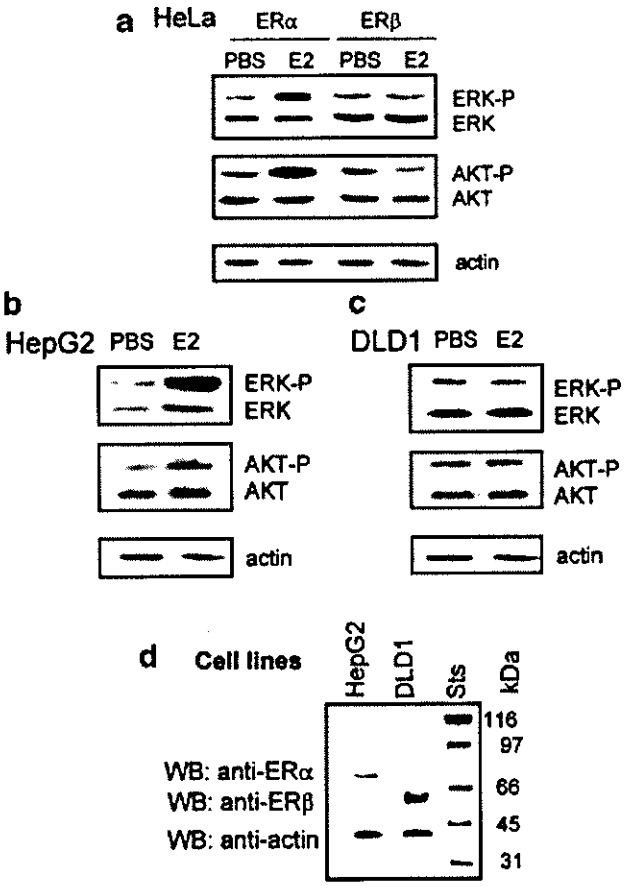

Fig. 3. Signal transduction pathways activated by E2. Western blot analysis of phosphorylated and un-phosphorylated ERK and AKT were performed, as described in "Materials and Methods," on unstimulated (PBS) and 15 min E2-treated (10 nM) HeLa cells transfected with human ERα or human ERβ expression vectors (**part a**) or HepG2 cells (**part b**) or DLD1 cells (**part c**). Western blot analysis of ERα and ERβ levels were performed in HepG2 cells and DLD1 cells (**part d**). The amount of protein levels were normalized by comparison with actin expression. Typical blot of three independent experiments. For details see the text.

prevented the rapid (15 min) E2-induced p38/MAPK phosphorylation also in ER-transfected HeLa cells (Fig. 4c). Furthermore, the E2 inactive stereoisomer, 17α-estradiol, failed to induce p38/MAPK phosphorylation (Fig. 4c), whereas the E2 cell membrane impermeable, E2-BSA (Zheng et al., 1996; Marino et al., 2003), affected the p38/MAPK activation comparably to E2 (Fig. 4c). Altogether these data imply a membrane ER in the rapid and specific E2-induced activation of p38/MAPK signaling.

Cross-talk between proliferative and apoptotic signal transduction pathways and role of E2-induced p38/MAPK

The ability of E2 to induce p38/MAPK phosphorylation even in the presence of ERα was surprising and did not clarify the putative involvement of this kinase in the E2-induced apoptosis. Thus, we asked whether ERK/MAPK and PI3K/AKT cross-talk with p38/MAPK. None of the specific pathway inhibitors used (i.e., Ly 294002 and U 0126) prevented the E2-induced p38/MAPK phosphorylation in ERα-transfected HeLa cells (Fig. 5a) suggesting that the activation of this pathway was parallel and independent on ERK and AKT activation. On the contrary, the cell pre-treatment with the same inhibitors rescued the activation of the pro-apoptotic caspase-3 (Fig. 5b) as well as completely prevented E2-induced, anti-apoptotic, Bcl-2 accumulation (Fig. 5c). The same results were obtained in HepG2 cells (Fig. 5 d, e, and f) further indicating that E2–ERα-induced ERK and AKT activation negatively modulates the apoptotic signals. To direct evaluate the role of p38 in these effects in some experiments cells were pre-treated with the specific p38/MAPK inhibitor, SB 203580 (5 μM), before E2 stimulation. A block of p38/MAPK phosphorylation was evidenced while no effect was present on Bcl-2 levels in both cell lines considered. Note that, caspase-3 cleavage induced by E2 in the presence of U0126 was prevented by the pre-treatment of HepG2 cells with p38/MAPK inhibitor, SB 203580 (30 min) (Fig. 5g). However, the cell pretreatment with the signaling pathways inhibitors alone did not modify the p38/MAPK phosphorylation or caspase-3 and PARP cleavage.

Finally, the pre-treatment of ERβ-transfected HeLa cells with the specific p38/MAPK inhibitor, SB 203580, completely prevented the formation of the caspase-3 active fragment (Fig. 6a) and the cleavage of PARP (Fig. 6b) linking the p38/MAPK activation directly to the apoptosis. As expected, E2 induced p38/MAPK-dependent caspase-3 activation in ERβ-containing DLD1 cells (Fig. 6c) sustaining a pivotal role of the signaling activated by E2–ERβ complex (i.e., prolonged p38/MAPK phosphorylation) in inducing the apoptotic cascade.

DISCUSSION

E2 is known to support cell survival or induce cell death/apoptosis depending on the cell context (Song et al., 2001; Song and Santen, 2003). The mechanism(s) underling these opposite E2 effects could involve the classical/transcriptional mechanism of ER isoforms which, as ligand-dependent transcription factors, modulate the transcription of E2-induced target genes. In addition to this accepted model for the E2 action mechanism, emerging evidences indicated that rapid/non-genomic signaling molecules originating from the cell membrane are involved at least in E2–ERα-induced cell proliferation/survival (Castoria et al., 1999; Razandi et al., 1999, 2000a; Kousteni et al., 2001; Marino et al., 2001, 2002, 2003). These evidences prompted us to examine the non-genomic signaling mechanism(s) generated by the E2–ERβ complex and to compare the role(s) played by these rapid signals with those generated after E2–ERα binding. Although the different functions of ERα versus ERβ on cell proliferation/apoptosis balance has been suggested (Matthews and Gustafsson, 2003; Weihua et al., 2003), the contribution of signal transduction pathways generated by each isoform on these E2-induced cellular functions has not been yet clarified. Therefore, we chose the ER-devoid HeLa cells as experimental model. The transiently transfected HeLa cells allow us to discriminate the effect of each ER isoforms, without the mutual interference, in a E2-induced proliferation model (Marino et al., 2001, 2002). Furthermore, to avoid any dilemma due to the receptors over-expression, some experiments were performed in parallel in two different cancer cell lines which express endogenous ERα (HepG2) (Marino et al., 2001, 2002, 2003; Moon et al., 2004) or ERβ (DLD1) (Fiorelli et al., 1999; Di Leo et al., 2001).

In these experimental conditions, E2 induced different effects on cell growth or apoptosis in dependence on ER isoform present. While the E2–ERα complex activated multiple signal transduction pathways committed to

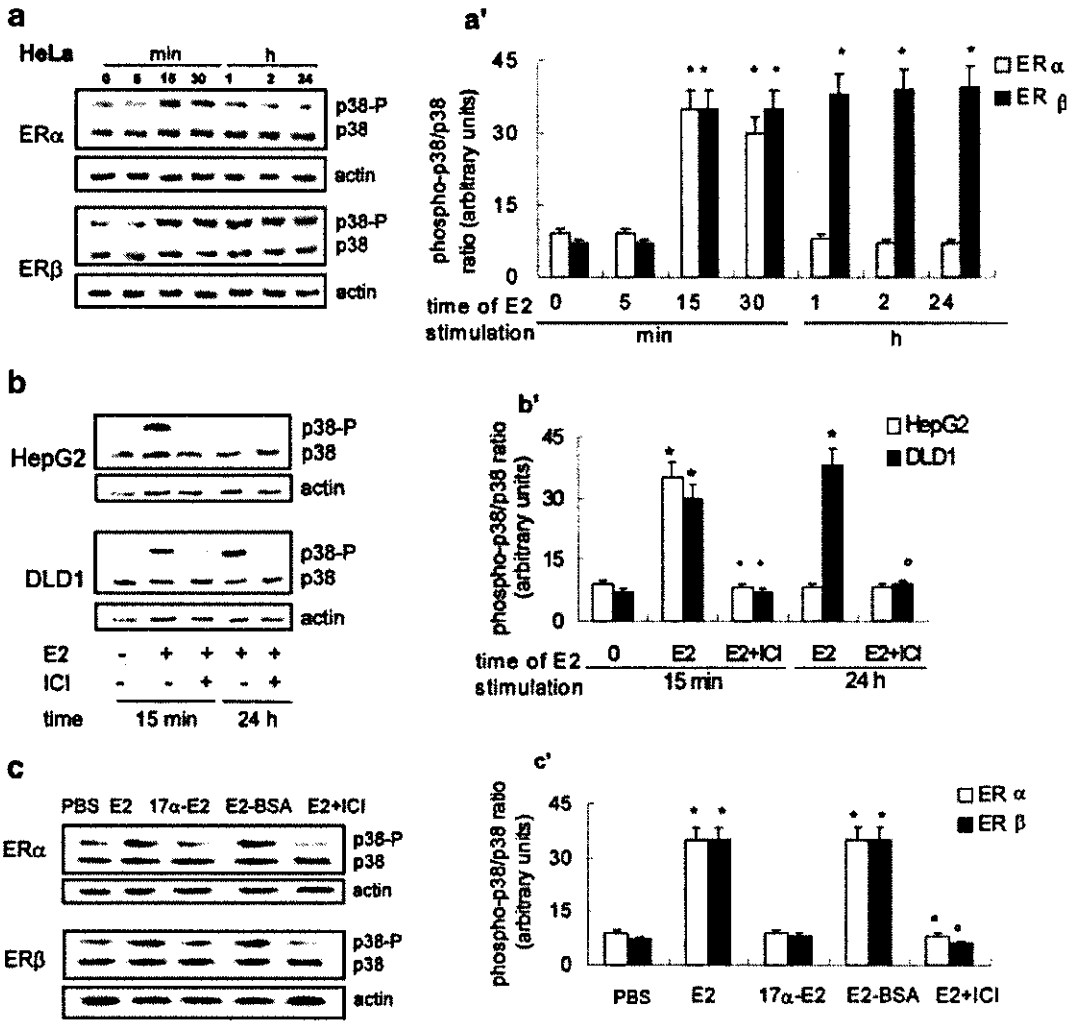

Fig. 4. Effect of E2 on p38/MAPK activation. Time course analysis of p38/MAPK phosphorylation was performed, as described in "Materials and Methods," on untreated (0, –) and E2-treated (10 nM) HeLa cells transfected with human ERα or human ERβ expression vectors (**parts a** and **a'**) or HepG2 or DLD1 cells (**parts b** and **b'**) at the indicated times. The amount of protein levels were normalized by comparison with actin expression. Parts (a) and (b) show the typical blot of three independent experiments; parts (a') and (b') show the data obtained by densitometric analysis, mean values ± SD. $P < 0.001$, calculated with Student's *t*-test, compared with respective un-stimulated (0, –) values (*) or with E2-stimulated values (°). For details see the text. Western blot analysis of p38/MAPK phosphorylation was performed, as described in "Materials and Methods," in un-stimulated (PBS) or 15 min E2- (10 nM) or 17α-estradiol- (17α-E2; 10 nM) or 17β-estradiol-BSA-(E2-BSA; 10 nM) treated HeLa cells transfected with human ERα or human ERβ expression vectors. In some experiments cells were pretreated with ICI 182,780 (ICI) (1 μM) before E2 stimulation. The amount of protein levels were normalized by comparison with actin expression. **Part (c)** shows the typical blot of three independent experiments; **part (c')** shows the data obtained by densitometric analysis, mean values ± SD. $P < 0.001$, calculated with Student's *t*-test, compared with respective un-stimulated control (PBS) values (*) or with E2-stimulated values (°). For details see the text.

both cell cycle progression and apoptotic cascade prevention, the E2–ERβ complex induced the rapid and persistent phosphorylation of p38/MAPK, which in turn, drove cells into the apoptotic cycle.

In the E2-stimulated ERα-containing HepG2 cells, we previously demonstrated that E2 enacted the rapid, non-genomic, and membrane starting signal transduction pathways which, in turn, worked cooperatively to achieve cell proliferation. In particular, E2-induced PKC-α was strongly related to DNA synthesis, but was not involved in cyclin D_1 transcription. On the contrary, E2-induced ERK/MAPK and PI3K/AKT pathways were strongly involved in both DNA synthesis and cyclin D_1 transcription (Marino et al., 2002, 2003). Present results clearly indicate, in well accordance with the literature (Razandi et al., 2000b; Kousteni et al., 2001), that these latter pathways have also a critical role in E2 action as a survival agent. While this work was in progress, Fernando and Wimalasena (2004) demonstrated that the prolonged activity of AKT was required to maintain the BAD phosphorylation decreasing its pro-apoptotic effect. In addition, we demonstrate that the E2-induced rapid activation of PI3K/AKT pathway is necessary to increase the level of the anti-apoptotic protein Bcl-2 and to avoid the cleavage of caspase-3 and the induction of apoptotic cascade. Beside AKT-mediated signaling, E2 can also signal through ERK/MAPK pathway. This pathway precedes and modulates AKT phosphorylation (Marino et al., 2003). In fact, the pre-treatment of HepG2 cells with U 0126 (ERK/MAPK inhibitor) rapidly increased the levels of the tumor-suppressor, PTEN, impairing the E2-induced AKT phosphorylation (Marino et al., 2003).

Work of the last years has established that expression and function of component of death machinery are under control of signaling pathways (see Rapp et al., 2004 and literature therein). ERK/MAPK as well as PI3K/AKT cascades cooperate in cellular protection. ERK/MAPK

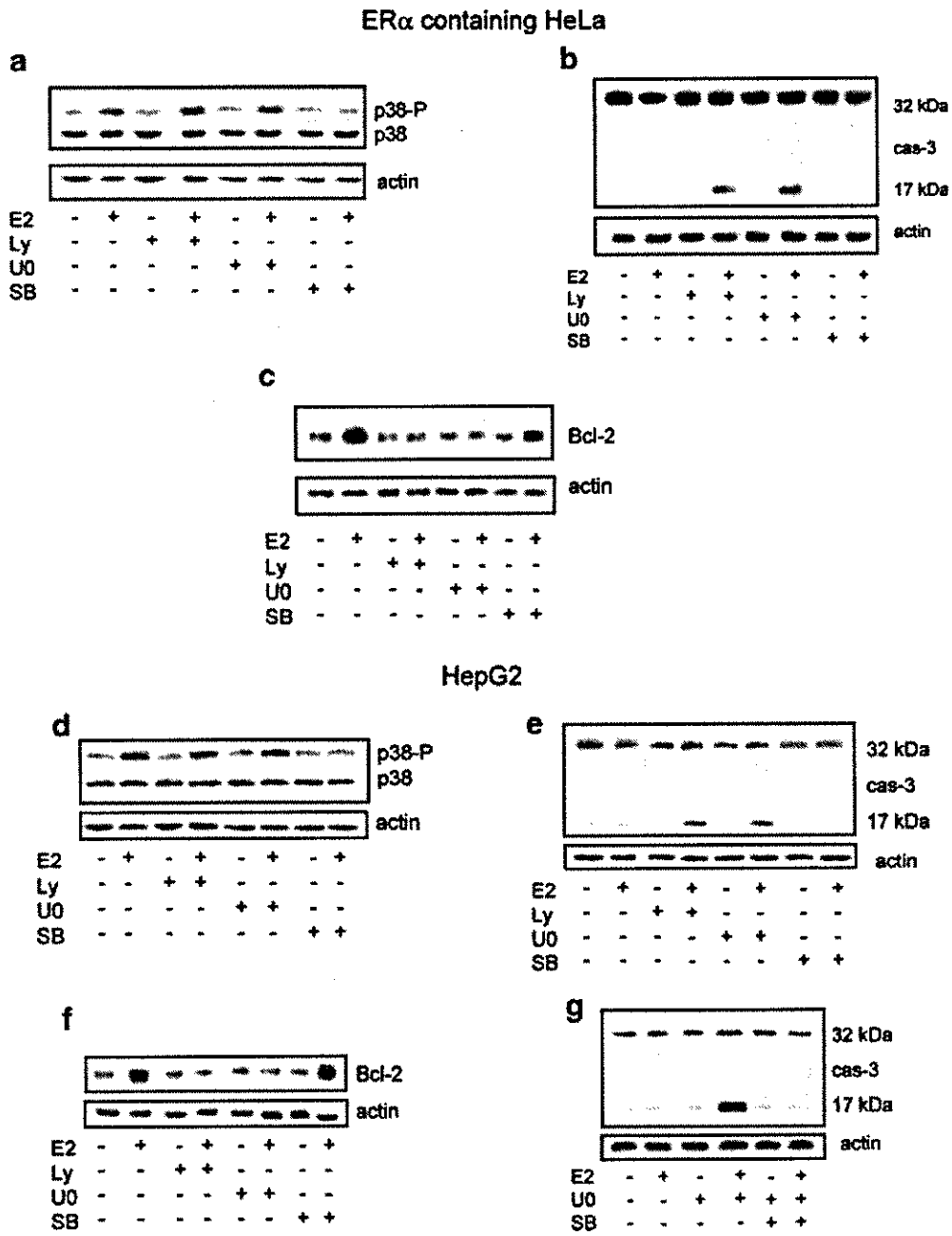

Fig. 5. Cross-talk among E2-induced ERK/MAPK, PI3K/AKT and p38/MAPK activation. Western blot analysis of p38/MAPK (**parts a** and **d**), caspase-3 (**parts b**, **e**, and **g**), and Bcl-2 (**parts c** and **f**) were performed, as described in "Materials and Methods," on un-stimulated (−) or E2-treated (10 nM) (15 min for p38 phosphorylation, 24 h for caspase-3 and Bcl-2 detection) HeLa cells transfected with human ERα expression vector or HepG2 cells. When indicated 10 μM U 0126, Ly 294002 (15 min) or 5 μM SB 203580 (30 min) (ERK/MAPK, PI3K/AKT, and p38/MAPK pathway inhibitors, respectively) were added before E2 administration. The amount of protein levels were normalized by comparison with actin expression. Typical blot of three independent experiments. For details see the text.

control cell survival by targeting Bcl-2 to the mitochondria membranes (Tamura et al., 2004) and together with PI3K/AKT may up-regulate the expression of Bcl-2 (Rapp et al., 2004). Furthermore a direct role of PI3K/AKT in caspase-3 inhibition has been recently reported after polyamine depletion (Zhang et al., 2004). Bcl-2 overexpression, in turn, decreases intracellular Ca^{++} level which can activate p38/MAPK and caspase cascades (Song et al., 2004). Our results, for the first time, show that steroid hormones may regulate this pathway. In fact, the ERK/MAPK and the PI3K/AKT pathways, rapidly activated by the E2–ERα complex, cooperatively enhance the expression of the anti-apoptotic protein (Bcl-2), block the parallel activation of the p38/MAPK, reduce the pro-apoptotic caspase-3 activation, and promote the G1/S transition via the enhancement of cyclin D_1 expression (Marino et al., 2002, 2003; present data).

One of the main findings in this study is the different signal generated by the E2–ERβ complex. There is 96% amino acid identity between the DNA-binding region (C domain) of ERα and ERβ, but in the ligand-binding region (E domain) the homology is only 53% (Kuiper et al., 1998; Matthews and Gustafsson, 2003). As the E domain of ERα is sufficient to elicit non-genomic actions (Marino et al., 2002; Razandi et al., 2002; Acconcia et al.,

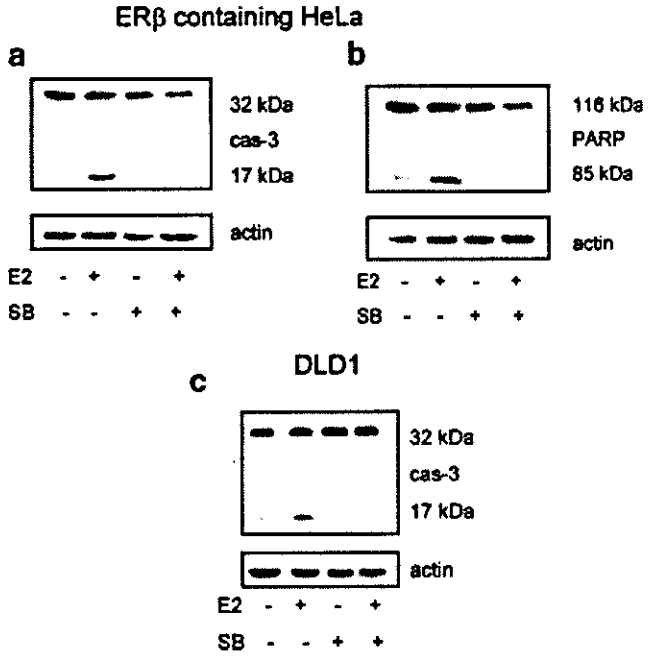

Fig. 6. Involvement of p38/MAPK in pro-apoptotic cascade activation. Western blot analysis of caspase-3 (**parts a** and **c**) and PARP (**part b**) activation were performed, as described in "Materials and Methods," on un-stimulated or 24 h 17β-estradiol-treated (E2; 10 nM) HeLa cells transfected with human ERβ expression vector or in DLD1 cells. When indicated 5 μM SB 203580 (p38/MAPK cascade inhibitor) was added 30 min before E2 administration. The amount of protein levels were normalized by comparison with actin expression. Typical blot of three independent experiments. For details see the text.

2004), it is most likely that different sets of signal transduction proteins may be activated by ERα and ERβ upon E2 binding. Besides these differences the two receptors display a different tissue localization and a different role in proliferation. For example, E2–ERα is a proliferative factor in the uterus, and the uterus of ERβ null mice is hypersensitive to the proliferative action of E2; the co-expression of both ER isoforms is rare during the proliferative phase of mammary gland cells typical of pregnancy, whereas more than 90% of ERβ-expressing mammary gland cells do not proliferate (Weihua et al., 2003); ERβ is abundantly expressed in normal colonic mucosa, but declines in colon adenocarcinoma paralleling the tumor's dedifferentiation (Konstantinopoulos et al., 2003); a progressive decline of ERβ expression has been found in multistage mammary carcinogenesis (Roger et al., 2001) and prostate cancer (Horvath et al., 2001). Very recently it has been reported that the induction of ERβ expression reduces the growth of exponentially proliferating breast cancer cells with a parallel decrease in components of the cell cycle associated with proliferation, namely cyclin D_1, cyclin E, Cdc25A, $p45^{Kip2}$ and an increase in the Cdk inhibitor $p27^{Kip1}$ (Matthews and Gustafsson, 2003; Paruthiyil et al., 2004; Strom et al., 2004). Our data amplify these evidences by adding the ability of ERβ isoform to rapidly induce the persistent membrane starting activation of p38/MAPK without any interference on the survival proliferative pathways, thus impairing the cell cycle components activation.

However, we were surprised to find that the E2–ERα complex increased p38/MAPK phosphorylation. Recently, Lee and Bai (2002) reported that in ERα-expressing endometrial cells, E2 activates the p38/MAPK pathway, which in turn mediates the ERα phosphorylation on threonine-311, promoting the receptor nuclear localization and interaction with specific receptor coactivators. In line with this result the E2-induced p38/MAPK phosphorylation plays a multifunctional role in cellular E2-induced effects. As discussed above, the contemporary increase of Bcl-2 levels, mediated by ERK/MAPK and PI3K/AKT pathways, may decrease the Ca^{++} levels impairing the prolonged p38/MAPK activation (Song et al., 2004).

Ample evidence indicates that the p38/MAPK pathway serves an important role in stress and immune response (Han et al., 1994). Furthermore, p38/MAPK pathway has been associated with a significant slowing in cell proliferation (Han et al., 1994; Badger et al., 1996) and with the regulation of the apoptosis (Kang et al., 2003). In particular p38/MAPK can sensitize cells to apoptosis through the positive regulation of Fas/CD-95 and Bax expression which, in turn, activate caspase cascades (Porras et al., 2004). The E2 capacity in activating p38/MAPK has been reported in a few articles and linked to the preservation of form of ERα- and ERβ-containing vascular endothelial cells (Razandi et al., 2000a), or their migration and proliferation (Geraldes et al., 2003), or even their apoptosis (Mori-Abe et al., 2003). Zhang and Shapiro (2000) reported the ability of E2 to induce p38/MAPK phosphorylation and cell apoptosis in a clone of ERα stably transfected HeLa cells (HeLa-ER5), unresponsive to the E2 proliferative stimuli. The reason for these disparities is not clear but could be related to the divergences in the experimental models, colture condition, E2 treatment period, proliferative capacity, or cell line variability. The E2 stimulation of two cell lines containing endogenous (DLD1) or transfected (HeLa) ERβ demonstrates the ability of human ERβ to drive cancer cells to apoptosis via p38/MAPK-cascade.

In conclusion, besides its role as negative modulator of ERα activities, our findings indicate that ERβ directs the anti-proliferative effects of E2 sustaining the tumor suppressor functions of ERβ. Therefore, the expression of ERs could account for the E2-dependent modulation of cell proliferation. In particular, E2 promotes cell survival through ERα-non-genomic signaling and cell death through ERβ-non-genomic signaling. Thus, the E2 opposite effects in cells co-expressing ERα and ERβ could depend on the balance between the signals originated by each isoform. However, the appearance of new and different signals in the presence of either receptors can not be excluded and it is currently under active investigation in our laboratory.

ACKNOWLEDGMENTS

The generous gift of DLD1 cells from Dr. Aldo Cavallini (Biochemistry Laboratory, I.R.C.C.S.S. de Bellis, Scientific Institute for Digestive Diseases, Via della Resistenza, I-70013 Castellana Grotte, Bari, Italy) is acknowledged. The editorial assistance of Mr. Peter De Muro is also acknowledged. This work was supported by grants from FIRB 2001 and 2004 University "Roma Tre" to Maria Marino.

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Cancer Res (in press)

Estrogen Receptor-Binding Fragment-Associated Antigen 9 Is a Tumor-Promoting and Prognostic Factor for Renal Cell Carcinoma

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Financial Support: This work was supported by the Ministry of Health, Labor and Welfare Japan, the Ministry of Education, Culture, Sports, Science and Technology Japan.

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Conflict of interests: none

Running Title: EBAG9 as a Tumor-Promoting Factor for Renal Cell Carcinoma

Key words: EBAG9, renal cell carcinoma, tumor growth, siRNA

Nonstandard abbreviations: estrogen receptor-binding fragment-associated antigen 9 (EBAG9); renal cell carcinoma (RCC)

ABSTRACT

The estrogen receptor-binding fragment-associated antigen 9 (EBAG9) has been identified as a primary estrogen-responsive gene in human breast cancer MCF7 cells. High expression of EBAG9 has been observed in invasive breast cancer and advanced prostate cancer, suggesting a tumor-promoting role of the protein in malignancies. Here we show that intratumoral administration of small interfering RNA (siRNA) against EBAG9 exerted overt regression of tumors following subcutaneous implantation of murine renal cell carcinoma (RCC) Renca cells. Overexpression of EBAG9 did not promote the proliferation of culture Renca cells, however, the inoculated Renca cells harboring EBAG9 (Renca-EBAG9) in BALB/c mice grew faster and developed larger tumors compared with Renca cells expressing vector alone (Renca-vector). After renal subcapsular implantation, Renca-EBAG9 tumors significantly enlarged compared with Renca-vector tumors in BALB/c mice, whereas both Renca-EBAG9 and Renca-vector tumors were developed with similar volumes in BALB/c nude mice. No apparent difference was observed in specific cytotoxic T cell responses against Renca-EBAG9 and Renca-vector cells, nonetheless, the number of infiltrating $CD8^+$ T lymphocytes was decreased in Renca-EBAG9 subcapsular tumors. Furthermore, immunohistochemical study of EBAG9 in 78 human RCC specimens showed that intense and diffuse cytoplasmic immunostaining was observed in 87% of the cases and positive EBAG9 immunoreactivity was closely correlated with poor prognosis of the patients. Multivariate analysis revealed that high EBAG9 expression was an independent prognostic predictor for disease-specific survival ($P = 0.0485$). Our results suggest that EBAG9 is a crucial regulator of tumor progression and a potential prognostic marker for RCC.

INTRODUCTION

Estrogen receptor-binding fragment associated gene 9, EBAG9, is an estrogen-responsive gene that we previously identified in MCF-7 human breast carcinoma cell line using a CpG-genomic binding site cloning method (1). EBAG9 protein, whose molecular size is 32 kDa by Western blot analysis, is expressed in estrogen target organs as well as several other tissues such as brain, liver, and kidney (2). The protein expression of EBAG9 is estrogen-inducible, as it has been shown in ovariectomized mice treated with 17β-estradiol administration (2). The physiological function of EBAG9 has not been well defined, yet the molecule may be implicated in cancer pathophysiology, with several lines of evidence of the protein expression in malignancies including breast (3), ovarian (4), prostate (5), and hepatocellular carcinomas (6). In prostate cancer (5), EBAG9 expression significantly correlated with advanced pathologic stages and high Gleason score ($P = 0.0305$ and $P < 0.0001$, respectively), suggesting the abundance of EBAG9 may relate to the progression of malignant tumors.

In the present study, we investigated whether EBAG9 expression is critical in tumor development of renal cell carcinoma (RCC). RCC that comprises the majority of kidney cancer is one of the ten most common malignancies in industrialized countries (7). The prognosis of patients with advanced RCC is poor, as 5-year survival rate is < 5% (8), and the treatment of metastatic RCC remains a difficult clinical challenge. Development of new and alternative modalities of diagnosis and therapy for RCC is a clinical requisite. We used murine syngeneic renal adenocarcinoma model of Renca cells in this study and investigated whether gene silencing or overexpression of EBAG9 influences Renca cell growth and/or *in vivo* tumorigenesis. Administration of small interfering RNA (siRNA) against EBAG9 regressed subcutaneous Renca tumors. The proliferation of culture Renca cells constitutively expressing EBAG9 was not basically different from control Renca cells, whereas EBAG9-expressing cells grew faster in BALB/c mice and developed larger tumors. The tumor-promoting effect of EBAG9 in Renca tumors may relate to the suppression of antitumor immunity, as intratumoral $CD8^+$ T lymphocytes were reduced in renal subcapsular Renca tumors. The tumorigenic relevance of EBAG9 in Renca models further extended to clinicopathological significance of the molecule in human RCC. EBAG9 immunoreactivity was closely correlated with poor prognosis of the patients and it was an independent prognostic predictor for disease-specific survival. Our findings demonstrate that EBAG9 is a tumor-promoting factor and a potential prognostic marker in RCC.

MATERIALS AND METHODS

Reagents. Rabbit anti-EBAG9 polyclonal antibody was generated against a fusion protein of glutathione-S-transferase and EBAG9 (2). Rabbit polyclonal anti-human CD3 antibody (DAKO Cytomation, Carpinteria, CA), rat anti-mouse CD4 (L3T4)(clone RM 4-5) and rat anti-mouse CD8a (Ly-2)(53-6.7) monoclonal antibodies (BD Pharmingen, San Diego, CA), and anti-β-actin monoclonal antibody (Sigma, St. Louis, MO) were commercially purchased. Human EBAG9 cDNA was cloned into a mammalian expression vector pcDNA3 (Invitrogen,

Carlsbad, CA).

Tumor Cells. Renca is a spontaneously arising murine renal cell carcinoma and was prepared as previously described (9, 10). Tumor cells were maintained in RPMI1640 containing 10% fetal calf serum and antibiotics.

Mice. BALB/c mice and BALB/c nu/nu mice (Nisseizai, Tokyo, Japan), which were syngeneic to Renca cells, were kept under specific-pathogen-free conditions and fed dry food and water. All mice used for experiments were male at the age of 5 weeks.

Patients and Tissue Preparation. We investigated 78 tissue samples of renal cell carcinoma obtained from patients (14 females and 64 males) who underwent radical or partial nephrectomy at Tokyo University Hospital between the years 1990 and 1995. Patient information was retrieved from the review of patient charts. Staging and grading of the tumors were performed according to the 1997 International Union Against Cancer TNM classification and WHO histopathological typing, respectively (11). The mean age of this population was 54 years old (26-76 years old) and the mean follow-up period was 60 months (2-78 months). For 32 patients with advanced tumors (pT2 or greater), adjuvant therapy was performed including immune therapy (n = 30), radiation (n = 5) and surgery for metastatic diseases in lung, colon, and pancreas (n = 8). During the follow-up period, 55 patients (70.5%) survived without evidence of disease, 8 cases (10.3%) presented with tumor recurrence, and 15 cases (19.2%) died of disease. None died of other diseases.

Western Blot Analysis. Cells were lysed in RIPA buffer [50 mM Tris-HCl, pH8.0, 200 mM NaCl, 20 mM NaF_2, 2 mM EGTA, 1 mM dithiothreitol, 2 mM sodium vanadate, 0.5% v/v Nonidet P-40 supplemented with a protease inhibitor cocktail Complete (Boehringer Manheim GmbH, Mennheim, Germany)] and proteins were resolved by 12.5% SDS-PAGE, transferred to polyvinylidene difluoride membranes. Membranes were probed with rabbit anti-EBAG9 antibody or anti-β-actin monoclonal antibody.

Tumor Regression by EBAG9 siRNA. Small interfering RNA (siRNA) duplex that targets EBAG9 was generated by Dharmacon (Lafayette, CO). The target sequence of EBAG9 siRNA was 5'- AAG AAG AUG CAG CCU GGC AAG -3'. Scramble II Duplex (Dharmacon) was used as a non-targeting control siRNA that does not possess homology with known gene targets in mammalian cells. The GC content of Scramble II Duplex was 57.9%, which was identical to that of EBAG9 siRNA.

To investigate *in vivo* silencing effect of EBAG9 siRNA in Renca tumors, intratumoral injection of siRNA duplexes was performed twice every week. Briefly, Renca cells (1×10^4 cells) were implanted in the flank of BALB/c mice. Tumor size measured weekly with a micrometer in two dimensions, and tumor volume was estimated according to the formula: $(\text{smallest diameter})^2 \times (\text{longest diameter})$. When the volumes of tumors reacheds 300 mm^3, siRNA duplexes (10 μg) were injected directly into tumors twice every week, along with 4 μl of GeneSilencer (Gene Therapy System, San Diego, CA) dissolved in 0.1 ml of Opti-MEM (Gibco BRL, Gaithersburg, MD). Mice were sacrified 4 weeks after treatment.

Generation of Renca Cells Stably Expressing EBAG9. Renca cells were transfected with an expression vector pcDNA3 including human EBAG9 cDNA or vector alone using

Lipofectamine (Gibco BRL, Gaithersburg, MD). G418-resistent cells were selected and several independent clones were isolated.

Reverse Transcription PCR. Total cellular RNA of Renca cells was extracted using ISOGEN reagent (NIPPON GENE, Tokyo, Japan) and first-stand cDNA was generated from 5 μg of total cellular RNA using a reverse transcriptase Omniscripit RT™ (QIAGEN, Tokyo, Japan) and random hexamers. To validate the expression of exogenous human EBAG9, reverse transcription (RT) PCR was performed using specific primers for human EBAG9 (sense: 5'-GCTACACAAGATCTGCCTTT-3', antisense: 5'-CTTCTTCATTAGCCGTTGTG-3'). The amplification was performed for 35 cycles at 62°C for annealing, using AmpliGold Taq polymerase (Perkin-Elmer).

***In Vivo* Tumor Challenge.** For subcutaneous implantation, transfected Renca cells (1 x 10^4 cells/ mice) suspended in 0.1 ml of complete medium were injected in the flank of BALB/c mice. Tumor volume was calculated weekly. In survival analyses, Renca-bearing mice were followed up for 14 weeks after implantation.

For renal subcapcular implantation, tumors cells (1 x 10^4 cells/ mice) suspended in 0.1 ml of complete media were inoculated into the subcapsule of the left kidney of BALB/c wild-type and nude mice. Mice were sacrificed 25 days after implantation and tumors were exicised.

Cell Proliferation Assay. Cells were seeded at a density of 1-3 x 10^5 cells/dish into 10-cm dishes and hemocytometer counting was performed every two days. Doubling time during exponential growth was determined by a formula: [incubation time (h) x $\log_{10}2$]/ [$\log_{10}$(cell number at sampling period) - $\log_{10}$(plating cell number)](12).

Proliferation assays were performed using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt (WST-8) reagent (Nacalai, Kyoto, Japan)(13). The assay is based on the conversion of the MTT-like tetrazolium salt WST-8 to a water-soluble formazan by metabolically active cells, provides a quantitative determination of viable cells. Cells were seeded in 96-well plates at an initial density of 625-5,000 cells/well. At one hour after inoculation, cells were transfected with either EBAG9 siRNA or Scramble II Duplex (100 ng/well) using GeneSilencer reagent (Gene Therapy Systems). Assays were performed on days 0, 2, and 4. For cells cultured up to day 4, medium was once exchanged on day 2. Spectrophotometric absorbance at 450 nm (for formazan dye) was measured with absorbance at 620 nm for reference.

Cytotoxicity Assay. Renca-EBAG9 or Renca-vector cells were used as target cells. Splenocytes of Renca-bearing BALB/c mice were stimulated for 5 days *in vitro* with irradiated Renca cells at a splenocyte: tumor cell ratio of 20:1 in the presence of 1,000 IU/ml interleukin-2, and used as effector CTLs. Target cells were incubated with effector CTLs at various E/T ratios in a final volume of 200 μl for 18 h at 37°C. Lactate dehydrogenase release from cells with a damaged membrane was examined using CytoTox-ONE Reagent (Promega, Madison, WI) and fluorescence was measured with an excitation wave length of 560 nm and an emission wave length of 590 nm. Experiments were performed in triplicate.

Immunohistochemistry. Immunohistochemical studies were performed using the streptavidin-biotin amplification method with horseradish peroxidase detection. Paraffin

sections of tumors were blocked in 0.3% H_2O_2 (30 min) and in 10% FCS (30 min), incubated overnight with specific antibodies against CD3, CD4, or CD8a for Renca tumors (1:20 dilution), or with purified rabbit anti-EBAG9 antibody for human RCC (1:40 dilution). Sections were incubated with biotinylated rabbit ani-rat IgG or anti-rabbit EnVison$^+$ reagent (Dako), developed by diaminobenzidine (Sigma) and counterstained with hematoxylin (Sigma). Negative controls were performed for each slide, using nonimmune IgG.

In Renca experiments, numbers of tumor infiltrating-lymphocytes (TILs) positive for CD3, CD4, or CD8 expression were microscopically examined in the high power field (HPF) of view at a magnification of 400X (14). BALB/c mouse spleen specimen was used as a positive control.

In RCC examination, immunoreactivity (IR) scores of EBAG9 expression were determined by two pathologists according to percentages of positive cells. Human breast cancer section (DAKO) was used as a positive control. Positivity was 0-4% for IR score 0 (negative), 5-24% for 1+, 25-49% for 2+, 50-100% for 3+. Sections that had ≥ 25% positive cells but apparent lower intensity compared with positive controls were scored as IR score 1+. IR scores 1+, 2+, and 3+ were defined as positive staining. If IR scores were different between two pathologists, the average IR score was adopted. If several types of histology were included in one section, IR score of predominant histology was utilized.

Statistical Analyses. Comparisons between different groups of Renca samples were analyzed with non-parametrical Mann-Whitney U test. The associations between EBAG9 immunoreactivity and clinicopathological characteristics were evaluated by student-*t* test or Fisher's exact probability test. Disease-specific survival was computed by Kaplan-Meier method and the curves were compared by log-rank test. Multivariate analysis of prognostic factors was performed using Cox-proportional hazard regression model. Computations were done with the StatView 5.0J software (SAS Institute Inc., Cary, NC). All *P* values are two-sided and evaluated as significant if < 0.05.

RESULTS

Gene silencing of EBAG9 suppressed *in vivo* tumor growth of Renca cells. To determine the role of EBAG9 in tumor growth of renal cancer cells, we investigated the effects of synthesized siRNA duplexes targeting EBAG9 on subcutaneous tumor models of Renca cells implanted in syngeneic BALB/c mice. Intratumoral injection of EBAG9 siRNA reduced the protein levels of endogenous EBAG9 compared with the levels of EBAG9 in parental Renca cells or in the Renca tumor treated with control scrambled siRNA duplexes (Fig. 1A). Under the treatment of scrambled siRNA, subcutaneously implanted Renca cells developed prominent tumors, whereas the injection of EBAG9 siRNA suppressed tumor growth of Renca cells (Fig. 1B and 1C). After 4-week treatments, the volume of tumors with EBAG9 siRNA treatment was significantly smaller than that with scrambled siRNA (3,854 ± 665 mm^3 *versus* 6,315 ± 1,053 mm^3, n = 5; $P = 0.0472$). We infer that tumor growth is modulated by EBAG9 expression, implicating EBAG9 as a tumor-promoting factor in renal carcinoma.

Generation of Renca cells stably expressing EBAG9. To explore whether constitutive EBAG9 expression influences tumor growth, we generated Renca cells stably expressing human EBAG9. We selected two Renca-EBAG9 cell clones #3 and #4 that express human EBAG9 mRNA as comfirmed by RT-PCR using human EBAG9 specific primers (Fig. 2A, top panel). The amounts of EBAG9 proteins in Renca-EBAG9 cells were ~2.0 fold increased compared with those in parental Renca cells and Renca-vector cell clones #1 and #2, that were transfected with pcDNA3 empty vector (Fig. 2A, bottom panel). In terms of cell growth rate, doubling time of culture Renca-EBAG9 cells was not significantly different from that of Renca-vector cells (Fig. 2B). Proliferation of Renca cells was further analyzed by a colorimetric MTT-like assay using a tetrazolium monosodium salt WST-8 that is converted to a water-soluble formazan by metabolically active cells (Fig. 2C). Neither EBAG9 overexpression nor RNA interference against EBAG9 did not significantly influence the growth of Renca cells. Moreover, EBAG9 overexpression did not influence the incorporation of BrdU in culture Renca cells (data not shown). The results suggest that stable expression of EBAG9 itself does not accelerate the proliferation of culture tumor cells.

EBAG9 promotes *in vivo* tumor growth of Renca cells. In spite of little difference of propagation abilities between Renca-EBAG9 cells and Renca-vector cells in culture, Renca-EBAG9 cells subcutaneously implanted into BALB/c mice developed more than 4-fold larger tumors compared with Renca-vector cells at 4 weeks after inoculation (Fig. 3A and 3B). Mean tumor volumes at 4 weeks were 1,712 ± 506 mm^3 for Renca-EBAG9 cell clones #3 and #4 *versus* 366 ±110 mm^3 for Renca-vector cell clones #1 and #2 ($P = 0.0055$, Fig. 3B).

In terms of prognosis of mice harboring Renca tumors, 23.5% of mice with Renca-vector cells (n = 17) survived on day 100 after tumor challenge whereas only 5.6% of mice with Renca-EBAG9 cells (n = 18) survived at the same period (Fig, 3C, $P = 0.0412$ by log-rank test). Systemic metastases, including tumor dissemination into peritoneum and distant metastases of lung and liver, were reasons for death in all deceased cases.

EBAG9 suppresses host immune surveillance. To determine whether aberrant EBAG9 expression in Renca cells affects the local immune responses in tumors, we implanted Renca-EBAG9 cells or Renca-vector cells under the renal capsule of BALB/c mice and immunodeficient BALB/c nude mice. Both Renca cell lines formed macroscopic tumors in all of the cancer-bearing hosts by Day 25 (Fig. 4A). In conventional BALB/c mice, Renca-EBAG9 tumors grew significantly larger compared with Renca-vector tumors (Fig. 4A and 4B). Mean volumes of tumors on Day 25 in BALB/c mice were 856 ± 162 mm^3 (n = 19) for Renca-EBAG9 clones #3 and #4 *versus* 149 ± 59 mm^3 (n = 18) for Renca-vector clones #1 and #2 (Fig. 4B, $P < 0.0001$). In immunodeficient BALB/c nude mice, both Renca-vector cells and Renca-EBAG9 cells developed extensive tumors compared with tumors in BALB/c mice and there was no significant difference in tumor volumes between Renca-vector cells and Renca-EBAG9 cells (Fig. 4A and 4B). Mean volumes of tumors on Day 25 in BALB/c nude mice were 2,215 ± 227 mm^3 (n = 18) for Renca-EBAG9 clones #3 and #4 versus 1,802 ± 240 mm^3 (n = 23) for Renca-vector clones #1 and #2 (Fig 4B, $P = 0.118$). These results may suggest that aberrant EBAG9 expression in Renca cells hampers a local primary immune response that retards the growth of

tumors, rather than potentiates the intrinsic tumorigenicity of the tumor cells.

To investigate whether the progression of Renca-EBAG9 tumors depends on a reduced sensitivity of the cells to tumor-specific CTLs, we performed cytotoxicity assays. Effector CTLs were derived from splenocytes of Renca-bearing BALB/c mice, after 5-day restimulation with Renca cells in the presence of interleukin-2. (Fig. 4C). Renca-EBAG9 cells and Renca-vector cells were equally lysed by tumor-specific CTLs, suggesting that EBAG9 expression itself does not affect the sensitivity of Renca cells to CTL lysis.

To assess whether EBAG9 modulates the subtype-specific reactivity of T lymphocytes against tumors, we examined the numbers of TILs in renal subcapsular Renca tumors developed in BALB/c mice (Fig. 4D). No significant differences in numbers of $CD3^+$ and $CD4^+$ T cells were observed between Renca-vector and Renca-EBAG9 tumors, whereas the number of $CD8^+$ T cells in Renca-EBAG9 tumors was significantly decreased compared with that in Renca-vector tumors ($P < 0.05$).

Expression of EBAG9 protein in human RCC tumors. The finding that EBAG9 modulated the growth of Renca tumors led us to the notion whether the molecule contributes to the progression of renal cell carcinoma in human tissues. EBAG9 expression was evaluated immunohistochemically in 78 RCC whole tissue specimens including normal lesions. In non-carcinomatous lesions, a weak and scattered immunostaining of EBAG9 was observed in the cytoplasm of the mesangial cells (Fig. 5A) as well as on the luminal surface of the renal tubular cells (data not shown). The levels of EBAG9 expression in normal renal tissues corresponded to IR score 0. In RCC tumors, 10 of 78 cases (13%) had negative immunoreactivity of EBAG9, whereas 68 of cases (87%) showed EBAG9 positivity. In regard to EBAG9-positive RCC tumors, the cancer cells generally retain intense and diffuse staining patterns in the cytoplasm or on the membrane (Fig. 5B, C, and D). The levels of EBAG9-positivity were IR score 1+ for 18 RCC tumors (23%), 2+ for 31 tumors (40%), and 3+ for 19 tumors (24%). With respect to RCC histology, clear cell tumors displayed an intense membrane staining as well as a diffuse cytoplasmic staining of EBAG9 (Fig 5B, IR score 2+). Sarcomatoid tumors showed an intense and frequent cytoplasmic immunoreactivity (Fig 5C, IR scores 3+). Lung metastatic tumors showed the highest EBAG9 staining, predominantly in the cytoplasm (Fig 5D, IR score 3+).

A significant association between EBAG9 immunoreactivity and clinicopathological parameters was observed in RCC patients (Supplementary Table 1). EBAG9 positivity (IR score >1+) was significantly correlated with advanced pathological tumor stages, positivity of vascular infiltration, and non-clear cell histology ($P = 0.0017$, $P = 0.0109$, and $P = 0.0126$, respectively).

In Kaplan-Meier analysis of the RCC patients, those in which the tumor had high EBAG9 immunoreactivity (IR score 3+) showed a shorter disease-specific survival (Fig. 6) compared with patients showing low or negative EBAG9 immunoreactivity (IR score 0–2+). The 5-year disease-specific survival in cases with EBAG9 IR score 3+ was 55% whereas 91.2% of patients with low or negative EBAG9 immunoreactivity were alive during the same period.

In univariate Cox proportional hazards analysis for 5-year disease-specific survival, established prognostic factors including infiltration, pathological stage, and metastatic status are

the most significant univariate parameters of survival (Supplementary Table 2; $P < 0.0001$ for all). Lower EBAG9 immunoreactivity as well as negativity of lymph node status or vascular infiltraion are also involved in significant univariate survival predictors ($P = 0.0007$, 0.0002, and 0.0003, respectively). In multivariate Cox proportional hazards analysis, negative metastatic status is the most significant predictor of survival (Supplementary Table 3; $P < 0.001$; relative risk, 42.53) Notably, high EBAG9 immunoreactivity is associated with disease-specific death in multivariate analysis ($P = 0.0485$; relative risk, 5.09). These results indicate that high immunoreactivity of EBAG9 is a potential poor prognostic parameter in RCC patients.

DISCUSSION

The present study shows the first evidence regarding a tumor-promoting role of EBAG9 *in vivo*. We showed that Renca tumors overexpressing EBAG9 had a much aggressive phenotype with poorer prognosis as compared to Renca tumors expressing empty vector, although the effects of EBAG9 on culture cell proliferation was relatively minimal. EBAG9 immunoreactivity was detected in most of human RCC samples and high amounts of EBAG9 protein may associate with poor prognosis of the patients. Our findings suggest that EBAG9 is a tumor-promoting factor in RCC yet does not function as an essential oncogene by itself. The present results lead us to the notion that EBAG9 potentiates tumor growth by altering tumor microenvironment.

Decreased local immune responses may one of the critical machanisms that change tumor microenvironment. In antitumor immunity, T lymphocyte-mediated immune surveillance is thought to be a principal host defense mechanism (15). Although tumors such as RCC are immunogenic and could be targeted by tumor-specific CTL or natural killer cells, antitumor immune reactions are not completely effective to reject tumor cells so that tumors continue to grow progressively (16). In our cytotoxicity assay, there was no significant difference of CTL lysis between Renca-EBAG9 and Renca-vector cells, suggesting that overexpression of EBAG9 may not particularly alter the presentation of tumor-associated antigens or the levels of MHC class I molecule expression. In TIL assay, however, $CD8^+$ T cells seemed to be specifically reduced by aberrant EBAG9 expression. We suspect that generation of immunosuppressive factors or apoptosis activation may result in the reduction of $CD8^+$ TIL, leading to hamper antitumor immunity.

The alteration in cell surface glycosylation could be implicated in the modulation of tumor microenvironments (17, 18). It has been recently shown that tumor-associated ganglioside expression in human RCC cells suppresses nuclear factor-κB activation in T cells and mediates T-cell apoptosis (19, 20). RCC display increased levels of gangliosides including GM2, GM1, and GD1a (21) as well as several disialogangliosides (22), which may inhibit the function of antigen presenting cells (23) or modulate tumor vascularization (24). It has been recently shown that tumor-associated *O*-linked glycan antigens Tn and TF were expressed in transfected cells expressing RCAS1 (receptor-binding cancer antigen expressed on SiSo cells)(25), whose cDNA has been found to be a homolog of EBAG9 (26).

Another possible explanation is that EBAG9 may stimulate angiogenesis by up-regulating growth factors or cytokines. There are literatures that vascular endothelial growth factor (VEGF) could be involved in RCC tumor progression. Mutations of the von Hippel-Lindau tumor suppressor gene, which are often observed in hereditary RCC and sporadic clear-cell RCC, result in overproduction of VEGF through a mechanism involving hypoxia-inducible factor α (27, 28). It has been recently shown that VEGF interferes with the development of T cells at pathologically relevant concentrations *in vivo* (29), thus the growth factor may contribute to tumor-associated immune deficiencies.

It has been generally accepted that tumor cells may escape from immune surveillance by expressing the EBAG9 homolog RCAS1, which targets RCAS1 receptor-expressing immune cells and induces apoptosis (26). Nakashima *et al.* identified the RCAS1 cDNA through expression cloning using the 22-1-1 monoclonal antibody that they originally generated (30). Engelsberg *et al.* recently showed, however, that the 22-1-1 epitope was distinct from the products encoded by RCAS1 cDNA, because RCAS1 protein was not recognized by the 22-1-1 antibody whereas the 22-1-1 antibody recognized the tumor-associated *O*-linked glycan antigens (25). They showed that their raised polyclonal antibody recognized a ~35 kDa protein, consistent with the immunoblotting results using our polyclonal antibody. On the contrary, putative RCAS1 protein recognized by the 22-1-1 antibody was identified as a ~80-kDa membrane molecule expressed on human uterine cancer cells (26, 30). Although there are a number of publications concerning RCAS1 in cancers from the point of view as the 22-1-1 antigen, we consider that a pathophysiological role of EBAG9 in tumor immunology needs to be properly evaluated. The present manuscript may provide new insights into an EBAG9-mediated *in vivo* function in cancer progression.

We have previously reported that the immunoreactivity of EBAG9 was mainly observed in the cytoplasm of normal epithelial cells with a granular staining pattern, or particularly in perinuclear regions (3, 5). In carcinoma tissues, an intense staining of the cell surface could be also observed such as in prostate cancer or hepatocellular carcinoma. The expression of RCAS1 immunoreactivity recognized by antibodies against recombinant RCAS1 was localized to perinuclear structures, suggesting that the protein is predominantly distributed in the Golgi system (25). Given that EBAG9 is a Golgi-predominant protein that could be trafficking from the perinuclear regions to the cell surface membrane, it is likely that EBAG9 immunoreactivity could be observed in both cytoplasm and cell surface of carcerous tissues with the abundant expression of EBAG9. Notably, EBAG9 immunoreactivity in RCC with advanced stages such as sarcomatoid or metastatic tumors was cytoplasmic-predominant (Fig. 5C and 5D). Further studies using confocal or electron microscopic examination may elucidate the dynamic distribution of EBAG9.

As we showed that there are several types of cancer that intensely express EBAG9 and the expression levels of EBAG9 may relate to advanced tumor grades (3-6), it is likely that the tumor-promoting effect of EBAG9 is a general event in malignancies regardless of their estrogen dependency. We also observed the lack of association between sex and EBAG9 expression in human RCC in our clinicopathological study (Supplementary Table 1). Thus,

EBAG9 could be a therapeutic target for various tumors constitutively expressing the molecule.

In summary, we demonstrate that EBAG9 is a tumor-promoting factor in both murine Renca RCC and human RCC. We propose that EBAG9 immunoreactivity is a new potential biomarker for prognosis of RCC and a treatment modality targeting EBAG9 will provide a novel therapeutic option for advanced RCC.

ACKNOWLEDGEMENTS

We thank T. Suzuki for her technical assistance.

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FIGURE LEGENDS

Fig. 1. Expression of EBAG9 siRNA suppresses tumor growth derived from murine renal cell carcinoma Renca cells in BALB/c mice. Subcutaneous primary Renca tumors were established by midflank injections of 10,000 tumor cells and intratumoral injections of either control scrambled siRNA or EBAG9 siRNA duplexes together with a transfection reagent GeneSilencer were performed twice a week in five mice per group when the initial tumor volumes reached 300 mm^3. Mice were sacrificed after 4-weeks siRNA administration and tumors were homogenized for protein extraction. *A*, Western blot analysis of lysates from *in vitro* culture Renca cells and tumor samples expressing either control scrambled siRNA or EBAG9 siRNA. *B*, Representative mice after 4-weeks siRNA treatment. Upper panel, mouse treated with control scrambled siRNA; Lower panel, mouse treated with EBAG9 siRNA. *C*, Tumor volume in EBAG9 siRNA-treated mice (n = 5) is reduced compared with control mice (n = 5). $^{*}P < 0.05$ at 4 weeks (EBAG9 siRNA *versus* scrambled siRNA).

Fig. 2. Overexpression of EBAG9 in Renca cells does not accerelate culture cell growth. *A*, RT-PCR analysis and Western blot analysis of Renca cells stably expressing human EBAG9 (Renca-EBAG9) or empty vector (Renca-vector). Upper panel, human EBAG9 mRNA is expressed in clones 3 and 4 of Renca-EBAG9 cells. Empty pcDNA3 vector was used as a negative control and pcDNA3 including EBAG9 cDNA as a positive control. Lower panel, EBAG9 protein is overexpressed in Renca-EBAG9 clones compared with Renca-vector clones or parental Renca cells. *B*, Doubling time of culture Renca cells. The numbers of cells in the exponential growth were counted every two days and doubling time was calculated according to a formula as described in Materials and Methods (n = 5 for each). *C*, Proliferative assay using WST-8 tetrasolium salt. Cells seeded into 96-well plates were transfected with control scrambled siRNA or EBAG9 siRNA duplexes (100 ng/well) and cell proliferation was evaluated on days 0, 2, and 4 (n = 3 for each). Absorbance at 450 nm (for formazan dye) was measured with absorbance at 620 nm for reference.

Fig. 3. Renca cells stably expressing EBAG9 develops large tumors in BALB/c mice. *A*, Representative mice 4 weeks after the inoculation of tumor cells. *B*, Volumes of tumors derived from Renca-EBAG9 cells are significantly larger compared with Renca-vector cells in BALB/c mice. Subcutaneous primary tumors were established by midflank injections of 10,000 tumor cells. $^{**}P < 0.01$ at 4 weeks (Renca-EBAG9 *versus* Renca-vector). Renca-vector #1, n = 16; Renca-vector #2, n = 6; Renca-EBAG9 #3, n = 6; Renca-EBAG9 #4, n = 8. *C*, Poorer prognosis of mice inoculated with Renca-EBAG9 cells compared with mice with Renca-vector cells. Disease-specific survival on day 100: $P = 0.0412$ (Renca-EBAG9 *versus* Renca-vector). Renca-vector #1, n =9; Renca-vector #2, n = 8; Renca-EBAG9 #3, n = 8; Renca-EBAG9 #4, n = 10.

Fig. 4. EBAG9 overexpression promotes renal subcapsular tumor growth by Renca cells in wild-type BALB/c mice. *A*, Representative tumors 25 days after the inoculation of tumor cells (10,000 cells). *B*, Volumes of Renca-EBAG9 tumors are larger than Renca-vector tumors in

BALB/c mice, whereas no significant difference of tumor volumes between Renca-vector and Renca-EBAG9 in BALB/c nude mice. $^{**}P < 0.0001$ on day 25 (Renca-EBAG9 *versus* Renca-vector). Renca-vector #1, n = 12; Renca-vector #2, n = 11; Renca-EBAG9 #3, n = 9; Renca-EBAG9 #4, n = 9. *C*, Lysis of Renca-EBAG9 and Renca-vector cells by tumor-specific cytotoxic T lymphocytes (CTLs). Splenocytes from Renca-bearing mice were cultured with Renca cells at a ratio of 20:1 pulsed with interleuikin-2 (1,000 U/ml) for 5 days. Lactate dehydrogenase release from cells with a damaged membrane was examined using CytoTox-ONE Reagent and fluorescence was measured with an excitation wave length of 560 nm and an emission wave length of 590 nm. *D*, Numbers of tumor infiltrating-lymphocytes positive for CD3, CD4, or CD8 immunostaining were microscopically examined in the high power field (HPF) of view at a magnification of 400X. BALB/c mouse spleen specimen was used as a positive control. $^{*}P < 0.05$ (Renca-EBAG9 *versus* Renca-vector).

Fig. 5. EBAG9 immunostaining in human kidney and renal cell carcinoma specimens. *A*, Normal kidney (IR score 0). EBAG9 is weakly expressed in the mesangial cells (arrow heads). *B*, Clear cell carcinoma (IR score 1+). EBAG9 is immunostained predominantly on the cell membrane in carcinous regions. *C*, Spindle cell carcinoma (IR score 3+). Intense immunostaining of EBAG9 is observed in the cytoplasm of sarcomatoid carcinous regions. *D*, Lung metastatic tumors (IR score 3+). Intense immunoreactivity of EBAG9 is observed in metastatic tumors with high IR scores. Bars represent 50 μm.

Fig. 6. Association of immunocytochemical staining for EBAG9 with disease-specific survival of 78 RCC patients. Five-year disease-specific survival of the patients with high EBAG9 immunoreactivity (IR score 3+, n = 19) was significantly worse than the patients with IR scores 0-2+ (n = 59) (55% *versus* 91%, $P = 0.0007$, by log-rank test).