proteins, coactivators, and corepressors, modulate the transactivation function of nuclear receptors via protein-protein interaction (37, 40). Among these coactivators, CBP-300/p300 (41) and members of the SRC-1 family such as TIF-2, SRC-1 and AIB1 (42–45), were found to interact with ERα in a ligand-dependent manner, resulting in an enhanced estrogen-dependent transcription activity. In our mammalian two-hybrid assay ERα interacted with MDM2 protein only in the presence of E2 (Fig. 4). Pull-down assay using GST-ERα with *in vitro* translated MDM2 protein showed the capability of their interaction also in the absence of E2 (Fig. 5). This result is consisted with a recent report doing almost identical pull-down assay (36). Whether the interaction between ERα and MDM2 in the living cells is ligand-dependent or independent can not be clarified in our experiments so far, since both *in vivo* and *in vitro* assay use the exogeneously expressed chimera-proteins in incomplete physiological condition. However at least, the presence of E2 seems to be more preferable condition for their interaction. Moreover, ERE-reporter assay in Saos-2 cells showed that MDM2 enhanced the ligand-dependent transcriptional activity of ERα, but did not affect the ligand-independent one (Fig. 3). This observation is consisted with our preliminary result that AF-2 domain of ERα, component for ligand-binding and ligand-dependent activation, is responsible for the interaction with MDM2. In addition to this, Liu *et al.* also reported that the mutant GST-ERα which lacked the AF-1 domain, component for ligand-independent function of ERα, could interact with MDM2 (36). These all findings indicate that the interaction between ERα and MDM2 must be important for the biology of breast cancer cell when it respond to E2 for the growth.

Recently, Nawaz *et al.* reported that E6-associated protein (E6-AP) directly interacts with nuclear hormone receptors, including ERα, and coactivates their transcriptional activity. The authors postulate a dual function of E6-AP as an ubiquitin-protein ligase (E3) and as a transcription coactivator (46). These dual functions of factors involving a ubiquitin-proteosome protein degradation pathway might be common between E6-AP and MDM2.

In summary, our findings presented here indicate a novel function of MDM2; MDM2 protein in breast cancer cells afforded a growth advantage in response to E2, in terms of the activation of ERα transcription. This may in turn provide the novel concept of cross talk between nuclear receptor and oncogene products in cancer cells, which could yield a clue to the role of MDM2 in the early stages of breast carcinogenesis.

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