Calmodulin-mediated Activation of Akt Regulates Survival of c-Myc-overexpressing Mouse Mammary Carcinoma Cells*

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c-Myc-overexpressing mammary epithelial cells are proapoptotic; their survival is strongly promoted by epidermal growth factor (EGF). We now demonstrate that EGF-induced Akt activation and survival in transgenic mouse mammary tumor virus-c-Myc mouse mammary carcinoma cells are both calcium/calmodulin-dependent. Akt activation is abolished by the phospholipase C-γ inhibitor U-73122, by the intracellular calcium chelator BAPTA-AM, and by the specific calmodulin antagonist W-7. These results implicate calcium/calmodulin in the activation of Akt in these cells. In addition, Akt activation by serum and insulin is also inhibited by W-7. EGF-induced and calcium/calmodulin-mediated Akt activation occurs in both tumorigenic and non-tumorigenic mouse and human mammary epithelial cells, independent of their overexpression of c-Myc. These results imply that calcium/calmodulin may be a common regulator of Akt activation, irrespective of upstream receptor activator, mammalian species, and transformation status in mammary epithelial cells. However, only c-Myc-overexpressing mouse mammary carcinoma cells (but not normal mouse mammary epithelial cells) undergo apoptosis in the presence of the calmodulin antagonist W-7, indicating the vital selective role of calmodulin for survival of these cells. Calcium/calmodulin-regulated Akt activation is mediated directly by neither calmodulin kinases nor phosphatidylinositol 3-kinase (PI-3 kinase). Pharmacological inhibitors of calmodulin kinase kinase and calmodulin kinases II and III do not inhibit EGF-induced Akt activation, and calmodulin antagonist W-7 does not inhibit phosphotyrosine-associated PI-3 kinase activation. Akt is, however, co-immunoprecipitated with calmodulin in an EGF-dependent manner, which is inhibited by calmodulin antagonist W-7. We conclude that calmodulin may serve a vital regulatory function to direct the localization of Akt to the plasma membrane for its activation by PI-3 kinase.

One of the fundamental etiologic processes in tumorigenesis is the ability of cancer cells to evade programmed cell death, or apoptosis (1). Potent cell survival signaling, in parallel with uncontrolled cell proliferation and other processes, ultimately leads to the development of a malignant tumor. In breast cancer, this pathologic outcome is strongly influenced by growth factors and/or hormones, which interact with their cognate receptors on mammary epithelial cells. Receptor-ligand interactions at the cell surface are propagated as cascades of signals through the cytoplasm, culminating in specific gene expression programs in the nucleus to define specific biological outcomes. The serine/threonine kinase Akt is considered a central player controlling cellular survival (2), apoptosis (3), and oncogenesis (4, 5). Akt is activated by growth factors and other stimuli through both phosphatidylinositol 3-kinase (PI-3 kinase)1-dependent and independent mechanisms (6–9). PI-3 kinase, a ubiquitous lipid kinase and upstream effector of Akt (10), has also been implicated in a variety of cellular functions, including survival and antiapoptosis (11, 12), growth and proliferation (13, 14), differentiation (15, 16), cytoskeletal rearrangement (17), translocation of glucose transporter GLUT4 (18, 19) and membrane ruffling (20). Upon growth factor stimulation, PI-3 kinase generates 3'-phosphorylated phosphoinositides, such as phosphatidylinositol 3,4-biphosphates and phosphatidyl inositol 3,4,5-triphosphates, at the plasma membrane. These phosphoinositides serve as binding anchors for the Pleckstrin homology domain of Akt and thus encourage translocation of Akt to the plasma membrane (10, 21–23). At the plasma membrane, Akt is phosphorylated at Ser-473 and Thr-308 and fully activated (24). In addition to a PI-3 kinase-dependent mechanism of Akt activation, a PI-3 kinase-independent mechanism(s) of Akt activation has also been reported. The prime candidate mediating this mechanism is calmodulin kinase kinase, which directly phosphorylates Akt in a calcium-dependent manner (6). Calmodulin, the allosteric regulator of calmodulin kinases, also regulates PI-3 kinase-dependent Akt activation, independent of the calmodulin kinase kinase and is known to control neuronal cell survival (25, 26) and GLUT4 translocation in 3T3-L1 adipocytes (27). Calmodulin binds to the β85α regulatory subunit of PI-3 kinase (28), but this binding does not result in the generation of phosphatidylinositol 3,4,5-phosphates, which are required for membrane targeting of Akt and for its subsequent activation (10). A calcium/calmodulin-dependent PI-3 kinase (hVPS34) cascade, responsible for phagosome maturation, has recently been reported (29). A consensus sequence in the p110 catalytic subunit of PI-3 kinase has been predicted to be the binding site of calmodulin, but no biochemical data currently exist to support this idea (30). Be-

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The abbreviations used are: PI-3 kinase, phosphatidylinositol 3-kinase; MMTV, mouse mammary tumor virus; FBS, fetal bovine serum; EGF, epidermal growth factor; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester; MAP, mitogen-activated protein; pAb, polyclonal antibody; PARP, poly(ADP-ribose) polymerase; PI, phosphatidylinositol; PEC, protein kinase C; EF-2, elongation factor 2; PLC, phospholipase C; EGFR, epidermal growth factor receptor.
cause mammary epithelial cells can release abundant calcium from intracellular stores in response to growth factors and survival ligands (31, 32), we hypothesized that calcium plays a major role in survival of these cells. Our laboratory has already reported that EGF-induced survival of c-Myc-overexpressing mammary carcinoma cells is mediated by activation of PI-3 kinase/Akt kinase (35). In search of a specific survival mechanism downstream of EGFR, which may be a therapeutic target in breast carcinoma, and to uncover any existing relationship between PI-3 kinase and calcium, we evaluated activation of Akt, we undertook further investigation of calcium- and PKC-dependent survival mechanism(s). Our new studies resulted in the identification of a calcium/calmodulin-dependent Akt activation and survival mechanism in these cells. In particular, EGF-induced Akt activation is mediated by calmodulin, the universal calcium sensor, resulting in cell survival. We have shown that calmodulin does not exert its effect directly at the PI-3 kinase level. We have further shown that an EGF-dependent complex forms between calmodulin and Akt. This mechanism probably transports Akt to the plasma membrane for its activation by a PI-3 kinase-dependent mechanism. Perturbation of this targeting mechanism by calmodulin antagonism leads to apoptotic cell death in tumorigenic mammary carcinoma cells. This novel mechanism may have broader implications in the regulation of breast cancer, GLUT4 translocation, and neuronal survival.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—Mammary tumor-bearing MMTV-c-Myc transgenic and MMTV-c-Myc/MIT-TGFα transgenic mice have been described previously (34–36). Mammary tumor-derived carcinoma cells from these mice were cultured in improved modified Eagle’s medium containing 2.5% fetal bovine serum (FBS), 10 ng/ml EGF, and 5 μg/ml insulin. The MMTV-c-Myc transgenic tumor cell lines Myc83, Myc9, and Myc7 were examined, and representative experiments were performed in Myc83 cell lines. Likewise, Myc75 was chosen as a representative cell line of MMTV-c-Myc/MIT-TGFα transgenic tumors. The Comma D cell line was previously derived from normal mammary epithelium (37–38); cells were cultured in improved modified Eagle’s minimum essential medium containing 5% FBS. Isolation and maintenance of non-tumorigenic immortal human mammary epithelial cell lines in 184A1N4 and 184A1N4-Myc were described previously (39, 40). A description of retroviral transfection, selection, and development of immortalized MCF 10A-LXSN and MCF 10A-c-Myc stable human mammary epithelial cell lines was published previously (41).

**Antibodies and Reagents**—Pharmacological inhibitors staurosporine, BAPTA-AM, GFF109203X, W-12, W-7, KN-62, KN-92, AG1487, and Rottlerin were purchased from Calbiochem. STO-609 was from Toecis Cookson Inc. Phospho-Akt (Ser-473), Phospho-Akt (Thr-308), phospho-p44/42 MAP kinase (Thr-202/Tyr-204) and phospho-EP2 (Thr-56) and Akt pAb were obtained from Cell Signaling Technology, Inc. Akt(1–20) goat polyclonal Ab, PARP pAb (H-250), and CaM I (FL-149) pAb were purchased from Santa Cruz Biotechnology, Inc. U-73122, goat polyclonal Ab, phospho-p38α/β, anti-phospho-Akt monoclonal antibody, clone 4G10, and anti-phospho-Akt monoclonal antibody (clone 4G10) were purchased fromUpstate Biotechnology. Hoechst stain was from Sigma. Phosphatidylinositol was purchased from Avanti Polar Lipids.

**Treatment with Pharmacological Inhibitors and Preparation of Whole Cell Lysates**—Seminfluent cell monolayers were serum-starved overnight and then incubated with inhibitors for indicated periods of time. Cells were stimulated with 10 nM EGF for 3 min or for the indicated time periods (as shown in the text and figure legends) (in time-course experiments) at 37 °C, and lysates were prepared in lysis buffer containing 20 mM Tris-base, pH 7.4, 1 μg Triton X-100, 5 μl EdTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 μM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml each of pepstatin, leupeptin, and aprotinin. Lysates were vortexed and centrifuged at 15,000 × g for 15 min at 4 °C. Protein concentrations of lysates were measured using BCA protein assay kit (Pierce) and UltraMark Microplate Imaging System (Bio-Rad).

**Immunoprecipitation and Western Blotting**—Immunoprecipitations and Western blotting were performed as described previously (42). In brief, 1 μg of antibody was added to 500 μg of clarified whole cell lysates and incubated for 1 h at 4 °C. 5 μl of protein A-agarose beads were added, and lysates were further incubated for 1 h. Beads were precipitated by centrifugation at 15,000 × g for 2 min and washed 3 times in lysis buffer. Bound proteins were released by boiling in SDS-PAGE sample buffer for 3 min. Proteins were resolved on SDS-PAGE and transferred to polyvinylidene difluoride (Immobilon-P, Millipore) membranes. Membranes were incubated in primary antibody for 2 h, followed by biotinylated secondary antibody for 1 h, and detected byVectastain ABC Elite kit (Vector Laboratories) and enhanced chemiluminescence (PerkinElmer Life and Analytical Sciences).

**Hoechst Staining and PARP Cleavage Assays**—Seminfluent growing cells were transferred to complete medium containing either 30 μM Wt-12 or Wt-7. After 36 h, all floating and adherent cells were collected. Samples were centrifuged for 8 min at 1000 × g for 4 °C. Supernatants were discarded, and cell pellets were suspended in 1X phosphate-buffered saline solution containing 0.3% formaldehyde and 2% Nonidet P-40 and stained with 10 μg/ml of Hoechst 33258 dye (Sigma) for apoptotic analysis. For each replicate, at least 500 cells were counted and evaluated for the presence of condensed nuclei and overall apoptotic appearance. For PARP cleavage assays, a third plate was treated with 50 μM of 5′-benzoyloxycarbonyl-Val-Asp (OMe)-fluoromethyl ketone, a broad-spectrum caspase inhibitor. After 16 h, adherent cells were trypsinized and lysed, and equal amounts of total proteins were resolved in SDS-PAGE and immunoblotted by anti-PARP antibody.

**PI-3 Kinase Assay**—PI-3 kinase assays were performed, using a modified protocol from Kapeller et al. (44). In brief, anti-phosphotyrosine and anti-phosphoAkt immunoprecipitates were washed twice in lysis buffer (phosphate-buffered saline, pH 7, 1% Nonidet P-40, and 100 μM vanadate), twice in a second buffer (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, 100 μM LiCl, and 100 μM vanadate), and finally twice in a third buffer (10 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 100 μM vanadate). Lipid kinase assays were performed on washed beads at 37 °C for 10 min in a reaction mixture containing 20 mM HEPES, pH 7, 40 μM ATP, 10 mM MgCl2, 100 μM vanadate, 0.2 μg/μl sonicated phosphatidylinositol (PI), and 20 μM of [γ32P]ATP (6000 Ci/mmol). At the end of the reaction, 80 μl of HCl (1 M) were added, followed by 160 μl of methanol/chloroform (1:1), and the organic layer was extracted and spotted on oxalate/EDTA-activated thin layer chromatography plates. Thin layer chromatography plates were resolved using a running buffer containing 60:40:11.3:2 chloroform/methanol/H2O/ammonium hydroxide.

**RESULTS**

**EGF-induced Akt Activation Is Inhibited by Staurosporine But Not by GF109203X**—Our previous observations implicated EGF-induced PI-3 kinase/Akt activation as a survival mechanism of MMTV-c-Myc mouse mammary carcinoma cells (henceforth called Myc83 cells) (33). We next wanted to investigate the possible upstream signaling mechanisms regulating this Akt kinase activation and cell survival. In a previous study with small cell lung cancer cells, c-Myc sensitized these cells to apoptosis during nutrient depletion (45). Under these conditions, PKC-β2 overexpression improved cell survival by protecting against c-Myc-induced apoptosis. PKCδ is also known to promote survival of small cell lung cancer cells (46). By analogy, we hypothesized that EGF-induced PKC activation, upstream of PI-3 kinase, might be responsible for Akt activation. To begin to test this idea, serum-starved Myc83 cells were incubated with staurosporine, a broad spectrum PKC inhibitor, as well as GF109203X, a specific inhibitor of PKC (all isomers) (47–49), for 30 min, followed by stimulation with 10 nM EGF for 3 min. Cells were lysed, and equal amounts of total protein-containing lysates were probed by activation specific anti-phospho Akt (Ser-473) antibody. Although 1 μM staurosporine completely inhibited EGF-induced Akt activation, 5 μM GF109203X had no effect (Fig. 1A, WB: P-Akt S-473). 5 μM GF109203X also failed to inhibit EGF-induced Akt activation in serum-starved, bitransgenic MMTV-c-Myc/MIT-TGFα mouse

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mammary carcinoma cells (henceforth called Myc75 cells) and non-malignant mouse Comma D cells (37–38) (data not shown). Staurosporine also caused significant inhibition of MAP kinase. However, GF109203X had no effect on MAP kinase activity (Fig. 1A, WB: P-MAPK). 5 μM GF109203X inhibited PMA-induced MAP kinase activation in all of these cell lines, indicating that it is active in these cells at this concentration (data not shown). To examine whether GF109203X inhibits Akt activation at later time points, the effect of both staurosporine and GF109203X on Akt and MAP kinase activation were examined in a time course experiment. Staurosporine inhibited Akt activation in a sustained manner, whereas GF109203X did not inhibit Akt activity at any time point tested (Fig. 1, B and C, WB: P-Akt). On the other hand, MAP kinase activity was significantly inhibited by staurosporine at 3 min, but substantial activity was regained at later time points (Fig. 1B, WB: P-MAPK).

Similar to results in the short term experiment, neither EGF-induced Akt activation nor MAP kinase activity was affected by GF109203X in the time course experiment (Fig. 1C, WB: P-Akt and WB: P-MAPK). Taken together, these data show that EGF-induced Akt activation is mediated by a staurosporine inhibitable-factor but not by PKC.

**Calcium Chelator BAPTA-AM and Calmodulin Antagonist W-7 Inhibit EGF-induced Akt Activation**—Staurosporine has been reported to inhibit a variety of kinases, including calcium/calmodulin kinase II (50) and PKC (51). Considering that mammary epithelial cells release abundant calcium from intracellular stores in response to EGF (31, 32), we hypothesized that a calcium-regulated signaling mechanism downstream of EGFR might be a potential effector of Akt activation. To test this idea, serum-starved cells were preincubated for 90 min with 10 μM BAPTA-AM, an intracellular calcium chelator (52–54), and stimulated with EGF. BAPTA-AM completely inhibited Akt activity and partially inhibited EGF-induced MAP kinase activity (Fig. 2A, WB: P-Akt S-473 and WB: P-MAPK), whereas incubation of the cells with 2 mM EGTA (external calcium chelator) affected activation of neither Akt nor MAP kinase (Fig. 2A, WB: P-Akt S-473 and WB: P-MAPK). Identical results were obtained in both Myc75 and Comma D cells (data not shown). These results suggest that EGF-induced release of calcium from intracellular stores is required for Akt activation in Myc75, Myc75, and Comma D cells.

To further examine whether calcium mediates its effect on Akt activation through the universal calcium sensor calmodulin, we used a selective calmodulin antagonist, W-7 (27, 55). Calmodulin antagonists have been used previously for inhibiting nerve growth factor- and brain-derived neurotrophic factor-induced Akt activation, resulting in neuronal cell survival (25, 26). As shown in Fig. 2B, 30-min pretreatment of 30 μM W-7, but not its inactive analogue W-12 (56), significantly inhibited EGF-stimulated Akt activation in Myc75 cells (WB: P-Akt S-473). Similar to W-7-treated Myc75 cells, Myc75 and Comma D cells also showed significant inhibition of their EGF-induced Akt activity (Fig. 3, B and C, WB: P-Akt S-473). The specificity of W-7 was confirmed by its ability to block EGF-induced dephosphorylation of elongation factor 2 (EF-2) (Fig. 3, A–C, WB: P-EF-2). EF-2 remains highly phosphorylated (Thr-56) in quiescent cells, thus inhibiting peptide chain elongation (57) and protein synthesis. Growth factors, such as insulin, cause dephosphorylation of EF-2 via calcium/calmodulin-dependent activation of calmodulin kinase III (formerly known as EF-2 kinase), resulting in peptide chain elongation. The calmodulin antagonist W-7 and the EF-2 kinase inhibitor Rotterlin both effectively inhibit this dephosphorylation. Calmodulin has been implicated in survival of neuronal cells (25, 26) and chicken lymphoma B cells (58), but we are unaware of any report indicating a role for calmodulin in mediating Akt activation linked to mammary epithelial cell survival. Although
calmodulin was shown to function upstream of Akt kinase leading to neuronal cell survival, the intermediate signaling mechanism is not completely understood.

We next examined the ability of calcium/calmodulin to activate Akt in a series of MMTV-c-Myc and MMTV-c-Myc/MT-TGFα transgenic mouse mammary tumor-derived cell lines, in addition to Myc83 and Myc75, respectively. W-7 but not W-12 (inactive analogue) inhibited activation of Akt, thus excluding any effect of clonal variation in this mechanism (data not shown). However, W-7 incubation did not inhibit EGF-induced MAP kinase activity. It is interesting that the basal level of activated MAP kinase, as detected by anti-phospho-MAP kinase immunoblotting, increased in W-7-treated Myc83 cells (Fig. 2B, WB: P-MAPK). This is in agreement with previous observations of down-regulation of Ras/Raf/ERK pathway by calmodulin and of activation of MAP kinase activity by calmodulin antagonists, observed in NIH 3T3 fibroblasts (43, 59).

To examine whether the effect of W-7 on Akt or MAP kinase activation is sustained, we preincubated Myc83 cells with 30 μM W-7 and then stimulated them with EGF for different time periods. Anti-phospho-Akt (S-473) immunoblotting of the lysates revealed sustained inhibition of Akt activity by W-7, compared with inactive analogue, W-12 (Fig. 2C, WB: P-Akt). In contrast, W-7 incubation resulted in a sustained MAP kinase activation (Fig. 2C, WB: P-MAPK). Sustained MAP kinase activation is linked to both proliferation and differentiation, depending upon the cell line. In PC-12 cells, nerve growth factor-mediated, sustained MAP kinase activation results in differentiation (60). In contrast, sustained calmodulin inhibition, in serum-starved fibroblasts cells, induces extracellular signal-regulated kinase 2 phosphorylation and p21cip1expres-
sion, leading to inhibition of cellular proliferation (59). Taken together, our data suggest that EGF-induced activation of Akt is mediated by calcium/calmodulin-dependent mechanism(s) and that calmodulin has opposing effects on sustained Akt and MAP kinase activities.

**Fig. 2.** Effect of intracellular calcium chelator, BAPTA-AM, and calmodulin antagonist W-7 on Akt activation in Myc83 cells. A, intracellular calcium chelator BAPTA-AM inhibits EGF-induced Akt activation. Myc83 cells were serum-starved overnight and incubated with MeSO (DMSO; lanes 1 and 2), 10 μM BAPTA-AM for 90 min (lanes 3 and 4), and 2 μM EGTA (lanes 5 and 6) for 90 min followed by 3 min of EGF stimulation. Lysates were probed for activated Akt (WB: P-Akt (S-473)) and activated MAP kinase (WB: P-MAPK). Blots were reprobed for total Akt (WB: Akt) and total MAP kinase (WB: MAPK), respectively. B, calmodulin antagonist W-7 inhibits Akt activation but up-regulates MAP kinase activation. Serum-starved cells were incubated with 30 μM W-7 (lanes 9 and 10) or inactive analogue W-12 (lanes 7 and 8) for 30 min. Cells were stimulated with 10 nM EGF for 3 min and processed for Akt and MAP kinase activation as described in Fig. 1A, C, sustained Akt inhibition and MAP kinase activation by calmodulin antagonist. Serum-starved Myc83 cells were preincubated with 30 μM W-7 (lanes 11–17) or inactive analogue W-12 (lanes 18–24) for 30 min, and stimulated with 10 nM EGF for indicated time period. Akt and MAP kinase activation were examined as described in Fig. 1B. Blots were reprobed by anti-tubulin antibody (WB: Tubulin) for consistent gel loading.

**Fig. 3.** Inhibition of Akt activation by calmodulin antagonist occurs in both tumorigenic carcinoma and non-tumorigenic mouse mammary epithelial cells. Tumorigenic cell lines Myc83 (lanes 1–4), Myca75 (lanes 5–8), and non-tumorigenic cell lines Comma D (lanes 9–12) were pre-incubated with either W-7 or W-12 for 30 min, stimulated with 10 nM EGF for 3 min, and processed for Akt activity exactly as described in Fig. 2B. An identical blot from each cell line was probed by anti-P-EF-2 (Thr-56) antibody (WB: P-EF-2).
Calmodulin Is a Common Central Regulator of Akt Activation, Irrespective of Ligands, Species, Tumorigenicity, and c-Myc Expression Status—To investigate whether calmodulin-mediated activation of Akt occurs in other mammary epithelial cell systems, we tested the c-Myc-overexpressing, non-tumorigenic human mammary epithelial cell lines 184A1N-Myc (39, 40) and MCF10A-c-Myc (41), along with their control counterparts 184A1N4 and MCF-10A-LXSN. In all the cell lines tested, W-7 inhibited EGF-stimulated Akt activation (Fig. 4, A and B, WB: P-Akt S-473). W-7 also specifically inhibited insulin- or FBS-induced Akt activation in Mbc83 cells (Fig. 4C, WB: P-Akt S-473). These observations suggest that calcium/calmodulin is a common regulator of Akt activation, irrespective of c-Myc expression status, species, tumorigenicity, and survival ligands in a variety of mammmary epithelial cell models.

Calmodulin-mediated Cell Survival Does Not Depend on Calmodulin Kinase Activation and Calmodulin Kinase II or III—To determine whether calmodulin mediates Akt activation via calmodulin kinase(s), we tested specific inhibitors of calmodulin kinases. Serum-starved Myc83 cells were incubated with 100 ng/ml STO-609 for 6 h, 10 μM KN-62 for 2 h, or 10 μM Rottlerin for 2 h, followed by stimulation with 10 nM EGF for 3 min. STO-609 is a potent inhibitor of calmodulin kinase kinase (61, 62), which is an upstream activator of calmodulin kinase I and calmodulin kinase IV (6, 63). KN-62 (inactive analogue KN-92) inhibits calmodulin kinase II (64, 65), and Rottlerin inhibits calmodulin kinase III (also known as EF-2 kinase). None of these compounds inhibited EGF-induced activation of Akt in Myc83 cells (Fig. 5, A–C, WB: P-Akt S-473).

Myce75 (data not shown), and Comma D cells (data not shown). Although calmodulin kinase III acts downstream of Akt kinase, and PDK-1-null cells have no calmodulin kinase III activity (67), we used Rottlerin to rule out the possibility of any feedback activation of Akt by calmodulin kinase III. These data suggest that neither calmodulin kinase, calmodulin kinase II, nor calmodulin kinase III transduces EGF-induced, EGFR-originated, and calmodulin-mediated signals to Akt.

Calmodulin Antagonist Does Not Inhibit EGF-induced PI-3 Kinase Activation—Our laboratory previously demonstrated that EGF-dependent survival signaling in Myc83 cells is PI-3 kinase-Akt dependent, because preincubation of cells with PI-3 kinase inhibitor LY294002, inhibited Akt activation, leading to apoptosis (33). Overexpression of constitutively active myr-Akt protected Myc83 cells from LY294002-induced apoptosis (33). Joyal et al. (28) have demonstrated that calmodulin binds to the p85α subunit of PI-3 kinase in a calcium-dependent manner. However, this interaction did not produce any phosphatidylinositol 3,4,5-trisphosphate and thus cannot recruit Pleckstrin homology domain-containing proteins, such as PDK1 or Akt, to the plasma membrane (10, 68). A calcium/calmodulin-mediated mechanism of PI-3 kinase (hVPS34) activation was reported recently that describes the mechanism that Mycobacterium tuberculosis employs to block phagosome maturation and to evade bactericidal agents (29). To investigate whether calcium/calmodulin-mediated Akt activation is linked to classic EGF-induced PI-3 kinase-Akt activation, mediated by phosphotyrosine-phosphatase III, we employed the specific EGFR tyrosine kinase inhibitor AG1478 (69), the calmodulin antagonist W-7, and the phospholipase C-γ inhibitor U-73122 in Myc83 cells. We then examined whether any of these pharmacological inhibitors affected the ability of p85α regulatory subunit to be co-immunoprecipitated with an anti-phosphotyrosine antibody.
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Serum-starved Myc83 cells were treated with 1 μM AG1478, 1 μM U-73122, or 30 μM W-7 for 30 min and then stimulated by EGF. Phosphotyrosine-containing proteins from the lysates were captured with an anti-phosphotyrosine antibody, followed by immunoblotting with anti-phosphotyrosine pAb. As shown in Fig. 6A, p85α was co-immunoprecipitated, in an EGF-dependent manner, by anti-phosphotyrosine antibody, but not when cells were pre-incubated with AG1478 (WB: p85α). Corresponding lysates from this experiment revealed that EGF-dependent Akt activation was strongly inhibited by AG1478 (Fig. 6A, WB: P-Akt). Myc83 cells express EGFR and ErbB2 (henceforth called ErbB) and do not express significant ErbB3 and ErbB4. These data confirm that calmodulin does not affect targeting of either functional PI-3 kinase or EGF-induced phosphotyrosine-associated PI-3 kinase activities in Myc83 cells.

Calmodulin Forms a Complex with Akt in an EGF-dependent Manner—To investigate whether calmodulin forms complexes with Akt in an EGF-dependent manner, calmodulin was immunoprecipitated from serum-starved and EGF-stimulated Myc83 cells, and immunoprecipitates were probed for bound Akt. As shown in Fig. 7, Akt was co-immunoprecipitated with calmodulin in EGF-stimulated Myc83 cells, and this binding was abolished by pretreatment with W-7 (WB: Akt). Lysates from the same experiment revealed a parallel inhibition of Akt kinase activities (Fig. 7, WB: P-Akt S-473, WB: P-Akt T-308). These data suggest that calmodulin forms an EGF-dependent complex by either direct or indirect binding with Akt.

Calmodulin Antagonist W-7 Induces Apoptosis in c-Myc-overexpressing but Not in Normal Mammary Epithelial Cells—To investigate whether calcium/calmodulin-mediated Akt activation contributes to mammary epithelial cell survival, Myc83, Mycα75, and Coma D cells were subjected to calmodulin antagonism by W-7, and cellular apoptosis was studied by PARP cleavage and Hoechst staining. In Myc83 and Mycα75 cells, W-7 treatment induced significant PARP cleavage after 16 h that could be rescued by N-benzoxycarbonyl-VAD-fluoromethyl ketone, a broad spectrum caspase inhibitor. This result confirmed that an early apoptotic program was initiated in Myc83 and Myc α75 cells in response to W-7 (Fig. 8A). These findings suggest that calmodulin-mediated Akt activation contributes to mammary epithelial cell survival.
incubated with 30 μM W-12 (lanes 1 and 2) or W-7 (lanes 3 and 4) for 30 min and induced with or without 10 nM EGF for 3 min. Lysates were immunoprecipitated with an anti-calamulin monoclonal antibody and then immunoblotted for Akt (WB: Akt). The blot was reprobed with an anti-calmodulin pAb to demonstrate consistent immunoprecipitation (WB: Calmodulin). Corresponding lysates from this experiment were immunoblotted by anti-Phospho Akt (Ser-473) (WB: P-Akt S-473), anti-phospho Akt (Thr-308) (WB: P-Akt Thr-308), and by an anti-tubulin antibody (WB: α-Tubulin).

**DISCUSSION**

In this communication, we have presented evidence for the existence of a unique mechanism of EGF-induced and calcium/calmodulin-mediated survival in mouse mammary carcinoma cells. EGF-induced activation of Akt was shown to be a prime survival pathway of MMTV-c-Myc transgenic mammary tumor-derived epithelial cells (33). We have shown that EGF-induced and PLC-γ-mediated release of calcium from intracellular stores results in a calcium/calmodulin-dependent activation of Akt and survival of these cells. Calcium/calmodulin-regulated Akt activation in mammary epithelial cells is mediated neither by calmodulin kinases (6) nor directly by a PI-3 kinase-dependent mechanism(s), as described previously for neuronal cells (25). Calmodulin binds to Akt in an EGF-dependent manner, potentially targeting functional Akt to the plasma membrane for its subsequent activation by a PI-3 kinase-dependent mechanism. Calmodulin-mediated Akt activation, therefore, is indirectly linked to a phosphataseroteinase-dependent, PI-3 kinase activation mechanism; perturbation of either mechanism by LY294002 (33) or calmodulin antagonist, W-7, induces apoptosis (in this study) in c-Myc-overexpressing mammary carcinoma cells. We also showed that calmodulin regulation of Akt kinase is common in a variety of mammary epithelial cells, irrespective of survival ligands (EGF, insulin, or FBS), c-Myc expression status, species (human or mouse), and tumorigenicity. Calmodulin antagonist specifically resulted in apoptosis of tumorigenic c-Myc-overexpressing mammary carcinoma cells but did not affect normal mammary gland-derived epithelial cells (i.e. Comma D), implying that calmodulin-mediated Akt activation is an integral part of the survival mechanism in certain tumorigenic cells.

Calmodulin is a universal calcium sensor and performs a myriad of biological functions including cell growth (76), cell cycle progression, proliferation (77, 78), trafficking (79), synaptic plasticity (80), and glucose transporter GLUT4 targeting to the plasma membrane (27, 81). Cellular incorporation of antisense calmodulin RNA and microinjection of calmodulin antibody leads to cell cycle arrest and inhibition of DNA synthesis (82). Recent publications have highlighted calmodulin’s role in modulating cell survival, upstream of Akt kinase, both by PI-3 kinase-dependent or -independent mechanisms. In particular, calmodulin and calmodulin kinase mediate membrane depolarization and, subsequently, cell survival in motor neurons and neuroblastoma cells by a PI-3 kinase-independent mechanism(s) (6, 83). Genetic studies also revealed that calcium/calmodulin, through calmodulin kinase, promotes Saccharomyces cerevisiae survival from pheromone-induced growth arrest (84). On the other hand, brain-derived neurotrophic factor- and neurophin-induced and calmodulin-mediated cell survival is considered to be mediated by PI-3 kinase-dependent Akt activation (25, 26). Although calmodulin was predicted to control generation of PI-3 kinase products in neuronal cells (25), the exact mechanism has not been addressed. Likewise, translocation of the GLUT4 glucose transporter to the plasma membrane in 3T3L1 adipocytes (27, 81) is regulated by W-7 in either of these assays (Fig. 8, A and B). Comma D cells previously showed a marked inhibition of EGF-induced Akt activation by W-7 (Fig. 3C). However, this compound had no effect on apoptosis. The simplest explanation for these opposite results is that additional event(s), independent of calmodulin-dependent Akt activation, are required to support survival mechanism of Comma D cells. A recent study demonstrated that immortalization of Comma D cells is independent of the EGF-PLC-PI-3 kinase-Akt signaling cascade (75). Additional future experiments are required to determine the possible contribution of calmodulin to Comma D cell survival.
Calcium (85), calmodulin, and Akt, although calmodulin's direct role might not be ascertained (27). In neuronal cells, a calmodulin antagonist inhibited Akt activation, and constitutively active Akt (gag-Akt) expressing neuronal cells escaped apoptosis induced by a calmodulin antagonist (25). In an analogous situation in 3T3-L1 adipocytes, a calmodulin antagonist inhibited insulin-induced Akt activation and GLUT4 translocation to the plasma membrane (27). An enhanced green fluorescent protein-Pleckstrin homology fusion protein also failed to translocate to the plasma membrane in the presence of a calmodulin antagonist. However, in both neuronal cells and 3T3-L1 cells, phosphoryosine-associated in vitro PI-3 kinase was not inhibited by a calmodulin antagonist (25, 27). It was suggested that calcium/calmodulin is probably required for proper in vivo targeting of PI-3 kinase to its substrate and calmodulin antagonist inhibits this process. As a result, PI-3 kinase products, such as phosphatidylinositol 3,4,5-triphosphates, are not produced, and Akt is not activated.

Our investigations are very similar to both of these observations, and we also observe EGF-induced Akt inactivation and apoptosis of Myc83 and Myco75 cells in the presence of W-7. Similar to neuronal and 3T3-L1 cells, phosphoryonosine- and p85a-associated PI-3 kinase activities were not inhibited by a calmodulin antagonist in vitro. In contrast, in our experiments, calmodulin antagonism could not inhibit ligand-induced association between tyrosine phosphorylated ErbB and p85, implying that targeting of PI-3 kinase to the plasma membrane is not affected. Because membrane targeting of p85 (α/β) alone is not always sufficient for full PI-3 kinase/Akt activity (86), it is possible that the effect of calmodulin on Akt activation is at or distal to PI-3 kinase in vivo. Our in vitro lipid kinase data demonstrate that the calmodulin antagonist W-7 has no effect at the PI-3 kinase level. However, we observe a calmodulin-Akt association in vitro in an EGF-dependent manner that can be disrupted by W-7. This suggests that calmodulin probably performs a trafficking function for Akt by increasing Akt availability to PI-3 kinase products at the plasma membrane. Specific inhibition of either PI-3 kinase activation by LY294002 (33) or of Akt trafficking by a calmodulin antagonist (this study), thus inhibits Akt activation in these cells. A recent study demonstrated that glial cell line-derived neurotrophic factor-induced neuronal survival is mediated by calcium/calmodulin's association with PI-3 kinase, resulting in Akt activation (87). It was shown that calcium-dependent binding of calmodulin to the p85 regulatory subunit induces PI-3 kinase activation, resulting in Akt activation. Previous observations indicate that calmodulin-p85 interaction does not produce phosphatidylinositol 3,4,5-triphosphate (28), a prime ligand for binding to Akt Pleckstrin homology domain for subsequent Akt activation. In view of this, it is not clear how calmodulin-p85 interaction and its associated PI-3 kinase activity resulted in Akt activation. Calmodulin is up-regulated in Myc83 cells (88), and constitutively activated Akt-expressing neuronal cells escape calmodulin antagonist-induced apoptosis (25). It is established that Akt needs to be plasma membrane-targeted for its activation. However, it is still not clear how Akt is transported to the plasma membrane from the cytoplasm. Because calmodulin forms a complex with Akt, it is more likely that calmodulin regulates Akt targeting and its consequent activation downstream of PI-3 kinase (Fig. 9).

EGF and related ligands, such as TGFα, β-cellulin, and amphiregulin, as well as the ErbB family receptors, have enormous influence on normal mammary development. In addition, dysregulation of either ligands or their receptors is frequently observed in breast cancer (89–92). Calmodulin is up-regulated in a variety of transgenic mouse mammary tumor models, including MMTv-c-Myc (88). Our data demonstrate that calmodulin is a major contributory factor in Akt activation and cellular survival in c-Myc-overexpressing mouse mammary carcinoma cells. Furthermore, EGF-induced Akt activation was also strongly inhibited by W-7 in the human breast cancer cell line MCF-7 (data not shown), indicating that this mechanism is prevalent in human cancer cell lines that do not overexpress c-Myc. Previously, inhibition of calmodulin with W-7 and W-13 in the estrogen receptor-negative breast cancer cell line, MDA-MB-231, prevented colony formation in soft agar, suggesting that inhibition of calmodulin inhibits the transformation processes in certain human breast cancer cell lines independent of estrogen receptor status (93). Although we have shown that calmodulin forms a complex with Akt in an EGF-dependent manner, it is not known whether this interaction is direct or mediated by any auxiliary protein. The IQ motif is known to bind calmodulin in both a calcium-dependent and -independent manner (94). Based on hydrophathy, hydrophobic residue, residue charge and mass, α-helical class, and position of particular residue, numerous calmodulin-binding proteins have been described previously (calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html). In that context, Akt might potentially be a calmodulin-binding protein. In contrast, because EGFr-induced Akt activation is also inhibited by staurosporine, it is probable that a staurosporine-sensitive, auxiliary protein kinase, other than PKC, mediates calmodulin's association to Akt. Our future investigations are directed toward investigating the interaction of Akt with calmodulin and associated survival signaling. These findings could have biologic relevance for defining the phenotype(s) of c-Myc-overexpressing breast cancer.

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FIG. 9. A model of calcium/calmodulin-dependent Akt activation and survival in Myc83 cells. A probable model of calcium/calmodulin-induced Akt activation, and cell survival is presented. EGF-induced activation of ErbB tyrosine kinase produces phosphotyrosines, which can serve as binding ligands for Src homology 2-domain-containing proteins, such as the p85 regulatory subunit of PI-3 kinase and PLC-γ. The p85-p110 heterodimer is thus targeted to the plasma membrane, where PI-3 kinase is activated. Activated PLC-γ increases cytosolic calcium from intracellular stores. Calcium-bound activated calmodulin (CaM) associates with Akt and transports Akt to the plasma membrane, where Akt binds to PI-3 kinase products, such as phosphatidylinositol-3,4,5-triphosphate (PIP3), and is subsequently activated by phosphorylation. Inhibition of PI-3 kinase (by LY294002), PLC-γ (by U-73122), chelation of intracellular calcium (by BAPTA-AM), and inactivation of calmodulin (by W-7) all result in Akt inhibition and apoptosis in Myc83 cells.

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