Novel Role for RbAp48 in Tissue-Specific, Estrogen Deficiency-Dependent Apoptosis in the Exocrine Glands

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Although tissue-specific apoptosis in the exocrine glands in estrogen-deficient mice may contribute to the development of autoimmune exocrinopathy, the molecular mechanism responsible for tissue-specific apoptosis remains obscure. Here we show that RbAp48 overexpression induces p53-mediated apoptosis in the exocrine glands caused by estrogen deficiency. RbAp48-inducible transfectant results in rapid apoptosis with p53 phosphorylation (Ser9) and α -fodrin cleavage. Reducing the expression of RbAp48 through small interfering RNA inhibits the apoptosis. Prominent RbAp48 expression with apoptosis was observed in the exocrine glands of C57BL/6 ovariectomized (OVX) mice but not in OVX estrogen receptor $\alpha^{-/-}$, p53 $^{-/-}$, and E2F-1 $^{-/-}$ mice. Indeed, transgenic expression of the RbAp48 gene induced apoptosis in the exocrine glands but not in other organs. These findings indicate that estrogen deficiency initiates p53-mediated apoptosis in the exocrine gland cells through RbAp48 overexpression and exerts a possible gender-based risk of autoimmune exocrinopathy in postmenopausal women.

Estrogenic action has been suggested to be responsible for the strong female preponderance of many autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, and Sjögren's syndrome (50, 51). Recent evidence suggests that apoptosis plays a key role in the physiology and pathogenesis of various autoimmune diseases (2, 7, 19, 35, 42). We have demonstrated that estrogenic action influences target epithelial cells through Fas-mediated apoptosis in a murine model for Sjögren's syndrome (13). Recently, we found that tissue-specific apoptosis in the exocrine glands spontaneously occurring in estrogen-deficient mice may contribute to the development of autoimmune exocrinopathy (14). We speculate that antiestrogenic actions might be a potent factor in the formation of pathogenic autoantigens. It has been reported that the antiestrogen tamoxifen (TAM) induces cell death in the human breast cancer cell line MCF-7 (17). We observed a time- and concentration-dependent increase in apoptosis of mouse and human salivary gland cells ([MSG] mouse primary culture; [HSG] human cell line) treated with TAM but not in other cell lines (HT-29, Colo201, and Jurkat) (14).

Apoptosis can be initiated by many different factors, but activation of caspases, which are a special class of proteolytic enzymes, is always involved in this process. Activation of caspases may be achieved by several molecular pathways; the best known stimuli triggering the caspase cascade are stimulation of Fas or TNF receptors, release of cytochrome *c* from the cellular mitochondria, and exposure to granzymes, which are secreted by cytotoxic T cells (3, 12, 31, 37, 54). Detailed research on the mechanisms controlling caspase activity will pro-

vide better insight into the pathogenesis of autoimmune diseases with special reference to estrogen deficiency. In this study, we have focused on the molecular mechanisms responsible for tissue-specific apoptosis caused by estrogen deficiency and identified RbAp48 as a novel apoptosis-inducing gene exclusively in the exocrine glands. Retinoblastoma (Rb) protein is a multifunctional protein that binds to transcription factors and kinases to regulate both cell growth and apoptosis (11). Although recent data suggest that loss of Rb can cause apoptosis through derepression of basally inhibited extrinsic apoptotic pathway genes (20), no mechanism has provided a molecular explanation for RbAp48 in the induction of apoptosis.

MATERIALS AND METHODS

Cell culture and gene transfection. HSG, MSG, HT29, Colo201, HeLa, HepG2, SH-SY5Y, NEC14, THP-1, Jurkat, Raji, U937, and W138 cells were cultured in Dulbecco's modified Eagle medium (DMEM) or RPMI 1640 medium containing 10% fetal bovine serum at 37°C. HSG and MSG cells have been described elsewhere (38, 40). The following were used for cell cultures: 10⁻⁷ M TAM (Wako Pure Chemical, Osaka, Japan), 10⁻⁹ M β -2-estradiol (E2; Wako), 10⁻⁷ M staurosporin (Wako), paclitaxel (Wako), 1 μ M etoposide (Wako), 1 μ M ICI182780 (Wako), 25 ng/ml anti-Fas monoclonal antibody (MAb) (MBL, Nagoya, Japan), and 10 ng/ml recombinant human gamma interferon (R&D Systems, Minneapolis, MN). The RbAp48 gene inserted into the pCMV (2N3T) vector, a gift from D. Trouche, was transfected into the cells using FuGENE6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN). The RbAp48-stable cell line (RH0) from HSG cells in which RbAp48 expression was regulated by isopropyl-1-thio- β -D-galactopyranoside (IPTG), was established using a LacSwitch II Inducible Mammalian Expression System (Stratagene, La Jolla, CA). Briefly, the repressor vector (pCMVLacI) and RbAp48-inserted operator vector (pOPRVI/MCS) were cotransfected into HSG cells with FuGENE6, and the RbAp48 expression of hygromycin and G418-resistant transfectants was controlled by IPTG. For infection of adenovirus vector, RbAp48 gene-transfected MSG cells from p53 $^{-/-}$ or wild-type mice were infected with 100 multiplicities of infection of adenovirus vector including the p53 gene obtained from Toren Finkel (National Institutes of Health). MSG and mouse mammary glands (MMG) were removed, placed in DMEM containing 10% fetal calf serum (FCS) and 10 mM HEPES (pH 7.4), and rapidly minced. The mate-

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FIG. 1. Identification of the RbAp48 gene in salivary gland cell apoptosis. (A) A time-dependent increase in apoptotic HSG cells stimulated with TAM (10^{-7} M) was observed, and E2 (10^{-9} M) treatment inhibited apoptosis. Apoptotic cells were detected by flow cytometry using FITC-conjugated annexin V. (B) TAM-induced gene fragments cloned by differential display PCR were used as a probe by Northern blotting with mRNA from HSG cells treated with TAM (10^{-7} M) or E2 (10^{-9} M and 10^{-8} M). β -Actin mRNA was detected as an internal control. Each blot is representative of three independent experiments. (C) Analysis of RbAp48 mRNA expression was performed using total RNA from TAM-stimulated HSG and MCF-7 cells for 0 to 24 h. The graph is representative of three independent experiments. (D) The inhibitory effects of siRNA on TAM-induced apoptosis in HSG cells with siRNA (15 nM) of the RbAp48 construct but not in MCF-7 cells. After transfection of siRNA of RbAp48 and a fluorescence-labeled control gene, the cells were incubated with TAM (10^{-7} M) for an additional 24 h. Apoptosis was estimated by flow cytometric analysis using PE-conjugated annexin V. The graph is representative of three independent experiments. (E) A dose-dependent inhibition of siRNA (0 to 200 nM) of TAM-induced RbAp48 expression in HSG cells, not MCF-7 cells, was observed. The graph is representative of two independent experiments. (F) RbAp48 mRNA expression levels were analyzed using human tissue total RNA-blotted membrane. Message levels (arbitrary units) were quantified by BAS-2000II and expressed as the ratio of RbAp48 mRNA/ β -actin mRNA. The graph is representative of two independent experiments.

FIG. 2. RbAp48 overexpression in OVX B6 mice. (A) Increased RbAp48 expression in salivary and lacrimal gland tissues in OVX B6 mice from 0 to 3 weeks (age of mice, 4 to 7 weeks). Expression levels of RbAp48 in thymus, spleen, liver, and mammary glands from OVX B6 mice were constant. Western blot analysis was performed on proteins from tissue homogenates of OVX and sham mice. Blots were representative of three independent experiments. (B) Detection of RbAp48⁺ and TUNEL⁺ cells in salivary and lacrimal glands from OVX B6 and sham B6 mice at the age of 7 weeks. Immunohistochemical analysis of RbAp48 and in situ TUNEL assays were performed on the sections of salivary and lacrimal glands from OVX and sham mice. Images are representative of sections from five mice. The percentage of RbAp48⁺ and TUNEL⁺ cells in salivary and lacrimal glands was enumerated using a 10- by 20-mm grid covering an objective area of 0.16 mm². Data were analyzed in 10 fields per section and expressed as mean percent $\pm$ standard deviation of data from five mice. (C) TAM-induced apoptosis was associated with RbAp48 expression in

rials were then digested for 1 h at 37°C with 750 U/ml collagenase (Wako), 500 U/ml hyaluronidase type IV (Sigma), 1% bovine serum albumin, and 10 mM HEPES (pH 7.4) in DMEM. After digestion, they were filtered through a 70- μ m nylon mesh, centrifuged, and rinsed twice with DMEM containing 10% FCS. These cells were cultured in chamber slides (Nalge Nunc International, Denmark) at a density of 5×10^4 /well with DMEM containing 10% FCS. After cells were cultured for 24 h, the medium was changed to HuMedia-KG2 (Kurabo, Osaka, Japan).

Differential display analysis and Northern blotting. Total RNA was isolated from TAM-treated or nontreated HSG cells and reverse transcribed for differential display PCR with an RNImage kit (Gene Hunter, Nashville, TN). TAM-induced cDNA fragments were gel excised and subcloned for TA vector. The clones were screened with a cDNA library derived from mRNA of TAM-stimulated HSG cells. The screened clone was transformed to plasmid and sequenced. Expressions of RbAp48 mRNA were detected by Northern blot analysis using ^{32} P-labeled RbAp48 cDNA probe. Equal loading of the gel was confirmed by using β -actin cDNA probe. In addition, the human total RNA-blotted membrane (Biocain Institute, Inc., San Leandro, CA) was used for analysis of RbAp48 mRNA in various human tissues.

Apoptosis detection assay. Apoptosis was detected using the annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Genzyme Corp., Cambridge, MA). Briefly, after cultured cells were washed with phosphate-buffered saline, the cells were incubated with FITC-conjugated annexin V and propidium iodide (PI) for 10 min at room temperature in the dark. Binding buffer was added, and apoptotic cells were detected by flow cytometric analysis with an EPICS flow cytometer (Beckman Coulter, Inc., Miami, FL).

Mice. Estrogen receptor α -deficient (ER $\alpha^{-/-}$), p53 $^{-/-}$, E2F1 $^{-/-}$, or C57BL/6 (B6) mice were purchased from Taconic (Germantown, NY), Jackson Laboratory (Bar Harbor, ME), or Nihon Clea (Tokyo, Japan). These mice were subjected to ovariectomy (OVX mice) and/or to a sham operation (sham mice) at 4 weeks of age. At 0 to 3 weeks after OVX, all organs were evaluated by pathological or immunohistochemical analysis. To generate the RbAp48-transgenic (TG) mice, B6 mice were used to obtain fertilized eggs, and the gene fragment containing RbAp48 cDNA regulated by salivary gland-specific promoter (22) (provided by B. B. Larsen) was microinjected into the pronucleus of fertilized eggs to establish the transgenic lines. Histopathological analysis of all organs of RbAp48-TG mice screened by PCR was performed. All mice were maintained in our specific-pathogen-free facility.

siRNA of RbAp48. Small interfering RNA (siRNA) corresponding to the coding sequence +136 to +156 of the RbAp48 gene was synthesized by Hokkaido System Science (Sapporo, Japan) according to standard methods (23, 52) for the following: sense, CGAGGAAUACAAAUAUGGTT; antisense, CCAUAAUUUGUAUUCUCGTT. siRNA of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Ambion, Austin, TX) was used as a control. siRNA (0 to 50 nM) and 1 μ g of pCMV-green fluorescent protein (GFP) plasmid were cotransfected into HSG, MCF-7 cells, and the IPTG-controlled RbAp48-stable cell line (RH0) using a Silencer siRNA Transfection II Kit (Ambion) or FuGENE6 (Roche). At 24 h after cotransfection, RH0 cells were incubated with IPTG for an additional 24 h. GFP $^{+}$ apoptotic cells were detected by flow cytometry using phycoerythrin (PE)-conjugated annexin V.

E2F-1, ARF, and p53 siRNA. For siRNA of E2F-1, ARF, and p53, an siTrio Full Set (B-Brigde International, Sunnyvale, CA) was used for HSG cells. Briefly, each cocktail including the three RNA oligonucleotides listed below was transfected into cells with a Quick-Step Transfection Kit (B-Brigde International). Sequences of the oligonucleotide sets are as follows: for E2F-1, CCAACGUCCUUGAGGGCAUTT (sense), AUGCCCUCAAGGACGUUGTT (antisense), CUGCAGAGCAGAUGGUUAUTT (sense), AUAACCAUCUGCUCUGCAGTT (antisense), GGAAAGUGAGGGAGGGAGATT (sense), and UCUCCCUCCCUCACUUUCCTT (antisense); for ARF, GCUCACCUCUGGUGCCAAATT (sense), UACCAAGAAGACCGCGCACTT (antisense), GGGUUUUCGCGGUUCACAUTT (sense), AUGUGAACCACGAAAACCTT (antisense), GGGUUUUCGUGGUUACAUTT (sense), and AUGUGAACCACGAAAACCTT (antisense); for p53, GGAAACUACUCCUGAAAATT (sense), UUUUCAGGAAGUAGUUUCCTT (antisense), CUGGAAGACUCCAGUGGUATT (sense), UACCACUGGAGUCUCCAGTT (antisense), CUUAGUACCUAAA

AGGAAATT (sense), and UUCCUUUUAGGUACUAAGTT (antisense). Transfected cells were incubated with or without TAM, and confocal or flow cytometric analysis was performed.

Western blotting. Whole-cell extracts of HSG or RH0 cells were purified using radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride) supplemented with a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). After centrifugation for 20 min at 12,000 rpm at 4°C, the supernatant was extracted and used for samples. Also, to detect α -fodrin in organs, tissue samples from OVX and sham C57BL/6 mice were extracted as described above. Ten micrograms of each sample per well was used for 7.5 to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes, which were probed with anti-RbAp48, anti-Rb (p110 and p130), anti-Bad, anti-Bax, anti-ARF (p14 and p19), anti-cyclin D3 (BD Transduction Laboratories, Lexington, KY), anti-Mdm2, anti-E2F-1, anti-phospho-Rb (Sigma), anti-p53, anti-phospho-p53 Ab sampler kit (Ser6, Ser9, Ser15, Ser20, Ser37, Ser46, and Ser392; Cell Signaling Technology Inc., Beverly, MA), anti- α -fodrin (Affiniti, Mamhead, United Kingdom), and anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA) as the primary Abs, and anti- α -tubulin, GAPDH, or histone MAb (Sigma) as internal control. The nitrocellulose membranes were incubated with peroxidase-conjugated horse anti-mouse or rabbit immunoglobulin G (IgG; Vector Laboratories) as the secondary Ab. Protein binding was visualized with ECL Western blotting reagent (Amersham Corp., Arlington Heights, IL).

TUNEL assay. Apoptotic cells were detected in sections using the in situ terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) kit (Wako). Sections were incubated with proteinase K (20 μ g/ml) for 10 min and then presoaked in TdT buffer (0.5 μ M cacodylate, 1 mM CoCl $_2$, 0.5 μ M DTT, 0.05% bovine serum albumin, 0.15 M NaCl) for 10 min. Sections were incubated for 2 h at 37°C in 25 μ l of TdT solution, containing $1 \times$ terminal transferase buffer, 0.5 nmol of biotin-dUTP, and 10 U of TdT. After the TdT reaction, sections were soaked in TdT blocking buffer (300 mM NaCl, 30 mM Tris-sodium citrate-2-hydrate), incubated with horseradish peroxidase-conjugated streptavidin for 30 min at room temperature, and developed for 10 min in phosphate-buffered citrate (pH 5.8) containing 0.6 mg/ml DAB (3,3'-diaminobenzidine-tetrahydrochloride-dihydrate). Nuclei were counterstained with hematoxylin. For confocal microscopic analysis, FITC-labeled UTP was used.

Caspase activity assay. Caspase activities were assayed using a caspase family colorimetric substrate set (BioVision Inc.). Briefly, 100 μ g of cytoplasmic lysates of RH0 cells was incubated with 200 μ M Ac-YVAD-pNA (caspase 1 substrate), Ac-VDVAD-pNA (caspase 2 substrate), Ac-DEVD-pNA (caspase 3 substrate), Ac-WEHD-pNA (caspase 5 substrate), Ac-VEID-pNA (caspase 6 substrate), Ac-IETD-pNA (caspase 8 substrate), and Ac-LEHD-pNA (caspase 9 substrate) at 37°C for 1 h. The absorbance of samples was read at 405 nm in a microtiter plate reader. The relative percent increase in caspase activity was determined by comparing these results with the level of the uninduced control.

Gel shift assay. Nuclear extracts were prepared from RH0 cells by a method previously described (29). Nuclear extracts containing 5 μ g of protein were incubated in 20 μ l of binding buffer (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM MgCl $_2$, 0.5 mM DTT, and 4% glycerol) with or without a cold competitor. The E2F-1 DNA probe, 5'-TCCGTAGTTTTCGCGCTTAAATTTGAGAAAGGGCGCAACTAGTC-3' (10,000 cpm) labeled with [γ - ^{32} P]ATP was added, and the samples were incubated at room temperature for 20 min. Reaction mixtures were separated in a 4% polyacrylamide gel and autoradiographed on X-ray film (Fujifilm, Kanagawa, Japan).

Immunohistochemical analysis. Immunohistochemical analysis of RbAp48 expression was performed on the sections of salivary and lacrimal glands from sham, OVX B6, RbAp48-WT (wild type), and RbAp48-TG mice. Paraffin-embedded sections were stained with anti-RbAp48 MAb (BD Transduction Laboratories) as the primary Ab. Protein binding was detected with an LSAB2 kit containing horseradish peroxidase (DAKO, Carpinteria, CA) and DAB as a substrate. The counterstaining of nuclei was performed with hematoxylin.

Confocal microscopy. Confocal microscopic analysis of RbAp48, E2F-1, ARF, and p53 expression, and TUNEL-positive cells was performed on the cultured cells, and frozen sections of salivary glands from sham, OVX ER $\alpha^{-/-}$, p53 $^{-/-}$,

MSG cells from B6 mice, and the inhibitory effects with siRNA of RbAp48 construct were observed by confocal microscopic analysis. The percentage of TUNEL $^{+}$ apoptotic cells was enumerated as described. Cont, irrelevant siRNA control. Images are representative of three independent experiments. (D) TAM-induced apoptosis was not associated with RbAp48 expression in the primary culture of MMG cells from B6 mice. TUNEL $^{+}$ apoptotic cells were enumerated as described. Images are representative of three independent experiments.

E2F1^{-/-}, RbAp48-WT, and RbAp48-TG mice using a Confocal Laser Microscan (LSM 5 PASCAL; Carl Zeiss, Germany). As the second Abs, Alexa Fluor 488–anti-mouse IgG heavy and light chain [IgG (H+L)], Alexa Fluor 568–goat anti-rabbit IgG (H+L), Alexa Fluor 488–donkey anti-rat IgG (H+L), Alexa Fluor 488–chicken anti-goat IgG (H+L), and Alexa Fluor 568–rabbit anti-goat IgG (H+L) were used. Nuclear DNA was stained with 4',6-diamidino-2-phenylindole dihydrochloride.

BrdU incorporation. MSG cells from RbAp48 TG and WT mice were stimulated with phorbol 12-myristate 13-acetate for 24 h, and 10 mM bromodeoxyuridine (BrdU) was incorporated for the last 2 h. Fixed and permeabilized cells were treated with DNase and stained with FITC-conjugated anti-BrdU antibody (BD Pharmingen, San Diego, CA). The polyvinylidene difluoride DNA synthetic activity was analyzed by flow cytometry.

RESULTS

Identification of TAM-induced gene. We found a time-dependent increase in apoptotic HSG cells stimulated with TAM, and E2 treatment inhibited the apoptosis (Fig. 1A). To identify gene products specific to TAM-induced apoptosis in the salivary gland cells, mRNAs from HSG cells treated with TAM and nontreated cells were analyzed by a differential display PCR method. From the samples isolated with the highest grade of differential expression, we analyzed the mRNAs from HSG cells treated with TAM and nontreated cells by a reverse Northern blotting technique (Fig. 1B). The sequence of TAM-induced mRNA corresponds (100%) to RbAp48. The expression of RbAp48 mRNA in HSG cells reached peak level at 2 h after stimulation with TAM, and then the level decreased, whereas increased expression of RbAp48 mRNA in MCF-7 cells was not observed (Fig. 1C). We confirmed the inhibitory effects of siRNA on TAM-induced apoptosis in HSG cells, not MCF-7 cells, with siRNA (5 to 50 nM) of RbAp48 construct (Fig. 1D). In addition, a dose-dependent inhibition of siRNA (0 to 200 nM) on TAM-induced RbAp48 expression in HSG cells, not MCF-7 cells, was observed (Fig. 1E). We next searched the tissue distribution of RbAp48 mRNA using human tissue total RNA-blotted membrane by Northern blot analysis. We found the highest level of expression of RbAp48 mRNA in the testis, which is consistent with the previous report (33), and the lowest was found in the parotid salivary gland (Fig. 1F), although the molecular mechanism by which the lowest RbAp48 mRNA is expressed in the parotid glands is unclear.

RbAp48 overexpression in estrogen-deficient mice. To confirm the in vivo overexpression of RbAp48 and apoptosis in estrogen-deficient B6 mice, OVX was performed on mice at the age of 4 weeks. Using Western blotting, we detected a time-dependent increase in RbAp48 in the salivary and lacrimal gland tissues from 0 to 3 weeks after OVX (at the age of 4 to 7 weeks) but not in other organs, including mammary glands (Fig. 2A). RbAp48⁺ and TUNEL⁺ apoptotic cells were detected by immunohistochemical analysis in the salivary and lacrimal gland sections from OVX B6 mice at the age of 7 weeks but not in sham mice (Fig. 2B). In vitro studies using primary cultured cells from B6 mice demonstrated that TAM-induced apoptosis was associated with RbAp48 expression in MSG cells but not in MMG cells (Fig. 2C and D). We confirmed the inhibitory effects of siRNA in MSG cells but not in MMG cells with siRNA of RbAp48 construct (Fig. 2C and D).

RbAp48 as a novel apoptosis-inducible gene. RbAp48 mRNA expression and apoptosis could be induced in HSG

cells stimulated with TAM and a pure antiestrogen, ICI182780, but not with other apoptotic stimuli such as staurosporin, paclitaxel, anti-Fas MAb, and etoposide (Fig. 3A). This indicates that induction of RbAp48 mRNA expression might be dependent on estrogen deficiency. To ensure the role of RbAp48 in various types of cells, RbAp48 was transiently transfected, and apoptosis was determined by flow cytometry using an annexin V-FITC apoptosis detection kit. Among the cells examined (HSG, MSG, MCF-7, HT-29, Colo201, HeLa, HepG2, SH-SY5Y, NEC14, THP-1, Jurkat, Raji, U937, and WI38), significant apoptosis was induced exclusively in the salivary gland cells transfected with RbAp48 of both human and mouse origin (Fig. 3B). Notably, apoptosis was induced by transfection with RbAp48 cDNA in MSG cells isolated from ERα^{-/-} mice, indicating that this signaling might act in the downstream of estrogen-ER binding. Apoptotic cells could not be induced by the transgene of RbAp48 cDNA but was induced by TAM in MCF-7 cells. We confirmed that the induction levels of RbAp48 are the same in the other cell lines including Jurkat and THP-1 as the HSG cells (Fig. 3C). We next generated and analyzed the RbAp48-stable cell line (RH0), which was an IPTG-inducible transfectant of RbAp48 in HSG cells, with a LacSwitch II Inducible Mammalian Expression System using repressor and operator vectors. Apoptosis was drastically induced in IPTG-treated RH0 cells in association with RbAp48 expression within 8 h (Fig. 3D). When we examined the effect of siRNA on RbAp48-induced apoptosis, the apoptosis in IPTG-treated RH0 cells was clearly inhibited by siRNA of RbAp48 but not by siRNA of GAPDH or an irrelevant control (Fig. 3E).

Molecular mechanisms for RbAp48-induced apoptosis. We next examined the molecular mechanisms responsible for RbAp48-induced apoptosis. We detected upregulation of phosphorylated Rb, cyclin D3, p14ARF, Bax, Bad, cytochrome *c* (Cyt *c*) and a cleavage product of α-fodrin (arrow) in IPTG-treated RH0 cells (Fig. 4A). Our previous report demonstrated that α-fodrin is a candidate autoantigen of primary Sjögren's syndrome (10). When nuclear extracts of IPTG-treated RH0 cells were analyzed by gel shift assay, DNA binding activity of E2F-1 was detected in RbAp48-induced apoptotic cells (Fig. 4B). E2F-1 protein was also detected in the nuclear extract by Western blotting (Fig. 4B). It has been proposed that the E2F-1 transcription factor serves as a link between the Rb/E2F proliferation pathway and the p53 apoptosis pathway by inducing the expression of p14ARF, a protein that regulates p53 stability (36). We next focused on the p53-dependent pathway, because MSG cells transfected with RbAp48 isolated from p53^{-/-} mice are apoptosis resistant (Fig. 4C). When Adp53-infected MSG cells were transfected with the RbAp48 gene, apoptosis was rapidly induced (Fig. 4C). Phosphorylated p53 (Ser9) was found by Western blotting after 2 to 4 h, but no other phosphorylated p53 (Ser15, Ser20, and Ser392) was detected (Fig. 4D). The phosphorylation of p53 in the other sites (Ser6, Ser37, and Ser46) was not observed (data not shown). We also confirmed a time-dependent downregulation of Mdm2 (Fig. 4D), which is important as a regulatory partner of p53 (47). Using Western blotting, we also detected increased p21 expression, a major player in the p53-mediated pathway, in IPTG-treated RH0 cells (Fig. 4E). p53 induces apoptosis by a multitude of molecular pathways, in addition to transactivation

FIG. 3. Role of RbAp48 in salivary gland cell apoptosis. (A) HSG cells were treated with antiestrogenic reagents (10^{-7} M TAM and 10^{-7} M ICI182780) and general apoptotic stimuli (staurosporin, paclitaxel, anti-Fas MAb, and etoposide). RbAp48 mRNA was quantitated with BAS-2000II, and message level was expressed as the ratio of RbAp48 mRNA/ β -actin mRNA; the percentage of apoptotic cells was detected by FITC-annexin V-PI. (B) The RbAp48 gene was transiently transfected into various cells using FuGENE6. At 48 h after transfection of pCMV-RbAp48 plasmid or pCMV (mock) plasmid, apoptotic cells were detected by FITC-annexin V-PI. Data are the means $\pm$ standard deviations of triplicate samples. The graph is representative of three independent experiments. (C) The levels of the induction ratio of RbAp48 were shown to be the same in the other cell lines including Jurkat and THP-1 as the HSG cells using Western blot analysis. The levels were expressed as the ratio of RbAp48/GAPDH protein. (D) Establishment of the RbAp48 stable cell clone. An increase in RbAp48 expression and apoptosis of IPTG-treated RH0 cells were observed in a time-dependent manner. Apoptotic cells were detected by FITC-annexin V-PI. Data are means $\pm$ standard deviations of triplicate samples. Expressions of RbAp48 and GAPDH as an internal control were detected by Western blot analysis. Graph and images are representative of four independent experiments. (E) Inhibitory effects of siRNA on RbAp48-induced apoptosis. IPTG-treated RH0 cells were cotransfected with siRNA (5 to 50 nM) of RbAp48 and pCMV-GFP. Apoptotic cells gated on GFP $^{+}$ were detected by PE-conjugated annexin V. Data are means $\pm$ standard deviations of triplicate samples. Graph is representative of three independent experiments.

FIG. 4. Molecular mechanisms responsible for RbAp48-induced apoptosis. (A) Contribution of cell cycle and mitochondrion-related molecules in RbAp48-induced apoptosis of RH0 cells. The lysates of IPTG-treated or nontreated RH0 cells were used to detect RbAp48, Rb (p110 and p130), phospho-Rb, cyclin D3, p14ARF, Bax, Bad, Cyt-c, the cleavage product of α -fodrin, and GAPDH for Western blot analysis. Blots are representative of three independent experiments. (B) Detection of E2F-1 transcriptional activity in RbAp48-induced apoptosis. The nuclear proteins of IPTG-treated RH0 cells were analyzed by gel shift assay with an E2F-1 binding DNA probe. To confirm the specific binding to the E2F-1 binding site, the mutant oligonucleotide and wild-type oligonucleotide as a competitor were used for this assay. E2F-1 protein was detected in the nuclear extract by Western blotting. Histone was used for an internal control. Blots are representative of three independent experiments. (C) Apoptosis of MSG cells from p53^{-/-} mice was not observed by RbAp48 gene transfection. Transfection with pCMV (Mock) was used as control. MSG (p53^{-/-}) cells were infected with Adp53 and incubated for 24 h. The infected cells were cotransfected with the RbAp48 gene and

of target genes, and it can elicit apoptosis by transcription-independent mechanisms (5, 28). Although apoptosis in response to p53 activation is often accompanied by caspase activation, the mechanisms underlying p53-induced caspase activation remain poorly understood. Caspase activities in RbAp48-induced apoptosis in HSG cells were assayed using a caspase family colorimetric substrate set. A significant increase in caspase 1 activity was detected with relatively elevated caspase 3 and 8 activity on RbAp48-induced apoptotic HSG cells (Fig. 4F). RbAp48-induced apoptosis in HSG cells was clearly inhibited by siRNA of E2F-1 and p53 but only moderately by siRNA of ARF (Fig. 4G).

RbAp48/E2F1/ARF-p53 pathway in the salivary glands. We evaluated the effects of RbAp48 overexpression and knock-down in primary MSG cells and documented the effects on E2F-1, ARF, and p53 protein levels in these cells. We demonstrated that overexpression of RbAp48 in MSG cells from B6 mice induced E2F-1, p19ARF, and phospho-p53 expression, and the inhibitory effect of siRNA of RbAp48 was observed from confocal microscopic analysis (Fig. 5A). We next examined whether TAM-induced apoptosis is associated with RbAp48 expression in MSG cells from B6 mice, compared with cells from ER^{-/-}, E2F1^{-/-}, and p53^{-/-} mice. By confocal microscopic analysis, we found that TAM-induced apoptosis was associated with RbAp48 expression in MSG cells from B6 mice but not from ER^{-/-}, E2F1^{-/-}, and p53^{-/-} mice (Fig. 5B). We further examined the effect of OVX on the expression of RbAp48, E2F-1, p19ARF, and phospho-p53 in MSG cells from B6, ER^{-/-}, E2F1^{-/-}, and p53^{-/-} mice. By double-labeled confocal microscopy, we found coexpression of RbAp48/E2F1, RbAp48/p19ARF, and RbAp48/p53 in MSG cells from OVX B6 mice but not from B6 mice (Fig. 5C). No differences in RbAp48/E2F1, RbAp48/p19ARF, and RbAp48/phospho-p53 expression levels were observed in MSG cells from non-OVX and OVX ER^{-/-}, E2F1^{-/-}, and p53^{-/-} mice (Fig. 5C).

Findings in RbAp48-transgenic mice. We constructed several lines of B6 background TG mice (39) expressing RbAp48 in the salivary glands using Lama promoter as described in Materials and Methods. Prominent expression of RbAp48 in the salivary glands from TG mice was determined at the age of 8 to 20 weeks by both immunohistochemistry and Western blotting (Fig. 6A and B). No difference in RbAp48 expression in the spleen was observed between TG and WT mice. A considerable number of TUNEL⁺ apoptotic epithelial duct cells were found in the salivary glands of RbAp48-TG mice but not WT mice at the age of 20 weeks (Fig. 6C). In addition, expression of E2F-1, p19ARF, and phospho-p53 was observed in the salivary

glands of RbAp48-TG mice but not WT mice (Fig. 6D). BrdU studies of TG mice with ectopic RbAp48 in the salivary glands demonstrated that cellular proliferation is barely affected (Fig. 6E). No pathological findings were observed in other organs of TG mice.

DISCUSSION

In this study, we demonstrated the first evidence that gender-based, tissue-specific apoptosis could be induced in the exocrine gland cells through RbAp48 overexpression with p53 phosphorylation. Indeed, RbAp48 overexpression with apoptosis was observed in the exocrine glands in OVX C57BL/6 mice, and transgenic expression of the RbAp48 gene induced tissue-specific apoptosis in the exocrine glands.

RbAp48, initially identified as a retinoblastoma binding protein (34), was characterized as a component of distinct nucleosome-modifying complexes, including the nuclear histone deacetylases (18, 25). In general, the functions of the RbAp48-like proteins in these complexes remain undetermined. It was reported that E2F-1 and RbAp48 are physically associated in the presence of Rb and histone deacetylase (26), suggesting that RbAp48 could be involved in transcriptional repression of E2F-responsive genes. The induction of apoptosis in various cell lines is accompanied by a shift in Rb from the hyper- to the hypophosphorylated form (49). Rb dephosphorylation, which has been shown to be required for apoptosis, occurs in the early stage of apoptosis (6). Loss of Rb function can induce p53-dependent apoptosis, but little is known about the mechanisms of Rb-regulated p53-dependent apoptosis. Recently, Lieman et al. provided evidence for a novel mechanism linking Rb-E2F to the extrinsic apoptotic pathway through inactivation of focal adhesion kinase and activation of caspase 8 (20). It has been proposed that the E2F-1 transcription factor serves as a link between the Rb/E2F proliferation pathway and the p53 apoptosis pathway by inducing the expression of p14ARF, a protein that regulates p53 stability. Recent observations have revealed that p53 can directly translocate to mitochondria and induce apoptosis in a transactivation-independent manner (21). In this study, we confirmed a time-dependent downregulation of Mdm2, which is important as a regulatory partner of p53 (47). In addition to regulation of p53, Mdm2 has been reported to stimulate E2F-1 transactivation by a mechanism that remains unclear. E2F-1 can signal p53 phosphorylation in the absence of p14ARF, similar to the observed modifications to p53 in response to DNA damage. p53 modification is found to be crucial for E2F-1-mediated apoptosis, and this apoptosis is compromised when E2F-1 is coexpressed with a p53 mutant

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pCMV-GFP, and then apoptosis was detected by PE-annexin V on GFP⁺ cells. Infection of adenovirus β -galactosidase was used as a control. Graphs are representative of five independent experiments. (D) Expression levels of p53, phospho-p53 (Ser9), and Mdm2 in IPTG-treated RH0 cells. Other phosphorylated p53 proteins (Ser15, Ser20, and Ser392) were not detected. GAPDH expression was used for an internal control. Blots are representative of three independent experiments. (E) Detection of increased p21 expression, a major player in the p53-mediated pathway, by Western blotting. Blot is representative of two independent experiments. (F) Caspase activities of IPTG-treated RH0 cells were analyzed by a caspase enzymatic activity assay. A significant increase in caspase-1 activity was detected with relatively elevated caspase 3 and 8 activity. The absorbance of samples was read at 405 nm in a microtiter plate reader and the relative percent increase in activity was calculated by comparing the absorbance of IPTG-treated cells with that of untreated cells. Data are means $\pm$ standard deviations of triplicate samples. The graph is representative of three independent experiments. (G) RbAp48-induced apoptosis in HSG cells was clearly inhibited by siRNAs of E2F-1 and p53 but only moderately by siRNA of ARF. Graphs are representative of three independent experiments.

FIG. 6. RbAp48 overexpression and apoptosis in TG mice. (A) RbAp48 overexpression in the salivary gland tissues of TG mice but not WT mice at the age of 20 weeks detected by immunohistochemistry. The percentage of RbAp48⁺ cells was enumerated as described above (legend of Fig. 2B). (B) RbAp48 expression in the salivary gland tissues at the age of 20 weeks of TG mice compared with age-matched WT mice as detected by Western blotting. No differences in the levels of RbAp48 expression in spleens of WT and TG mice were detected. GAPDH expression was used for an internal control. (C) TUNEL⁺ apoptotic epithelial duct cells were found in salivary glands of TG mice but not of age-matched WT mice at the age of 20 weeks. The percentage of TUNEL⁺ cells was enumerated as described above. Images are representative of five mice. (D) Expression of E2F-1, p19ARF, and P-p53 was observed by confocal microscopy in the salivary glands of RbAp48-TG mice but not WT mice. Images are representative of five mice. (E) BrdU studies of TG mice with ectopic RbAp48 in the salivary glands demonstrated that cellular proliferation is barely affected. The graph is representative of three mice.

FIG. 5. RbAp48/E2F1/ARF-p53 pathway in the salivary glands. (A) Overexpression of RbAp48 in MSG cells from B6 mice induced E2F-1, p19ARF, and phospho-p53 (P-p53) expression, and the inhibitory effect of siRNA (15 nM) of RbAp48 was observed by confocal microscopic analysis. Cont, irrelevant siRNA control. Images are representative of three independent experiments. (B) TAM-induced apoptosis was associated with RbAp48 in MSG cells from B6 mice but not from ER^{-/-}, E2F1^{-/-}, and p53^{-/-} mice as detected by confocal microscopy. Images are representative of two independent experiments. (C) Coexpression of RbAp48/E2F1, RbAp48/p19ARF, and RbAp48/p53 was detected by double-labeled confocal microscopic analysis in MSG cells of OVX B6 mice but not sham B6 mice. No differences in RbAp48/E2F1, RbAp48/p19ARF, and RbAp48/p53 expression levels were observed in MSG cells of non-OVX and OVX ER^{-/-}, E2F1^{-/-}, and p53^{-/-} mice. Photos are representative of two independent experiments.

lacking many N- and C-terminal phosphorylation sites (36). These findings suggest that p53 phosphorylation is a key step in E2F-1-mediated apoptosis. The transcription factor E2F-1 functions as a key regulator for both cell cycle progression and apoptosis. E2F-2-deficient T lymphocytes exhibit enhanced T-cell receptor-stimulated proliferation and a lower activation threshold, leading to the accumulation of a population of autoreactive T lymphocytes, which appear to be responsible for causing autoimmunity in E2F-2-deficient mice (24). E2F-1^{-/-} mice exhibit a defect in T lymphocyte development leading to an excess of mature T cells due to a maturation stage-specific defect in thymocyte apoptosis (8).

Our recent study suggests that antiestrogenic actions have a potent effect on the proteolysis of α -fodrin autoantigen in the salivary glands through upregulation of caspase 1 and caspase 3 activity (14). We found here a proteolysis of α -fodrin and a significant increase in caspase 3 activity in addition to the elevated caspase 1 and caspase 8 activity on RbAp48-induced apoptotic HSG cells. The fodrin α -subunit of various cells has been shown to be cleaved in association with apoptosis, in particular, due to upregulation of caspase 3 (4, 15, 48). Several reports have demonstrated that estrogen may play an inhibitory role in apoptosis in endothelial cells, breast cancer cells, cardiac myocytes, prostate cells, and neuronal cells (30, 32, 41, 43). Moreover, it has been noted that some enzymatic activities are elevated in postmenopausal women compared with normal healthy women (1, 27). Increased caspase levels seem to potentiate cell death in the presence of p53-generated signals that trigger caspase activation. Activated caspases digest many cellular proteins responsible for cell cycle regulation (e.g., Rb and Mdm2) (16), DNA damage recognition and repair [e.g., DNA-dependent protein kinase, p53, and poly(ADP-ribose) polymerase], and regulation of the cellular structure (e.g., actin, lamin, and fodrin) (44, 45). All these functional and structural protein modifications lead directly to apoptosis. Moreover, RbAp48 is found not only in histone deacetylase complexes but also in ATP-dependent remodeling complexes (9). Here we show that RbAp48 specifically activates E2F-1-mediated p53 phosphorylation in the salivary gland cells but not in many of the other types of cells examined. Thus, although the association of RbAp48 with nuclear transcriptional coactivators has not been described, there is abundant evidence that these histone binding factors interact with related classes of proteins (53, 46).

Taken together, our results demonstrate a direct molecular mechanism by which estrogen deficiency might promote p53-mediated apoptosis exclusively in exocrine gland cells through RbAp48 overexpression.

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