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Bcl-X_L mediates epidermal growth factor dependent cell survival in HC11 mammary epithelial cells

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ABSTRACT

Apoptosis is the predominant process controlling cell deletion during post-lactational mammary gland remodeling. The members of the Bcl-2 protein family, whose expression levels are under the control of lactogenic hormones, internally control this mechanism. Epidermal growth factor (EGF) belongs to a family of proteins that act as survival factors for mammary epithelial cells upon binding to specific membrane tyrosine kinase receptors. Expression of EGF peaks during lactation and dramatically decreases in the involuting mammary gland. Though it was suggested that the protective effect of EGF is mediated through the phosphatidylinositol-3-kinase (PI3K) or MEK/ERK kinases activities, little is known about the downstream mechanisms involved on the anti-apoptotic effect of EGF on mammary epithelial cells; particularly the identity of target genes controlling apoptosis. Here, we focused on the effect of EGF on the survival of mammary epithelial cells. We particularly aimed at the characterization of the signaling pathways that were triggered by this growth factor, impinge upon expression of Bcl-2 family members and therefore have an impact on the regulation of cell survival. We demonstrate that EGF provokes the induction of the anti-apoptotic isoform Bcl-X_L and the phosphorylation and down-regulation of the pro-apoptotic protein Bad. The activation of JNK and PI3K/AKT signaling pathways promotes the induction of Bcl-X_L while AKT activation also leads to Bad phosphorylation and down-regulation. This protective effect of EGF correlates mainly with the up-regulation of Bcl-X_L than with the down-regulation of Bad. In fact, HC11 cells unable to express *bcl-X*, die even in the presence of EGF. In this context, Bcl-X_L emerges as a key anti-apoptotic molecule critical for mediating EGF cell survival.

1. Introduction

Apoptosis is a tightly regulated and highly efficient process. It occurs throughout the development and growth of the mammary gland, as was described by several authors. The most dramatic changes due to programmed cell death occur during post-lactational involution, when the glandular secretory tissue of the lactating mammary gland is remodeled [1–3]. Particularly, it has been reported that 72 h involuting glands show reduced size, low milk content, and a restructured tissue organization with high amounts of dead cells and the presence of immune infiltrates.

Apoptosis can be triggered by diverse stimuli that ignite signals converging into a common cell death machinery. This process is controlled at several intracellular check points. One of these is mainly regulated by different members of the Bcl-2 family, which can play opposite functions on programmed cell death. This family of proteins is defined by the presence of up to four conserved motifs known as Bcl-2 homology domains (BH1–BH4). In addition to Bcl-2, a number of other proteins like Bcl-X_L, Bcl-w and Mcl1, have an anti-apoptotic effect. On the other hand, the members of the pro-apoptotic group can be divided into two subgroups: the Bax-subfamily consisting mainly of Bax and Bak, which contain domains BH1, BH2 and BH3 and the members of the BH3-only subfamily, which possess only the BH3 motif, like Bad, Bid, Bim, etc. [4].

Mammary gland tissue expresses a number of different Bcl-2 relatives including Bcl-X (the most abundant), Bax, Bak, Bad, Bcl-w, Bfl-1 and Bcl-2 [5,6]. These proteins play a critical role in the control of apoptosis in the mammary epithelium and their expression levels are under the control of lactogenic hormones. While Bcl-2 is expressed in the nonpregnant and early pregnancy mammary gland, the expression of Bax remains high during pregnancy, is down-regulated during lactation, and is again up-regulated at the start of involution [5]. In this sense, stimulation of *bcl-2* expression and suppression of *bax*, were suggested as key events in the anti-apoptotic action of prolactin in mammary epithelial cells [7,8]. On the other hand, Bak, Bad, Bcl-w, and Bfl-1 are also up-regulated during involution [5].

The activity of some members of the Bcl-2 family may also be regulated by phosphorylation. In particular, phosphorylation of Bad on residues Ser-112, Ser-136, Ser-155 and Ser-170 promotes its binding to 14-3-3 proteins, and its subsequent cytosolic sequestration. Importantly,

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these modifications prevent Bad from associating with Bcl-2 or Bcl-X_L on the mitochondrial outer membrane, leaving these proteins free to exert their anti-apoptotic function [9]. In normal human breast cells, Bad levels are relatively high relative to other organs [10], suggesting that Bad might have a special role in this mammary gland.

Epidermal growth factor (EGF) belongs to a family of growth factors that interact with specific membrane receptors with tyrosine kinase activity, Erb1 among them (reviewed in [11]). Expression of EGF is low in the mammary gland from virgin and pregnant animals, but increases dramatically towards the end of pregnancy, peaks during lactation and significantly decreases in the involuting mammary gland [12,13]). According to several lines of evidence it was suggested that members of the EGF family are survival factors for mammary epithelial cells [14–16]. In this sense, it was demonstrated that mammary tumor cell lines established from mice over-expressing the *c-myc* oncogene, undergo apoptosis if cultured in the absence of EGF [15,16]. This protective effect of EGF is blocked when the cells are grown in the presence of specific inhibitors of EGF-receptor tyrosine kinase activity, the phosphatidylinositol-3-kinase (PI3K) or MEK/ERK kinases [15–17].

HC11 is mouse mammary epithelial cell line, broadly used as a model for studying mammary cell differentiation and apoptosis. These cells express several members of the tyrosine-kinase receptor family. Particularly, the dimerization and tyrosine-phosphorylation of ErbB2 and EGFR (Erb1) in EGF-stimulated HC11 cells was previously demonstrated [18]. EGF induces proliferation in this cell line and protects the cells from apoptosis if cultured in serum free medium (SFM) [14]. The mechanisms whereby EGF influences apoptosis on this cell type have not been clarified yet, although different options have been suggested [19].

Several studies have also addressed the relevance of the AKT pathway in mediating cell survival [20]. Activation of AKT has been found to block apoptosis induced by growth factor withdrawal and loss of anchorage. Targets for AKT phosphorylation implicated in determining cell survival, have also been identified in different systems [21,22]. AKT can block caspase-9 activation [23], induce the sequestration of the transcription factor Forkhead, involved in regulating Fas ligand expression [24] and phosphorylate the pro-apoptotic protein Bad leading to its deactivation [25]. As for mammary gland, AKT activity peaks during pregnancy and lactation, and decreases significantly during mammary involution [20]. Although AKT activation by growth factors or lactogenic hormones has been well documented, only a few specific apoptotic targets have been identified in mammary epithelial cells. It was reported that constitutive activation of the PI3K/AKT pathway suppresses the transcriptional up-regulation of the BH3-only protein Bmf during anoikis [26]. Besides, several reports suggest AKT dependence for the phosphorylation of Bad, i.e. the apoptotic effect of TGF-β in bovine mammary epithelial cells results in a decrease in AKT activity that correlates with a decline in the levels of phosphorylated Bad [27].

The role of the JNK pathway in controlling cell death or survival in the mammary gland is still controversial. In this sense, Faraldo et al. demonstrated that a low level or absence of proliferation and an induction of MEC apoptosis in involuting glands correlates with lack of JNK activation [28]. On the contrary, Small et al. reported that the over-expression of the MAPK phosphatase 1 (MPK-1) decreased activation of JNK and this effect correlates with apoptosis decrease in human mammary epithelial and breast carcinoma cells [29].

Here, we analyzed the mechanisms involved on the effect of EGF upon expression of Bcl-2 family members in HC11 mammary epithelial cells. We demonstrated that the addition of EGF provokes both, the induction of the anti-apoptotic isoform Bcl-X_L and the phosphorylation and down-regulation of the pro-apoptotic protein Bad; while Bcl-2 and Bax protein levels remain unchanged. The analysis of the signal transduction pathways involved in this effect showed that despite the fact that ERK1/2, JNK and PI3K/AKT kinases are activated upon EGF addition; only JNK and PI3K/AKT activities seem to be sufficient to mediate the EGF dependent inhibition of cell death. In fact, activation of PI3K/AKT by EGF correlates with an increase in Bcl-X_L and a decrease in Bad while JNK seems to participate in the control of programmed cell death by inducing Bcl-X_L. HC11 cells transfected with siRNA against *bcl-X* were unable to survive in response to EGF.

In this way, our results together support the idea that while EGF triggers a variety of responses upon the Bcl-2 family of proteins in HC11 cells, induction of Bcl-X_L emerges as a key event in mediating EGF dependent cell survival.

2. Materials and methods

2.1. Reagents

Recombinant Human EGF was purchased from Invitrogen (Cat. No. 13247-051, Carlsbad, CA, USA). PD98059, SP600125, LY294002, H89 and MG132 inhibitors were purchased from Calbiochem (San Diego, CA, USA). For in vitro assays 1000× solutions were prepared. Stock solutions of kinase inhibitors were prepared in dimethyl sulfoxide. RPMI 1640 medium and Fetal Bovine Serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Cell cultures and treatments

HC11 cells, derived from BALB/c mouse normal mammary glands (gently provided by Dr. Nancy Hynes, Friedrich Meischer Institute, Basel, Switzerland) were grown in RPMI 1640 growth medium (GM) supplemented with 10% FBS containing insulin (5 μg/ml) (Sigma Aldrich, St. Louis, MO, USA), penicillin (100 IU/ml) (Invitrogen, Carlsbad, CA, USA) and streptomycin (100 μg/ml) (Invitrogen, Carlsbad, CA, USA). Serum-free medium (SFM) was prepared with RPMI 1640 medium supplemented only with penicillin (100 IU/ml) and streptomycin (100 μg/ml). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Induction of apoptosis by serum withdrawal

Cells were trypsinized and plated in p100 plates in the conditions described above. When the cultures reached confluence, the medium was removed; the cells were washed once with PBS and maintained in SFM during 1–2 h for starvation. After this period either SFM containing EGF (100 ng/ml) in the presence or absence of different kinase inhibitors PD 98059 (20 μM); SP 600125 (50 μM); LY 294002 (10 or 50 μM) or H89 (10 μM) was added. The cells were then incubated for the time course described on each experiment.

2.4. Crystal violet staining

The number of cells still attached to the culture plate at the end of each treatment was evaluated by crystal violet staining. HC11 cells were plated in 96-multiwell plates and cultured until confluence as described above. Apoptosis was induced by replacing GM with SFM and cells were maintained under these conditions during 96 h. Then, cells were fixed with 100 μl ice-cold glutaraldehyde (1.1% in PBS) for 15 min at 4 °C. Samples were washed three times by submersion in de-ionized water, air-dried, and stained for 20 min with 100 μl of crystal violet 0.1% in 200 mM phosphoric acid, pH = 6. After careful aspiration of the crystal violet, extensive washing with de-ionized water eliminated the excess dye. Samples were then air-dried and treated with 100 μl of acetic acid 10% during 30 min at room temperature. Optical density was measured at 590 nm with a multiplate spectrophotometer (Benchmark, Bio-Rad, Hercules, CA, USA).

2.5. DNA fragmentation

DNA from apoptosis induced confluent HC11 cells was isolated according to the method described by Herrmann et al. [30]. Briefly,

HC11 cells were plated in p100 plates, incubated in GM at 37 °C until confluence and then switched to SFM as described above. After 48 h cells were gently resuspended in 0.1 ml lysis buffer (50 mm Tris, pH 7.5, 20 mm EDTA, and 1% Nonidet P-40) for 1 min. After centrifugation at 4000 ×*g* for 5 min at room temperature the supernatant (containing the fragmented DNA) was collected in another tube. The pellet (nuclear DNA) was then resuspended in 0.1 ml lysis buffer. Both fractions were incubated at 56 °C for 2 h in the presence of 5 μg/μl ribonuclease A and 1% SDS. After ribonuclease A treatment, 5 μg/μl proteinase K was added to each sample and further incubated at 37 °C overnight. DNA was precipitated by the addition of 75 μl of 10 M ammonium acetate and 450 μl absolute ethanol. After precipitation, DNA was resuspended in 20 μl distillated water, electrophoresed in 1.5% agarose gels, and visualized under UV light.

2.6. Caspase-3-like activity assay

Caspase-3-like activity was measured. In brief, HC11 cells were plated in p60 plates, incubated in GM at 37 °C until confluence and then switched to SFM as described above. After 4 h, treated cells were lysed in 50 mM Tris–HCl buffer pH 7.4 containing 1 mM EDTA, 10 mM EGTA, 10 μM digitonin, 0.5 mM PMSF, 1.54 μM aprotinin, 14.58 μM pepstatin and 63.86 μM benzamidine, for 30 min at 37 °C. Cell lysates were clarified by centrifugation and 150 μl of the resultant supernatant (100–200 μg protein) was incubated with 146 μl of incubation buffer (100 mM HEPES pH 7.5, 20% glycerol, 0.5 mM EDTA and 5 mM dithiothreitol (DTT)) and 4 μl of the substrate acetyl-Asp-Glu-Val-Asp-7-amino-4 p-nitroanilide (Ac-DEVDpNA) (100 μM) at 37 °C for different times (4 and 12 h). Blanks were also run, containing either the substrate or the cell lysate alone, to deduce in each case. Caspase-catalyzed release of the chromophore *p*NA from the substrate was measured at 405 nm with a multiplate spectrophotometer (Benchmark, Bio-Rad, Hercules, CA, USA) and the cleavage activity was expressed as *p*NA absorbance units/mg protein. Protein concentration was determined by the method of Bradford [31] using bovine serum albumin as a standard.

2.7. Isolation of mitochondrial and cytosolic fractions

8 h starved confluent HC11 cells were lysed in MSHE buffer (0.22 M mannitol, 0.07 M sucrose, 0.5 mM EGTA, 2 mM HEPES/KOH, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, pH 7.4). The homogenate was centrifuged 10 min at 1000 ×*g* (pellet = crude nuclear extract) followed by 20 min at 10,000 ×*g* (pellet = mitochondria, supernatant = cytosol). Mitochondrial fractions were resuspended in MSHE buffer. The purity of both, mitochondrial and cytosolic fractions was assessed by western blot with antibodies against complex I and β-tubulin, respectively. Protein content was determined by the Bradford method.

2.8. RNA analysis

Cells were resuspended in denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.1 M β-mercaptoethanol and 0.5% sarcosyl) and total RNA was extracted by the single step method [32]. RNase protection analysis was performed as described previously [33]. For preparing the *bcl-X* probe, plasmid pGLD3 was digested with Hinf I and transcribed by T3 RNA polymerase. The full-length transcript size of the *bcl-X* riboprobe was 294 nucleotides, and the protected fragment for *bcl-X*$_L$ was 237-bp long. The *gapdh* template pTRIGAPDH (Ambion, Austin, TX, USA) was digested with BglII and transcribed with T3 RNA polymerase. The probe length was 359 nucleotides, and the size of the protected fragment was 316 bp. [α-32P]CTP (GE Health Care, Piscataway, NJ, USA) radiolabeled RNA probes were prepared using a kit according to the instructions of the manufacturer (Promega, Madison, WI, USA). The probes were coprecipitated with RNA samples and dissolved in hybridization buffer, denatured at 95 °C for 10 min, and hybridized at 52 °C for 18 h. After digestion with RNases A and T1 followed by digestion with proteinase K, the samples were precipitated, denatured, and subjected to electrophoresis on a 5% denaturing acrylamide gel. Quantification was performed with ImageJ 1.34s software (Wayne Rasband, National Institutes of Health, USA. http://rsb.info.nih.gov/ij/). In all of the cases, the quantitation was normalized against *gapdh* signal.

2.9. Protein analysis

Total proteins were extracted from HC11 cells in RIPA protein extraction buffer (50 mM Tris–HCl, 150 mM NaCl, 1% triton, 0.25% sodium deoxycholate, 1 mM EDTA pH 7.4) supplemented with protease (Protease inhibitor cocktail set I, Calbiochem, San Diego, CA, USA) and phosphatase inhibitors (1 mM NaF, 1 mM Na_2VO_4). The lysate was centrifuged at 14,300 ×*g* and 4 °C for 10 min, and the pellet discarded. Cleared lysates were combined with SDS sample buffer (50 mM Tris pH 6.8, 1% SDS, 0.1% bromphenol blue, 10% glycerol, 100 mM DTT). For western blot samples were boiled for 5 min and electrophoresed for 3 h at 100 V in 15% or a 12%, SDS-polyacrylamide gel, transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) by electroblotting in transfer buffer containing 20% methanol (vol/vol), 0.19 M glycine, 0.025 M Tris-base (pH 8.3) at 300 mA for 1.5 h at 0 °C. Blots were blocked 1 h at room temperature in TBS (20 mM Tris–Cl, pH 7.5, 500 mM NaCl) containing low-fat powered milk (5%) and Tween 20 (0.1%). The incubations with primary antibodies were performed at 4 °C for 12 h in blocking buffer (3% skim milk, 0.1% Tween, in Tris-buffered saline). The membranes were then incubated with the corresponding counter-antibody and the proteins evidenced by enhanced chemiluminescence detection (ECL+Plus System, GE Health Care, Little Chalfont, Buckinghamshire, UK). As primary antibodies the following were used: α-Bcl-X$_{L/S}$ (sc-634); α-Bax (sc-493); α-Bcl-2 (sc-492); α-P-ERK1/2 (sc-7383); α-ERK1/2 (sc-154); α-P-JNK (sc-6254); α-JNK (sc-474-G); α-P-p38 (sc-7973); α-p38 (sc-535-G); α-AKT (sc-1618); α-cytochrome *c* (sc-13156); α-ubiquitin (sc-8017); α-tubulin (sc-9104) and α-actin (sc-1616-R) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and α-P(Ser112)-Bad (#9296); α-P(Ser136)-Bad (#9295); α-Bad (#9292) and α-P-AKT (Cell Signaling Technology, Beverly, MA, USA) and α-Complex I NDUFA9 (421344) (Molecular Probes Inc., Eugene, OR, USA). As secondary antibodies the following were used: a peroxidase-labeled α-rabbit IgG; α-mouse IgM or α-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or α-mouse IgG (Bio-Rad, Hercules, CA, USA). Densitometric analysis of protein levels was performed with ImageJ 1.34s software (Wayne Rasband, National Institutes of Health, USA. http://rsb.info.nih.gov/ij/).

2.10. siRNA synthesis and transfection

Desalted DNA oligonucleotides were purchased at Invitrogen (Carlsbad, CA, USA). (i) T7, 5′-TAATACGACTCACTATA G-3′. (ii) Bcl-X; sense, 5′-AAATATCCTTTCTGGGAAAGCTATAGTGAGTCGTATT A-3′; antisense, 5′-AAGCTTTCCCAGAAAGGATACTATAGTAGGTCGTATTA-3′. Control of inespecific sequence; sense, 5′-AAAATTTAGCGCAGCAT-TACCTATAG TGAGTCGTATTA-3′; antisense, 5′-AAGGTAATGCTGCGC-TAAATCTATAGTGA GTCGTATTA-3′. The oligonucleotide-directed production of small RNA transcripts with T7 RNA polymerase has been described [34]. For each transcription reaction, 1 nmol of each oligonucleotide was annealed in 50 μl of TE buffer (10 mM Tris–HCl pH 8.0, and 1 mM EDTA) by heating at 95 °C; after 2 min, the heating block was switched off and allowed to cool down slowly to obtain dsDNA. Transcription was performed in 50 μl of transcription mix: 1× T7 transcription buffer (40 mM Tris–HCl pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl and 2 mM spermidine), 1 mM rNTPs, 0.1 U yeast pyrophosphatase (Sigma Aldrich, St. Louis, MO, USA), 40 U RNAsin (Promega, Madison, WI, USA) and 100 U T7 RNA Polymerase

(Promega, Madison, WI, USA) containing dsDNA 200 pmol as template. After incubation at 37 °C for 2 h, 1 U RNAse free-DNAse (Promega, Madison, WI, USA) was added at 37 °C for 15 min. Sense and antisense RNAs generated in separate reactions were annealed by mixing both crude transcription reactions, heating at 95 °C for 5 min followed by 1 h at 37 °C to obtain 'T7 RNA polymerase synthesized small interfering double stranded RNA' (T7 siRNA). The mixture (100 µl) was then adjusted to 0.2 M sodium acetate pH 5.2, and precipitated with 2.5 vol ethanol. After centrifugation, the pellet was washed once with 70% ethanol, dried, and resuspended in 50 µl of RNAse free water.

For liposome-mediated siRNAs transfection HC11 cells were seeded into 6-well plates 24 h prior to treatment. Transfection of T7 siRNAs was carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as directed by the manufacturer for adherent cell lines. 0.5 or 2 µl of T7 siRNAs were applied per well. Cells were harvested and lysed 48 h later for Bcl-X_L protein level analysis. For cell survival experiments cells were switch to GM or keep in SFM for 48 h after siRNA transfection and the number of cells still attached to the culture plate was evaluated by crystal violet staining as described above adjusting the volumes to 6-well plates.

2.11. Statistical analysis

Results were expressed as means ± standard error or means ± standard deviation, as it is indicated in the figure legends. For Bcl-X_L studies, ANOVA followed by Student–Newman–Keuls post hoc test was used to determine statistically significant differences ($p<0.05$). For crystal violet staining and caspase-3-like activity, one-way ANOVAs followed by Tukey's multiple comparisons tests were used to detect significant differences among treatments. Statistical analyses were performed with STATISTICA 6.0 (StatSoft, Inc.). Differences were regarded as significant at $p<0.05$. Before statistical analysis data were tested for normality and homoscedasticity using Lilliefors and Bartlett's tests, respectively.

3. Results

3.1. EGF inhibits apoptosis through the activation of JNK and PI3K

The EGF dependent activation profiles of the protein kinases ERK1/2, JNK, p38 MAPK and AKT was analyzed by Western blot of the phosphorylated forms on extracts obtained from HC11 confluent cells treated with EGF for 5 min to 4 h on SFM. Fig. 1A shows that EGF induces a transient increase in the phosphorylation levels of ERK1/2 and JNK between 5 and 15 min after its addition (lanes 3 to 5, first and third panels, respectively). The activity of both enzymes was not affected by the absence of serum (compare lanes 1 and 2). On the other hand, p38 kinase presents a relatively high basal level of phosphorylation, which is a consequence of the absence of serum in the culture (fifth panel, lane 2); however, the phosphorylation degree of p38 is not modified by the addition of the growth factor (fifth panel,

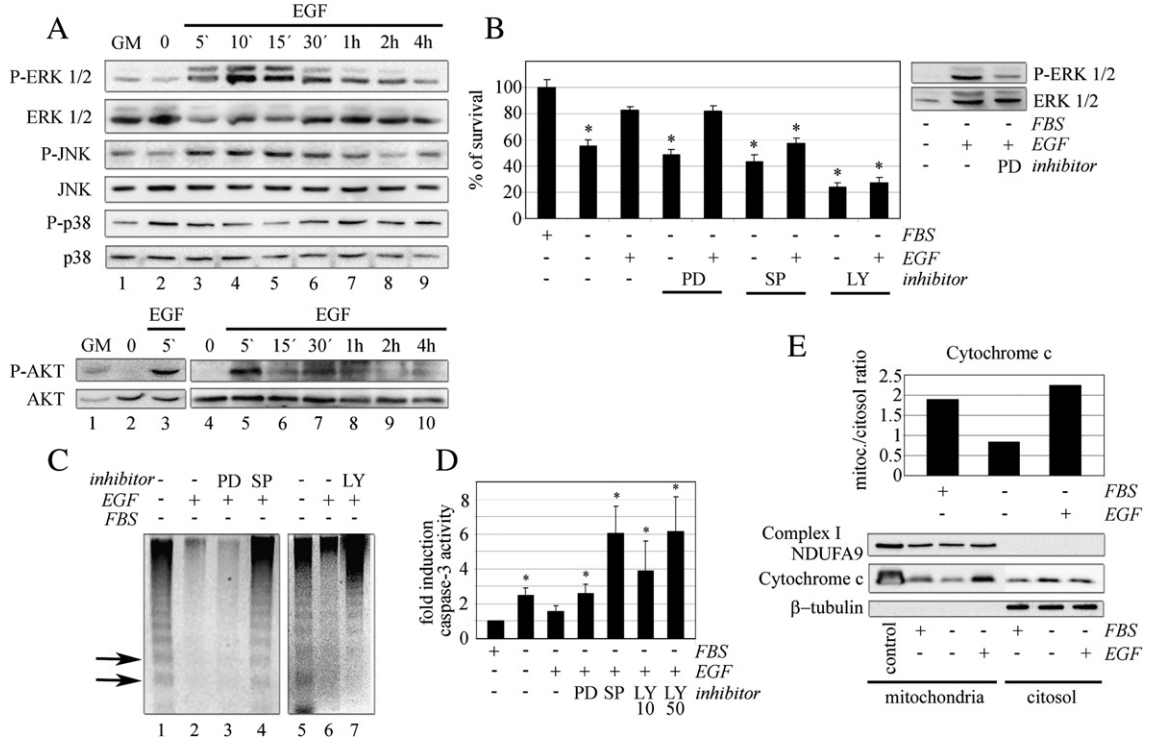

Fig. 1. EGF reverts apoptosis induced by cell density and SFM in HC11 cells by a mechanism that involves JNK and PI3K kinase pathways. Confluent HC11 cells were starved during 1 h on SFM and then stimulated with EGF (100 ng/ml) from 5 min to 4 h. Equal amounts of protein from each sample were analyzed by western blot. Membranes were probed with antibodies against (A) phosphorylated ERK1/2 and total ERK1/2 (first set, upper panels), phosphorylated JNK and total JNK (middle panels), phosphorylated p-38 and total p-38 (first set, lower panels) and phosphorylated AKT (second set, upper panels) and total AKT (second set, lower panels). Results are representative of three independent experiments. (B) Confluent HC11 cells were kept on SFM with the addition of EGF (100 ng/ml) and/or kinase inhibitors as indicated: PD98059 (20 µM), SP600125 (50 µM), LY294002 (50 µM). Viability was assessed by crystal violet staining assays performed after 96 h treatment and is expressed as percentage of the internal control (with FBS). Bars indicate Mean ± S.E. of five replicates from three independent experiments ($n=15$). *=$p<0.05$. (C) Agarose gel electrophoresis of a DNA ladder from confluent HC11 cells treated as in (B) for 48 h. The gel is representative of three independent experiments. (D) Caspase-3 activity was determined upon 4 h of treatment by measuring the proteolysis of Ac-DEVDpNA during 12 h. Specific activity was calculated as A405/mg protein under the standard incubation conditions and expressed as fold induction against the control. The means ± S.E. from three independent experiments are shown. *=$p<0.05$. (E) Cytochrome *C* release was tested by western blots performed on mitochondrial and cytosolic fractions of HC11 cells treated as in (C). Western blots against Complex I NDUFA9 protein and β-tubulin were performed to confirm the efficiency of fraction recovery. Bands corresponding to Cytochrome *c* were scanned and quantitated. The bars indicate the ratio between the band corresponding to the mitochondrial fraction and the band from the cytoplasmic fraction for each treatment. The gel is representative of three independent experiments.

lanes 3 to 9). AKT activation seems also to be extremely transient – between 5 to 10 min – (Fig. 1A, lower set).

After 96 h in SFM the number of viable cells fell down to 55.6±4.6%. However, cell viability was restored to 82.5±2.7% in the presence of EGF (100 ng/ml) (Fig. 1B, lane 3). To test the involvement of the different kinase pathways in this protective effect we used specific kinase inhibitors for MEK/ERK1/2 (PD 98059, PD); JNK (SP 600125, SP) and PI3K/AKT (LY 294002, LY). Results show that only SP and LY blocked EGF mediated protection (Fig. 1B, columns 7 and 9) and that in SFM, both kinase inhibitors, SP and LY, decreased cell viability per se (columns 6 and 8). These results suggest a putative role for both, JNK and PI3K/AKT pathways on EGF-induced cell survival. Conversely, MEK/ERK1/2 specific inhibitor PD (columns 4 and 5), although effective in blocking ERK1/2 phosphorylation (insert to the right in Fig. 1B) is unable to affect EGF-mediated cell survival (81.8±4.3%) or the SFM-induced cell viability (48.4±3.9%).

To analyze whether the protection conferred by EGF on HC11 cell viability is related to inhibition of apoptosis, we performed DNA fragmentation assays. HC11 confluent cells were incubated in SFM with or without EGF plus the kinase inhibitors PD, SP and LY. Fig. 1C shows that after 48 h, a laddering of DNA was detected in cells incubated on SFM alone (lanes 1 and 5) that considerably decreased by addition of EGF (lanes 2 and 6). This protective effect was still present when cells were incubated with PD but not observed in the presence of the kinase inhibitors, SP and LY (lanes 3, 4 and 7 respectively).

We next observed that EGF inhibits the activation of caspase 3 provoked by serum depletion (Fig. 1D, columns 2 and 3). While this EGF effect was blocked by all the kinase inhibitors tested, PD (column 4), SP (column 5) and LY (columns 6 and 7), the caspase 3 activity turned out significantly high in the presence of these two last inhibitors which indicates a per se effect of these drugs.

Taken together, these results suggest that even though three kinase pathways are activated upon EGF addition, only JNK and PI3K/AKT signaling are relevant on the EGF-dependent cell death protection.

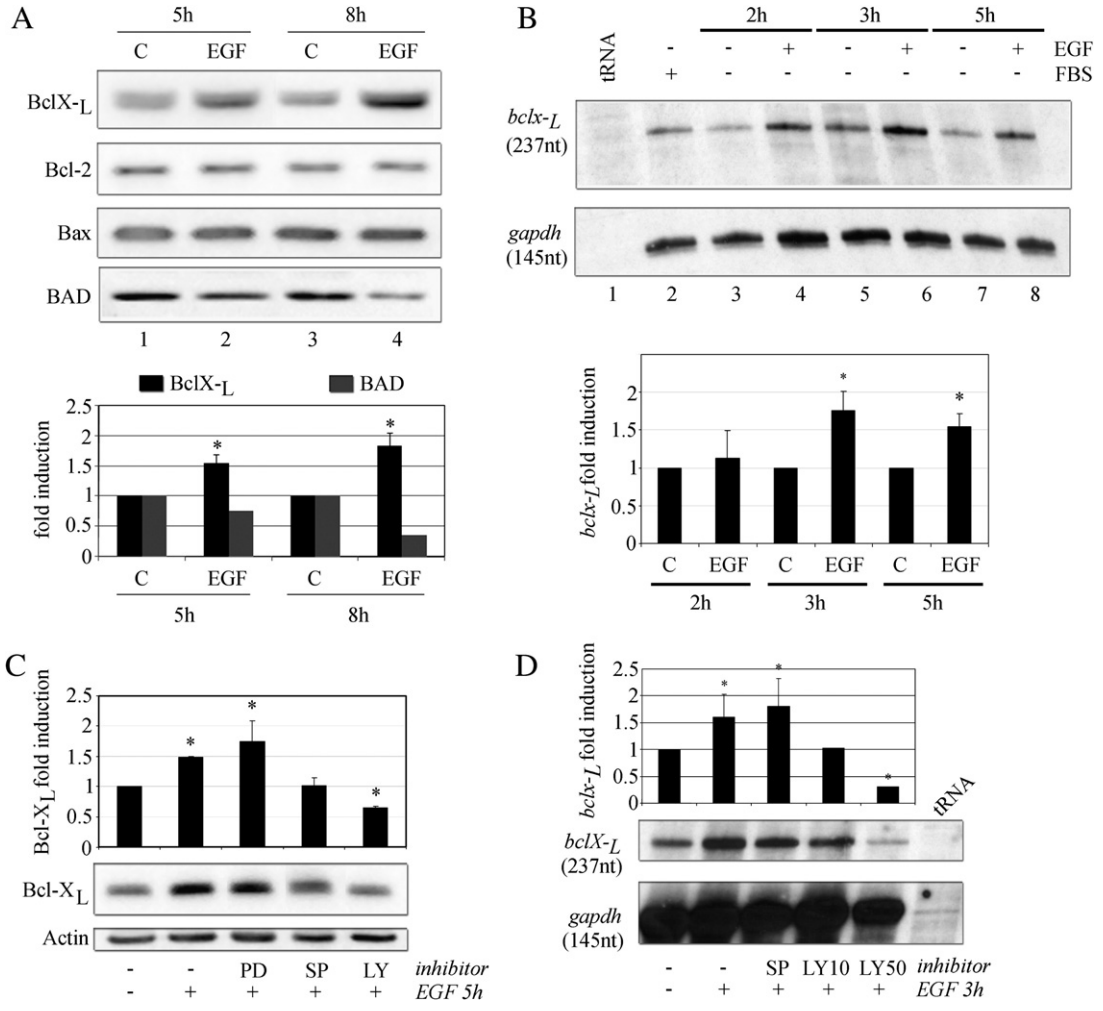

Fig. 2. EGF increases Bcl-X_L expression by JNK and PI3K kinase pathways and down-regulates Bad. (A) Confluent HC11 cells were starved during 1 h on SFM and then stimulated with EGF (100 ng/ml) for 5 and 8 h. Cell lysates were analyzed by western blot. Membranes were probed with antibodies against Bcl-X (upper panel), Bcl-2 (middle upper panel), Bax (middle lower panel) and Bad (lower panel) proteins. Representative gels from three independent experiments are shown. Bcl-X_L and Bad protein expression are expressed as fold induction relative to the controls. The means±S.E. from three independent experiments are shown (A, column graph). *=$p<0.05$. (B) RNAse protection assay of total RNA harvested from confluent HC11 cells starved during 1 h on SFM and then stimulated with EGF (100 ng/ml) for 2, 3, and 5 h. The protected fragments of *bcl-X_L* and *gapdh* are shown. tRNA is used as a negative control and medium with FBS as a positive control. A representative gel out of three independent experiments is shown. Values were normalized to *gapdh* and *bcl-X_L* fold induction is shown in the column graph at the bottom. The means±S.E. from three independent experiments are shown. *=$p<0.05$. *nt*, nucleotides. (C) Confluent HC11 cells were pre-incubated with kinase inhibitors as indicated and then EGF (100 ng/ml) was added for 5 h. Cell lysates were analyzed by western blot. Membranes were probed with antibodies against Bcl-X and actin proteins as indicated. Representative gels from three independent experiments are shown. Bcl-X_L levels normalized to actin are expressed as fold induction relative to the control. The means±S.E. from three independent experiments is shown (bar graph). *=$p<0.05$. (D) RNase protection assay of total RNA harvested from confluent HC11 cells pre-incubated with PD98059 (20 μM), SP600125 (50 μM) and LY294002 (10 or 50 μM) for 1 h in SFM as indicated and then treated with EGF(100 ng/ml) for 3 h is being shown. The protected bands of long *bcl-X* isoform and *gapdh* are shown. tRNA is used as a negative control. A representative gel out of three independent experiments is shown (lower panel). Values were normalized to *gapdh*. The means±S.E. from three independent experiments are shown for *bcl-X_L* fold induction (upper panel). *=$p<0.05$. *nt*, nucleotides.

3.2. EGF prevents Cytochrome C release from the mitochondria

Western blot analysis using an antibody against Cytochrome *C* was performed upon extracts of mitochondrial and cytosolic fractions of HC11 lysates. Effective fractionation was tested using antibodies against the Complex 1 protein NDUFA9 and β-tubulin, (Fig. 1E). The ratio of protein present in mitochondria compared to cytosol was calculated (columns in the upper panel) after quantification of the western blots developed with anti-CytC antibodies. We observed that incubation in SFM resulted in a dramatic release of Cytochrome *C* from the mitochondria as shown by a reduction of this ratio, which is restored to normal levels by the addition of EGF.

3.3. EGF increases Bcl-X_L and down-regulates Bad expression

Western blot assays were performed in order to analyze the expression levels of the members of the Bcl-2 family in HC11 confluent cells treated with EGF in SFM for 5 or 8 h. Results shown in Fig. 2A indicate that HC11 cells treated with EGF exhibit significantly higher protein levels of Bcl-X_L as compared with cells incubated in SFM alone (1.6±0.2 and 1.9±0.2 fold induction for 5 and 8 h, respectively) (first panel lanes 2 and 4). On the opposite way, Bad levels decrease upon EGF addition (0.76 and 0.36 fold induction for 5 and 8 h, respectively) (fourth panel lanes 2 and 4). Neither Bcl-2 nor Bax proteins change their levels upon EGF addition on HC11 confluent cells (Fig. 2A, second and third panels, respectively).

EGF-mediated *bcl-X_L* up-regulation was also observed in RNAse protection assays performed in HC11 cells treated with EGF (Fig. 2B). Serum withdrawal results in a considerable decrease in the messenger level of the *bcl-X_L* isoform (lane 3). This effect is reverted upon EGF addition, that restores and increases *bcl-X_L* levels to 1.76±0.26 fold after 3 h of treatment (Fig. 2B, lane 6). We conclude that EGF increases both the messenger and protein levels of *bcl-X_L* in HC11 confluent cells in a way compatible with its anti-apoptotic effect suggesting that up-regulation of Bcl-X_L would play a key role in EGF-mediated cell survival.

In order to study the participation of different kinase pathways on the effect of EGF on *bcl-X_L*, we treated HC11 confluent cells with EGF in SFM during 5 h in the presence of the different pharmacological inhibitors. Fig. 2C shows that the increase in Bcl-X_L protein levels triggered by EGF is completely blocked when cells are co-incubated with SP or LY but not with PD. The analysis of *bcl-X* transcripts shows that while LY diminishes *bcl-X_L* messenger levels in a dose-dependent manner, the JNK inhibitor does not affect EGF mediated increase in *bcl-X_L* mRNA (Fig. 2D).

3.4. EGF induces phosphorylation of Bad

Inactivation of Bad involves its phosphorylation [9]. We analyzed whether there is a correlation between the phosphorylation degree of Bad and the decrease on its expression levels. Fig. 3A depicts a time course of both, Bad phosphorylation degree and protein levels in confluent HC11 cells treated in SFM with or without EGF. The increase

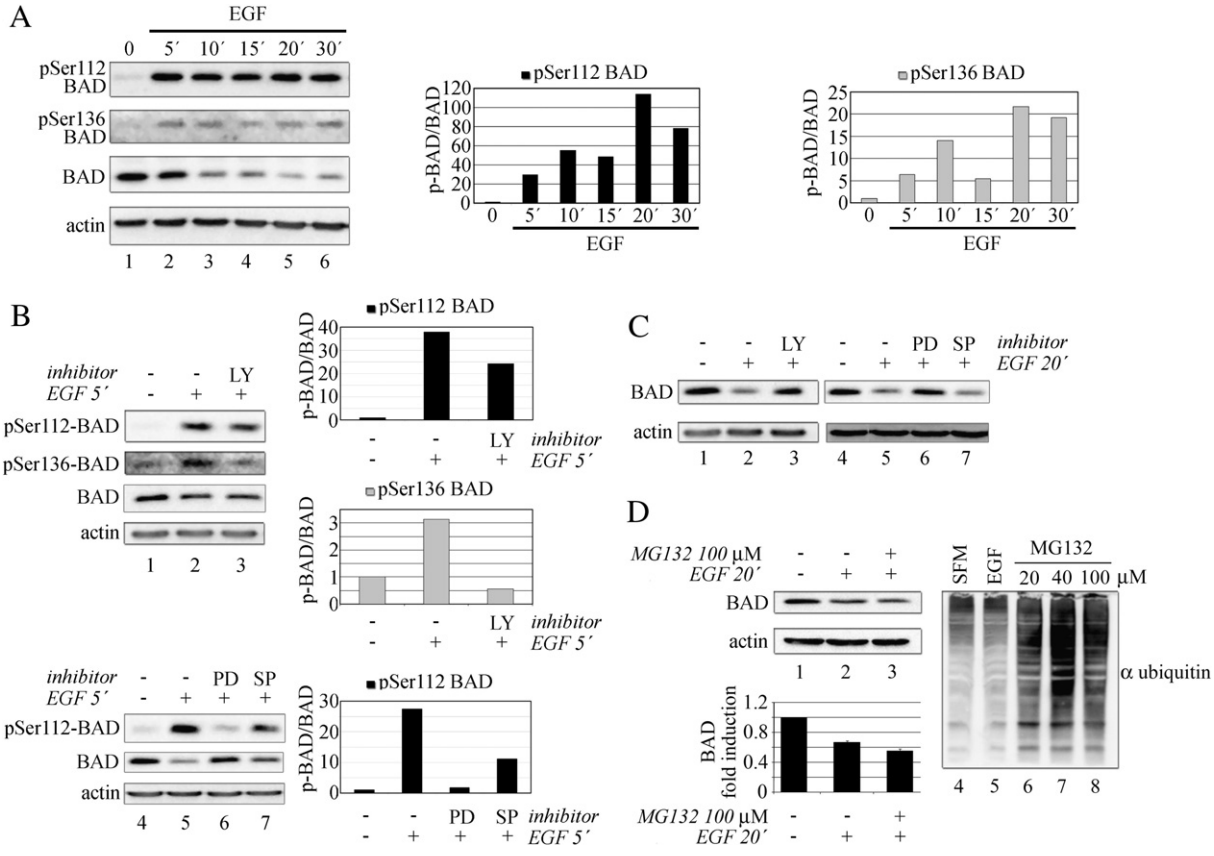

Fig. 3. Bad protein is phosphorylated at Ser112 and Ser136 and down-regulated after EGF treatment independently of the 26S proteasome degradation system. (A) Confluent HC11 cells were starved during 1 h on SFM and then stimulated with EGF (100 ng/ml) for 5 to 30 min. Cell lysates were analyzed by western blot with antibodies against: phosphoBad-Ser112, phosphoBad-Ser136, total Bad and actin. Levels of p-Bad/Bad from one representative out of three independent experiments are shown and represented as fold induction relative to the control. (B) and (C) Confluent HC11 cells were pre-incubated with kinase inhibitors as indicated and then stimulated with EGF (100 ng/ml) for 5 min (B) and 20 min (C). Cell lysates were analyzed by western blot. Membranes were probed with antibodies against phosphoBad-Ser112, phosphoBad-Ser136, total Bad and actin as indicated. Relative levels of p-Bad/Bad from one representative out of three independent experiments are shown. (D) Confluent HC11 cells were pre-incubated for 20 h with MG132 proteasome inhibitor as indicated and then stimulated with EGF (100 ng/ml) for 20 min. Cell lysates were analyzed by western blot. Membranes were probed with antibodies against total Bad, actin and ubiquitin as indicated. Relative levels of Bad from three independent experiments are shown.

in Bad phosphorylation levels was already evident 5 min after EGF addition in both residues, Ser-112 and Ser-136, previously described as targets of ERK1/2 or PKA respectively. In addition, EGF induces a decrease in total Bad protein levels upon 10 min of treatment, reaching a minimum after 20 min of incubation (lanes 3 to 6). Thus, our results show a steady increase in the ratio of phosphoBad/Bad at both target residues (bar graphics).

In order to identify the kinase pathway/s mediating EGF-dependent Bad phosphorylation, we made use of the set of kinase inhibitors. Fig. 3B shows that LY blocks Ser-136 without affecting Ser-112 phosphorylation (upper set, lane 3) while PD inhibits Ser-112 phosphorylation (lower set, lane 6). Both inhibitors are also able to block the EGF-mediated decrease in Bad levels after 20 min of incubation (Fig. 3C, lanes 3 and 6, respectively). On the other hand, though SP partially inhibited the EGF dependent Bad Ser-112 phosphorylation (Fig. 3B lower set, lane 7) it was not able to restore Bad down-regulation (Fig. 3C, lane 7).

According to a recent report, Bad phosphorylation may affect this protein's stability by ubiquitylation and proteasomal degradation [35]. In order to test this possible mechanism, we performed experiments in the presence of a proteasome inhibitor, MG132. Fig. 3D shows that the decrease in Bad levels is still observed in confluent HC11 cells treated with EGF (Fig. 3D, lane 2) despite MG132's ability to accumulate ubiquitylated proteins (Fig. 3D, right panel). These results suggest that, in our conditions, the down-regulation of Bad is independent of the 26S proteasome degradation system.

3.5. Putative role of Bcl-X_L in EGF-dependent cell survival

Together the results show that the protective effect of EGF correlates mainly with the up-regulation of the anti-apoptotic protein Bcl-X_L rather than the down-regulation of Bad, as the inhibition of JNK by SP blocks both, Bcl-X_L up-regulation and cell survival without preventing Bad decrease. To test the role of Bcl-X_L as a mediator in EGF dependent cell survival we performed small interfering RNA (siRNA) assays. Fig. 4A shows the effect on *bcl-X* expression upon increasing amounts of the specific siRNA. In fact, the addition of 50 pmol of siRNA is enough to down-regulate the expression of Bcl-X_L around 50%. The specificity of the effect is validated treating cells with an unspecific siRNA (Fig. 4B). Then we analyzed the effect of EGF on cell survival in the presence of *bcl-X* and control siRNAs. We performed two approaches: 1) under apoptotic conditions (cells cultured on SFM) (Fig. 4C, left panel) and 2) on survival conditions (cells cultured on GM) (Fig. 4C, right panel). After 48 h in SFM the number of viable cells fell down to 47 ± 3.6% and could not be recovered by the presence of EGF (100 ng/ml) when the cells were incubated with *bcl-X* siRNA (Fig. 4C, left panel). However, *bcl-X* siRNA was unable to decrease the cell viability upon cells grown on complete media (with FBS) (Fig. 4C, right panel). Together these results suggest the relevance of Bcl-X_L in mediating EGF survival in an apoptotic context.

4. Discussion

Many cellular processes are modulated by phosphorylation/dephosphorylation of molecular components, activating cascades of protein kinases upon determined stimuli. Among factors promoting growth and proliferation in mammary cells, EGF exerts its action by interacting with EGFR, a member of the ErbB receptor family. This interaction promotes cell survival and is involved in all the major mechanisms controlling cell death (reviewed in [36]). In this sense, survival factors such as Ras, PI3K/AKT, and Bcl-X_L/-2 are activated by ErbB signaling in several systems [36]. However, the mechanism by which this family of proteins prevents apoptosis has not been completely elucidated. As for mammary epithelial cells it has been demonstrated that EGF, IGF-I, and basic fibroblast growth factor (bFGF) act as survival factors [13]. In fact, excess activation of signaling downstream of ErbB1, has been linked to the development of breast cancer and to chemotherapeutic resistance [37].

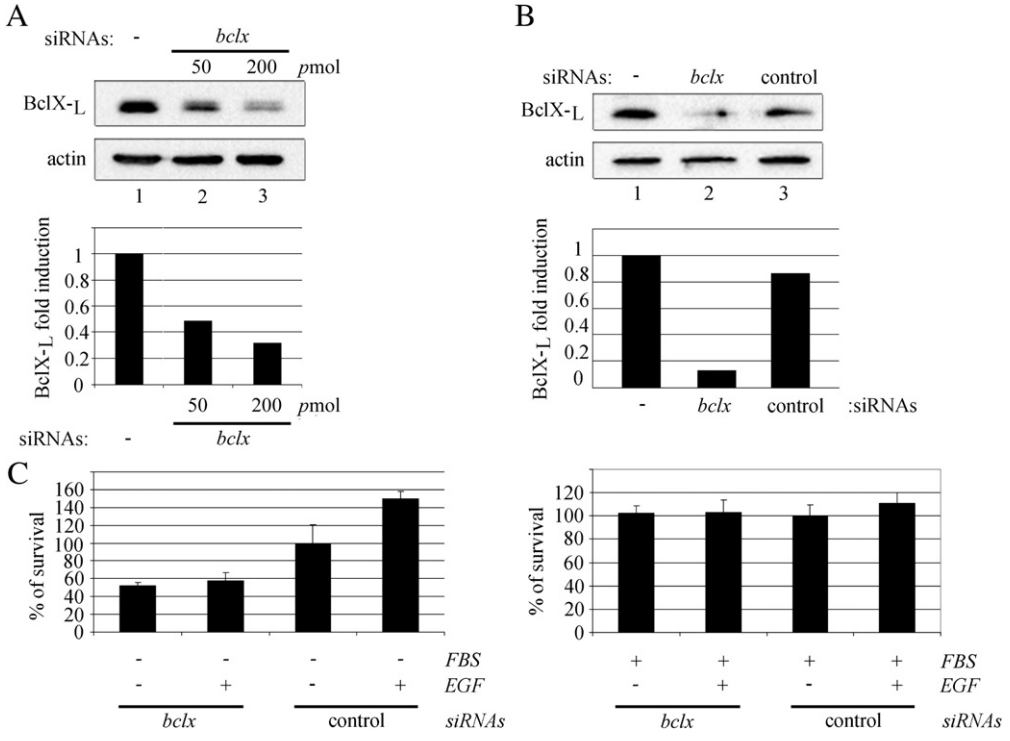

Fig. 4. Bcl-X_L is involved on EGF-dependent cell survival. (A) and (B) HC11 cells were seeded into 6-well plates 24 h prior to treatment and siRNAs against *bclx* [50 pmol and 200 pmol for (A) and 200 pmol for (B)] and control (200 pmol) were transfected. Cells were harvested 48 h later and lysates were analyzed by western blot with antibodies against: Bcl-X and actin. Relative levels of Bcl-X_L normalized to actin from three independent experiments are shown. (C) HC11 cells were seeded into 6-well plates 24 h prior to treatment and transfected with 200 pmol of siRNAs as indicated. Cells were switched to GM or kept in SFM for 48 h after transfection and viability was assessed by crystal violet staining assays. Results are expressed as percentage of the internal control (control siRNA without EGF). Bars indicate Mean ± S.E. of two replicates from three independent experiments ($n = 6$).

The experiments summarized in this paper support the notion that EGF prevents apoptosis in HC11 cells by activating different signal transduction pathways. Our results showed that EGF treatment results in the activation of MEK/ERK, PI3K and JNK pathways. The involvement of p38 kinase activity in the mechanism of action of EGF was ruled out as our results showed high levels of phosphorylated p38 in cells cultured in SFM regardless of the presence of EGF. Noteworthy, an EGF-dependent activation of p38 kinase in HC11 cells has been recently reported [19]. This discrepancy could be attributed to a difference in cell culture conditions as we observed an increase in the phosphorylation degree of p38 when the cells were incubated during 1 h on SFM; while in the other report authors evaluated the effect of EGF in differentiated HC11 cells cultured in 10% FBS [19].

Our results show ERK1/2 is activated by EGF, but this signaling pathway would not be relevant in mediating its activity as a promoter of cell survival in our system. This is supported by the fact that while the ERK1/2 inhibitor prevents EGF-dependent inhibition of caspase-3 and Bad down-regulation, it does not affect the cell survival rate obtained in the presence of the growth factor. In this sense, Galbaugh et al. have suggested that the Ras/Mek/ERK pathway is mainly preventing the EGF-dependent repression of specific target genes involved in differentiation [19].

On the other hand, we demonstrate that both PI3K/AKT and JNK pathways are crucial in mediating the inhibition of apoptosis by EGF as their activation is involved in controlling the expression levels and phosphorylation degree of some members of the Bcl-2 family. The protective effect of EGF in HC11 cells includes an increase in the levels of the anti-apoptotic protein Bcl-X_L and the phosphorylation and down-regulation of the pro-apoptotic protein Bad, but no changes in Bcl-2 or Bax protein levels. Similar results were reported by Merlo et al., who showed that the sharp increase observed in Bax protein levels with increased cell density is independent of the presence of EGF. These authors have mentioned that Bcl-2 is slightly up-regulated upon EGF addition in confluent human MCF-10 cells [38].

According to our results EGF increases Bcl-X_L expression in two different ways, at least: 1) by increasing *bcl-X$_L$* transcripts levels through the activation of PI3K and; 2) through a mechanism mediated by both JNK and PI3K resulting in a rise in its protein levels. Thus, it is tempting to speculate that up-regulation of Bcl-X_L plays a key role in the survival of HC11 cells mediated by EGF.

A variety of soluble growth factors inhibit apoptosis by phosphorylation of Bad on multiple serine residues [9,39,40]. Activation of receptors that regulate PI3K result in phosphorylation of Bad on Ser-136 [41], whereas activation of the MAPK pathway phosphorylates Bad on ser-112 and Ser-155 [42]. Phosphorylation in these three residues promotes Bad inactivation by sequestration in the cytoplasm that prevents interaction with Bcl-X_L [9,39,43].

Upon EGF addition, Bad is phosphorylated by ERK1/2 in Ser-112 and by PI3K/AKT in Ser-136. Ser-112 can also be phosphorylated by PKA; however this would not be the case for EGF-dependent (Ser-112)-Bad phosphorylation as it was not inhibited by the PKA inhibitor, H89 (data not shown). Our results suggest that the activation of both kinase pathways ERK1/2 and PI3K are necessary for EGF dependent Bad phosphorylation and down-regulation. However, the ERK1/2 pathway is not involved in the regulation of cell viability by EGF.

Phosphorylation of Bad by AKT increases its interaction with 14-3-3 proteins and prevents the dimerization of Bad with Bcl-X_L, promoting cell survival [9,39,43]. An opposite effect of JNK-dependent phosphorylation of Bad at Ser-128 has been recently described in neurons [44]. In these cells, JNK-dependent phosphorylation of Bad inhibits the interaction of phosphoBad-(Ser-136) with 14-3-3 proteins, thereby promoting the apoptotic effect of Bad [44]. The activation of the JNK pathway plays a critical role in naturally occurring apoptosis during development as well as in pathological death associated with different diseases. Under most circumstances in non-malignant cells, JNK activation results in turn in the activation of pro-apoptotic members of Bcl-2 family and in cytochrome *C* release from mitochondria (reviewed in [45]). However, JNKs have also been implicated in enhancing cell survival and proliferation. In this sense, it has been reported that JNK can suppress apoptosis in IL-3-dependent hematopoietic cells via phosphorylation of Bad [46]. This kinase pathway seems to represent an archetype of contrariety in intracellular signaling [47].

Our results show that EGF-dependent activation of the PI3K/AKT pathway induces an increase in phosphoBad-(Ser-136) levels and a concomitant down-regulation of Bad protein. We also found that EGF decreases Bad levels even in the presence of the JNK inhibitor, supporting the notion that this effect is independent of Ser-128 phosphorylation.

The question arising from these results is: "What is the role of Bad phosphorylation in the protective effect of EGF?" as it does not seem to be sufficient in the prevention of apoptosis. Complete activation of the mitochondrial-tethered portion of glucokinase would require Bad phosphorylation at Ser-136 and Ser-155. One possibility is suggested by this recently reported metabolic function of Bad, that is independent of its pro-apoptotic activity [48].

Together our results describe a complex dynamic pattern by which EGF modulates cell survival (Fig. 5). Two signaling pathways activated by EGF promote the induction of Bcl-X_L while AKT activation also leads to Bad phosphorylation and down-regulation. However, the protective effect of EGF correlates mainly with the up-regulation of the anti-apoptotic protein Bcl-X_L.

Walton et al. [49] demonstrated that the deletion of the *bcl-X* gene from the entire mammary epithelial compartment (ducts and alveoli) did not compromise proliferation or differentiation of mammary ductal and alveolar epithelial cells in virgin mice and during pregnancy and lactation; however, epithelial cell death and tissue remodeling is accelerated in the *bcl-X* conditional knockout mice during the first stage of involution. Moreover, the ratio between the anti-apoptotic *bcl-X$_L$* isoform and the pro-apoptotic *bcl-X$_S$*, significantly decrease in involuting glands of wild-type mice [50], suggesting that Bcl-X is not essential during mammopoiesis but is critical for controlling apoptosis during involution. The fact that EGF circulating levels also decrease in involuting glands reveals a putative critical role for *bcl-X* as mediator in the EGF dependent epithelial cell survival. In this sense, siRNA assays performed against *bcl-X* support this hypothesis, as the EGF protective effect disappears in *bcl-X* deleted cells.

Thus, Bcl-X_L emerges as a key anti-apoptotic molecule critical for mammary oncogenesis and tumor progression. In fact, it was demonstrated that Bcl-X_L expression correlates with a decrease in cellular sensitivity toward a variety of chemotherapeutic reagents providing high cell resistance against them. According with these and

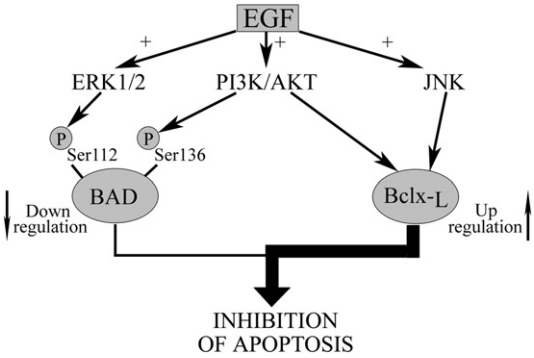

Fig. 5. Complex dynamic signaling mediates EGF-dependent cell survival. PI3K/AKT and JNK signaling pathways activated by EGF lead to up-regulation of Bcl-X_L. PI3K/AKT and ERK1/2 activation also leads to Bad phosphorylation on Serine136 and Serine112 respectively and Bad protein down-regulation. The cell protective effect of EGF correlates mainly with the up-regulation of the anti-apoptotic protein Bcl-X_L as noted by the thick lines.

our previous results [51], Bcl-X_L plays a key role as mediator in cell survival induced by such different stimuli as growth factors and glucocorticoids, both strong inhibitors of apoptosis in mammary epithelium. In this sense, the conditional deletion of the *bcl-X* gene from mouse mammary epithelium results in accelerated apoptosis during the first stage of involution. This phenotype is no further modified by the deletion of Bax protein [49]. In view of the new goals of clinical research, detailed studies of the mechanisms, signaling pathways and specific target genes involved in the regulation of cell survival in mammary epithelial cells will provide information that may be clinically relevant and influence therapeutic decisions for the development and use of new drugs.

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