Mouse Mammary Tumor Virus Suppresses Apoptosis of Mammary Epithelial Cells through ITAM-Mediated Signaling

Hyoungh H. Kim,^{a,c} Shannon M. Grande,^{b,c*} John G. Monroe,^{b,c*} and Susan R. Ross^{a,c}

Departments of Microbiology^a and Pathology and Laboratory Medicine^b and Abramson Cancer Center,^c Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA

Many receptors in hematopoietic cells use a common signaling pathway that relies on a highly conserved immunoreceptor tyrosine-based activation motif (ITAM), which signals through Src family tyrosine kinases. ITAM-bearing proteins are also found in many oncogenic viruses, including the mouse mammary tumor virus (MMTV) envelope (Env). We previously showed that MMTV Env expression transformed normal mammary epithelial cells and that Src kinases were important mediators in this transformation. To study how ITAM signaling affects mammary cell transformation, we utilized mammary cell lines expressing two different ITAM-containing proteins, one encoding a MMTV provirus and the other a B cell receptor fusion protein. ITAM-expressing cells were resistant to both serum starvation- and chemotherapeutic drug-induced apoptosis, whereas cells transduced with these molecules bearing ITAM mutations were indistinguishable from untransduced cells in their sensitivity to these treatments. We also found that Src kinase was activated in the MMTV-expressing cells and that MMTV-induced apoptosis resistance was completely restored by the Src inhibitor PP2. *In vivo*, MMTV infection delayed involution-induced apoptosis in the mouse mammary gland. Our results show that MMTV suppresses apoptosis through ITAM-mediated Src tyrosine kinase signaling. These studies could lead to the development of effective treatment of nonhematopoietic cell cancers in which ITAM-mediated signaling plays a role.

Immunoreceptor tyrosine-based activation motifs (ITAMs) are highly conserved sequences found in receptors involved in the activation, proliferation, survival, and differentiation of hematopoietic cells such as B and T lymphocytes, mast cells, platelets, and natural killer cells (17, 23, 37). The tyrosine residues found in the canonical motif DxxYxx(L/I)x6-12Yxx(L/I) are necessary and sufficient for signaling. After phosphorylation by intracellular Src family protein tyrosine kinases, the ITAM-associated tyrosines function as docking sites for SH2-containing signaling proteins such as Syk, which link receptor-initiated signals to downstream cellular responses. Mutation of the two Y residues to F in ITAM-containing proteins preserves protein structure but destroys their ability to signal (37).

A number of oncogenic viruses, some with tropism for nonhematopoietic cells, encode ITAM-containing plasma membrane-associated proteins that play a role in their ability to transform cells. These include Epstein-Barr virus (EBV) LMP2A (18, 35), Kaposi's sarcoma virus K1 (29, 30, 39), bovine leukemia virus gp30 (5, 8, 52), and mouse mammary tumor virus (MMTV) envelope (Env) (27, 41). Although of unknown significance, the spacing between the aspartate and tyrosine residue in the viral motifs usually differs from that found in cellular ITAMs [DYxx(L/I)x6-12Yxx(L/I)]. Most if not all of these viral proteins are believed to signal in a ligand-independent manner through Src pathways.

Src is the most widely studied member of the largest family of nonreceptor protein tyrosine kinases that includes Lyn, Fyn, Lck, Hck, Fgr, Blk, Yrk, and Yes (16). These kinases act on multiple pathways that regulate proliferation, motility and survival. At the molecular level, a variety of mechanisms are involved, including phosphorylation of numerous substrates on tyrosyl residues, as well as activation of the Ras/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, and STAT3-

dependent signaling pathways (34). Src family kinases also play an important role in cell survival pathways (44, 46).

We previously showed that expression of the ITAM-containing MMTV Env protein or an ITAM derived from the antigen receptor (BCR) was sufficient to cause morphological changes in normal mouse and human mammary epithelial cells *in vitro* (20, 27) and *in vivo* (41). Furthermore, we found Src kinases were important mediators of ITAM-induced transformation of mammary epithelial cells (20, 27). To understand how ITAM signaling affects survival of mammary epithelial cells, we utilized here mouse mammary epithelial cells transduced with a molecular MMTV provirus clone, as well as a cloned B-cell receptor ITAM; ITAM-mutated versions of both served as controls. These cells were analyzed for effects on differentiation, cell proliferation, and resistance to apoptosis. In addition, the effect of MMTV infection on mammary regression was also examined. We demonstrate that MMTV activates Src kinase through its ITAM and consequently suppresses apoptosis in mammary epithelial cells.

MATERIALS AND METHODS

Cell lines and reagents. NMuMG normal mouse mammary epithelial cell lines were purchased from the American Type Culture Collection (Rockville, MD). The NMuMG-HP and -HP^{YY} cells were previously described

Received 3 August 2012 Accepted 14 September 2012

Published ahead of print 26 September 2012

Address correspondence to Susan R. Ross, rosss@mail.med.upenn.edu.

* Present address: Shannon M. Grande, Department of Microbiology and Immunology, Harvard Medical School, Boston, Massachusetts, USA, and John G. Monroe, ITGR Diagnostics Discovery, Genentech, Inc., South San Francisco, California, USA.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.02029-12

(41). The construction of MAHB and its ITAM mutant variant and cloning into the MIGRI retroviral vector has been previously described (4). Cells were grown in Dulbecco modified Eagle medium with 5% fetal bovine serum, 10 μ g of insulin/ml, and penicillin (100 U/ml)-streptomycin (100 g/ml). All cell lines were cultured at a constant temperature of 37°C in a 5% CO₂ humidified atmosphere.

Three-dimensional morphogenesis. Cells were plated as single-cell suspensions on growth factor-reduced Matrigel (BD Biosciences, San Jose, CA) using the overlay method (3). Cells were maintained in culture for 2 weeks, and the medium was changed every 3 days. At 10 days after plating, the Matrigel-containing acini were embedded in OCT medium (Triangle Medical Sciences, Durham, NC), and 10- μ m-thick frozen sections were obtained. Samples were stained with hematoxylin and eosin (H&E) to identify the presence of lumen in the acinar structures.

Mice. BALB/c mice were purchased from the Animal Program of the National Cancer Institute. BALB/c mice infected with the MMTV-HP and -HP^{YY} viruses were previously described (41). All mice were housed according to the policies of the Institutional Animal Care and Use Committee of the University of Pennsylvania. Female mice were bred at 2 months of age. At 2 days postparturition, the litter size was adjusted to five to six pups. Mammary apoptosis was forced by weaning at peak lactation (day 15). Inguinal mammary glands were collected at early and late involution stages (day 2 and day 5 after weaning, respectively).

Tissue preparation. For histology and morphometry, mammary tissue was dissected from the number 4 inguinal mammary gland, embedded in OCT compound by freezing in liquid nitrogen-cooled isopentane, and stored at -80°C. Frozen sections were cut at 10 μ m on a Leica cryostat (GTI Microsystems) and fixed in ice-cold acetone for 10 min. Samples were stained with H&E to identify the presence of lumen in the acinar structures and photographed using an inverted microscope equipped with a Kodak digital camera. For each experiment, comparisons were always made between similar regions of the same mammary gland. Photographs of fourth inguinal mammary glands were taken at $\times 200$ magnification. The average lumen area was quantified for 10 to 15 lumens, five fields/gland/mouse ($n = 3$ mice/time point) using ImageJ (National Institutes of Health). For each sample, the results from five fields were averaged. The average apoptotic cells were counted from randomly selected 10 to 15 lumens each field. For each sample, the results from five fields were averaged.

Flow cytometry. Cells (5×10^5) were plated on 10-cm dishes. At 24 h after the initial seeding, the cells were incubated in serum-free medium for 24 h. Apoptosis was detected by using an Annexin V flow kit (BD Biosciences) according to the manufacturer's instructions. Annexin V-labeled cells were analyzed in a flow cytometer (FACSCalibur; BD Biosciences). The data was analyzed by FlowJo (Tree Star, Inc., Ashland, OR).

RNA analysis. Total RNA was isolated from cells and tissue by using an RNeasy minikit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Before the reverse transcription reactions, all RNA samples were treated with 1 U of DNase I (amplification grade; Invitrogen) for 10 min at room temperature in order to eliminate genomic DNA contamination. RNA was used to generate cDNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Reactions without the RT enzyme were used as negative controls. Quantitative real-time reverse transcription-PCR (RT-qPCR) was used for detecting MMTV, β -casein, MMP2, and SGP2 transcripts. All values were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Relative quantification using the comparative threshold cycle method ($\Delta\Delta C_T$) was applied for gene expression level analyses. The results were analyzed with the ABI 7900 HT sequence detection system (v2.3) software using a relative quantification approach ($\Delta\Delta C_T$) according to the manufacturer's instructions. The primers used in the present study were as follows: β -casein forward, 5'-AGAGGATGTGCTCCAGGCTA-3'; β -casein reverse, 5'-AATGACAGGCCCAAGAGATG-3'; SGP-2 forward, 5'-CGAAGATGCTCAACACCTCA-3'; SGP-2 reverse, 5'-TGTGATGGGGTCAGAGTCAA-3'; MMP2 forward, 5'-ACACTGGGACCT

GTCCTCC-3'; MMP2 reverse, 5'-GCGAAGAACACAGCCTTCTC-3'; GAPDH forward, 5'-CCCTTCATTGACCTCAACTACA-3'; and GAPDH reverse, 5'-CGCTCCTGGAGGATGGTGAT-3'.

Western blots. Cells and tissues were dissolved in radioimmunoprecipitation assay buffer with sonication. Western blots were probed with anti-MMTV, anti-gp52 antisera (41), and anti- β -casein (Abcam, Cambridge, MA). All Src antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). The species-appropriate horseradish peroxidase-conjugated secondary antibody was used, followed by detection with ECL reagents (Amersham Biosciences, Inc.). Anti- β -actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as a loading control.

Cell cytotoxicity assays. Cells (10^4) were seeded on 96-well plates and treated with the indicated doses of cisplatin, etoposide, paclitaxel, and vinorelbine (Sigma Chemical Co., St. Louis, MO) for 3 days. For the vinorelbine cytotoxicity study, 1 μ M PP2 (Sigma) was added. At the end of the treatment period, the relative cell number was determined by using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Rockville, MD) according to the manufacturer's instructions. The 50% inhibitory concentrations (IC₅₀s) were calculated using GraphPad Prism (v5.00; GraphPad Software, San Diego, CA).

Statistical analyses. GraphPad Prism for Windows was used in all statistical analyses. The cell cytotoxicity data was analyzed using two-way analysis of variance with Dunnett's post test. All other data were analyzed by using the Student *t* test.

RESULTS

Effect of ITAM expression on mammary epithelial cells. To study how ITAM signaling affects mammary cell transformation and apoptosis, we utilized two polyclonal populations of NMuMG mammary cells that were transduced with two different MMTV proviral molecular clones, one encoding a wild-type Env (Hybrid Provirus [HP]) and the other an Env with a mutated ITAM (HP^{YY}); both proviral clones have been previously described and are fully infectious *in vivo* and *in vitro* (41). MMTV protein expression in the transduced cell populations was confirmed by immunoblotting with anti-MMTV antisera (Fig. 1A). The population of HP-transduced cells had, on average, ~ 2 -fold-higher levels of MMTV RNA (data not shown) and protein (Fig. 1A) than the HP^{YY}-transduced cells. Because both lines produce infectious virus that is capable of reinfection, we were unable to develop stable clonal populations. In monolayer cultures, normal NMuMG mammary epithelial cells exhibit an aggregated growth pattern with cobblestone morphology (Fig. 1B). When plated at the same density, the MMTV-HP-transduced cells grew in a more spread-out pattern, and the cells themselves were more spindle-shaped, suggesting some loss of cell-to-cell adhesion. This change in morphology often represents hallmarks of epithelial-mesenchymal transition. This phenotype was reversed in the HP^{YY} cells. There was no difference in the growth rate of the different cultures (data not shown).

To confirm that these results were due to ITAM-mediated signaling and not to the different levels of MMTV expression, we also used NMuMG cells transduced with a retroviral vector expressing a recombinant protein consisting of the ITAM-containing cytoplasmic region, called MAHB (20). MAHB contains cytoplasmic domains of the cellular proteins Ig α and Ig β , which are normally expressed as a heterodimer associated with the B cell receptor. This protein is targeted to the inner leaflet of the plasma membrane, and a hemagglutinin (HA) tag separates the cytoplasmic domains of Ig α and Ig β . A signaling-deficient variant (MAHB^{YY}) in which only the ITAM tyrosines have been mutated (Y182F and Y193F in Ig α and Y195F and Y206F in Ig β) permitted us to assign

FIG 1 ITAM signaling causes morphological transformation of mammary epithelial cells. (A) Protein expression in MMTV HP-, HP^{YY}-, and BCR MAHB-transduced NMuMG mouse mammary epithelial cells. Western blots of cell extracts were analyzed using anti-MMTV (top panel) and anti-HA (bottom panel) antibodies. The arrow indicates the MMTV Env glycoprotein; the higher-molecular-weight band is the Gag polyprotein. (B) NMuMG cells transduced with MMTV HP- and BCR MAHB were grown in normal culture conditions. Images were captured at 70% confluence ($\times 200$ magnification). (C) Activation of Src kinase pathway in the MMTV HP- and BCR MAHB-transduced NMuMG cells was examined using anti-phospho Src kinase (Tyr416). The blots were also probed with the antibody specific to the inhibitory phosphorylation site (Tyr527) and with total anti-Src antibody. (D) NMuMG, MMTV-HP and -HP^{YY} cells grown in Matrigel. Images were captured at day 6 ($\times 200$ magnification). (E) MMTV HP-transduced NMuMG cells were grown in Matrigel with or without the Src kinase inhibitor PP2. Images were captured ($\times 200$ magnification) and acinar size was measured at day 6 of culture. Each point represents the size of a single colony. Abbreviations: Con, untransfected control cells; Vec, vector-transduced cells.

effects on cellular transformation to a signaling-competent ITAM. MAHB expression was confirmed by Western blotting, using anti-HA tag antibodies (Fig. 1A). The MAHB-transduced cells, but not cells expressing an ITAM-mutated version of the vector, also displayed a spindle-shaped morphology similar to that seen in cells expressing MMTV-HP (Fig. 1B).

In hematopoietic cells, ITAM signaling requires the activity of Src and Syk family kinases. Phosphorylation of ITAM tyrosines generates docking sites for SH2 domain-dependent recruitment and activation of Syk. Syk activation propagates this ITAM-initiated signaling cascade by phosphorylating downstream adaptor proteins and enzymes (38). Expression of the ITAM-containing cytoplasmic region of the MMTV Env or BCR is associated with Syk phosphorylation in NMuMG cells (20, 27). Src activity is regulated by tyrosine phosphorylation at two sites, but with opposing effects. Phosphorylation of Src at Y416 in the activation loop of the kinase domain upregulates enzyme activity, whereas phosphorylation of Y527 in the carboxy-terminal tail renders the enzyme less active (22). We tested whether the Src kinase pathway was activated by MMTV expression. The active form of Src kinase (phospho-Y416) was increased in NMuMG-HP cells but not in control or HP^{YY}-expressing cells (Fig. 1C). However, there were no changes in the inhibitory form of Src kinase (phospho-Y527), as well as total Src kinase. Activation of Src kinase was

also seen in MAHB-transduced but not in MAHB^{YY}-transduced cells (Fig. 1C).

Mammary epithelial cells recapitulate several aspects of mammary organogenesis when grown on an exogenous basement membrane such as laminin-rich Matrigel, including the formation of polarized, acinar-like spheroids with hollow lumens similar to terminal end buds and the basal deposition of basement components (13). Such cultures can also be used to distinguish epithelial cells from normal and tumorigenic tissue and to dissect the mechanisms by which known oncogenes perturb normal mammary gland growth and development. We previously showed that expression of the MMTV Env or the MAHB ITAM in normal mammary epithelial cells caused their morphological transformation when grown in three-dimensional but not two-dimensional cultures (20, 27). We next tested the transduced NMuMG cells in Matrigel cultures to determine whether MMTV infection caused morphological transformation of normal mammary epithelial cells. When grown in three-dimensional cultures, control NMuMG cells developed into spherical acinus-like structures with hollow lumens at day 6 (Fig. 1D). In contrast, the MMTV-HP-infected cells showed aggressive proliferation at day 2 and subsequently developed into larger asymmetric aggregates by day 6, often with cells filling the luminal space, a characteristic of the early neoplastic transformation of breast

FIG 2 MMTV-HP expression suppresses apoptosis. (A) MMTV HP⁻, HP^{YY}⁻, and BCR MAHB-transduced NMuMG cells were plated, and at 50% subconfluency, apoptosis was induced by serum starvation. PP2 was added to the HP- and MAHB-transduced cells at the beginning of serum starvation. (B) At 24 h of starvation, NMuMG-HP and HP^{YY} cells were collected, and apoptosis was measured by FACS analysis of cells stained with anti-Annexin V. (C) Extracts from MMTV HP⁻, HP^{YY}⁻, and BCR MAHB-transduced NMuMG cells analyzed by Western blot analysis at 24 h of starvation for the apoptosis marker cleaved PARP. (D) NMuMG-HP Cells were incubated with increasing concentrations of PP2 during the 24-h serum starvation. Apoptosis was analyzed using anti-cleaved PARP.

epithelium (Fig. 1D). Morphological transformation was not detected in the cells infected mutant form of MMTV-HP^{YY} (Fig. 1D). Because these structures arise from the proliferation of single cells, this suggests that the lower MMTV expression in the HP^{YY}-transduced cultures was not responsible for the morphological or biochemical differences but instead that these were due to the abrogation of signaling.

To determine whether the Src kinase pathway governs MMTV-induced transformation, we cultured cells with Src kinase inhibitor, PP2. Similar to what we previously reported for Env-expressing NMuMG cells, in Matrigel cultures, the aggressive morphological transformation of MMTV-HP-infected cells was completely reversed (not shown). The increased acinar diameters of the structures formed by MMTV-HP cells were also restored to those of the control cells by treatment with PP2 (Fig. 1E). Although PP2 may affect other kinases, these data, together with the known ITAM-mediated activation of the Src pathway and the increase in pSrc-Tyr416 phosphorylation seen in HP-

and MAHB-transduced cells, suggest that Src signaling is critical for the morphological changes in ITAM-expressing mammary cells.

MMTV expression suppresses apoptosis. Because Src family kinases are known to play an important role in cell survival pathways (44), we next tested whether activated Src kinase influenced apoptosis in MMTV-transfected cells. Apoptosis was induced by serum starvation. When subconfluent NMuMG or HP^{YY}-transfected cells were grown in serum-deprived media, large numbers of cells growing as monolayers rounded up and detached from the substratum (Fig. 2A). Flow cytometry data showed that 13.57% of the control cells were positive for the apoptosis marker, Annexin V (Fig. 2B). However, most of the HP-transduced NMuMG cells were still stably attached to the plate, and the apoptotic cell number was similar to the level of cells grown in normal medium. Similarly, MAHB- but not MAHB^{YY}-transduced cells were resistant to serum starvation-induced apoptosis (Fig. 2A).

FIG 3 MMTV-HP confers resistance to cytotoxicity in response to chemotherapeutic drugs. (A) Cells were incubated with the indicated concentrations of four different chemotherapeutic drugs for 3 days. (B) A total of 10^4 cells were seeded on 96-well plates and treated with various doses of vinorelbine with or without PP2 (1 μ M). At the end of the treatment period, the cell number was determined.

Apoptosis was also measured by examining the levels of cleaved poly(ADP-ribose) polymerase (PARP). Cleaved PARP was not detectable under normal growth conditions and increased with serum starvation (Fig. 2C). Its level was significantly lower in serum-starved MMTV-HP and MAHB cells compared to control cells. The apoptosis-resistant phenotype caused by MMTV expression was completely restored in the cells expressing the ITAM mutant MMTV-HP^{YY} and MAHB^{YY} constructs (Fig. 2C).

To determine whether MMTV ITAM-mediated signaling via the Src kinase pathway caused the apoptosis resistance, we also cultured cells in serum-free medium in the presence of the Src kinase inhibitor, PP2. PP2 treatment of MMTV-HP- and MAHB-expressing cells restored their sensitivity to serum starvation and induced apoptosis (Fig. 2A). Treatment with PP2 also induced the appearance of cleaved PARP in a dose-dependent manner in response to serum starvation in the MMTV-HP cells (Fig. 2D). PP2 did not affect apoptosis in the control cells (Fig. 2D).

MMTV-HP transduced cells are resistant to chemotherapeutic drugs. We next tested whether MMTV expression conferred resistance to apoptosis-inducing chemotherapeutic drugs. Four different clinically used anticancer drugs were tested: cisplatin, etoposide, paclitaxel, and vinorelbine. The survival of untransduced cells treated with cisplatin, etoposide, paclitaxel, and vinorelbine was very low. However, a significant number of NMuMG-HP cells survived these lethal concentrations of drugs (Fig. 3A). Indeed, vinorelbine was >5-fold less cytotoxic to NMuMG-HP cells (mean IC₅₀, 0.281 μ M) than control cells (mean IC₅₀, 0.046 μ M) (Fig. 3B).

Because we showed that the Src pathway was involved in ITAM-mediated apoptosis resistance, the effect of Src inhibition in combination with vinorelbine was examined. Although treatment with PP2 did not affect control NMuMG cells, it partially restored the cytotoxic effects of vinorelbine on NMuMG-HP cells (IC₅₀ of 0.075 μ M in the presence of PP2 versus an IC₅₀ of 0.281 μ M in the absence PP2) (Fig. 3B). This demonstrates that MMTV ITAM-mediated signaling through the Src pathway confers resistance to different inducers of apoptosis.

MMTV induces apoptosis-resistance in the involuting mouse mammary gland. We previously showed that virgin trans-

genic mice overexpressing either a full-length MMTV provirus or the MMTV Env alone in their mammary epithelial cells showed increased lobuloalveolar budding and that this altered morphology was not due to increased cell proliferation (41). ITAM-mediated signaling is known to induce antiapoptotic signals. We thus tested whether MMTV expression suppressed apoptosis in the mouse mammary gland, which could explain the altered morphology. We tested whether MMTV infection altered mammary gland morphology during mouse mammary gland involution, a time when widespread apoptosis is occurring. When pups are removed, the lactating mammary gland undergoes involution characterized by intensive apoptosis within 2 days, followed by a period of tissue remodeling concomitant with increased expression of proteases (33). Most of the epithelial cells are replaced by adipocytes (1).

BALB/c mice chronically infected with MMTV-HP and HP^{YY} were previously described; although there is no difference in the level of infection, HP^{YY}-infected mice develop mammary tumors with longer latency and lower incidence (41). Age-matched female mice were bred and mammary involution was forced by weaning at peak lactation (lactation day 15). At the time of weaning, the total mammary gland weight of all animals was the same (Fig. 4J). The lactating mammary glands mainly consisted of differentiated alveoli secreting milk with a branched ductal system surrounded by basement membrane and connective tissues; an extensive vascular and lymphatic network was also found (Fig. 4A, D, and G). This well-organized lobuloalveolar structures characteristic of lactating animals remained intact until 2 days after weaning (Fig. 4B, E, and H). At that time, the alveolus size was slightly increased in the control mammary tissue compared to peak lactation, possibly due to accumulation of milk in the lumen (Fig. 4K). However, compared to the control or HP^{YY}-infected mice, the mammary glands of the MMTV-HP-infected mice exhibited distended alveoli at involution day 2 (Fig. 4K). In addition, a number of apoptotic bodies were found in the lumens of wild-type and HP^{YY}-infected mammary glands at involution day 2 (Fig. 4B and H, box). However, MMTV-HP glands showed little evidence of epithelial apoptosis and shedding (Fig. 4E, box). When quantified, these apoptotic bodies were significantly reduced in the HP-in-

FIG 4 MMTV-HP-infected mammary glands show morphological evidence of delayed involution. (A to I) H&E-stained mammary glands from control (A to C), MMTV HP-infected (D to F), and HP^{YY}-infected (G to I) mice were examined at lactation day 15 (A, D, and G) and involution days 2 (B, E, and H) and 5 (C, F, and I) for morphological evidence of involution. By involution day 2, the alveoli are expanded, and numerous apoptotic cells are shed into lumen (arrows) in the control (B) and HP^{YY}-infected (H) glands. Fewer apoptotic cells were seen in the HP-infected glands (E). (J) The mammary gland weight of the number 4 inguinal mammary gland was measured at the indicated times. The gland weight was significantly increased in the HP-infected mouse at involution day 2. The mammary tissue weight gradually decreased throughout involution. (K) The average lumen size in the number 4 inguinal mammary gland was measured. The average lumen area was significantly increased in HP-infected mice at involution day 2. At involution day 5, the lumen area was greatly reduced, and the alveoli were collapsed and repopulated by adipocytes. (L) Apoptotic cell numbers in each lumen were counted. HP-infected mice showed significantly fewer apoptotic cells (**, $P \leq 0.01$).

infected mice compared to the wild-type or mutant virus-infected mice (Fig. 4L). By involution day 5, the total mammary gland weight and lumen size were greatly reduced in all of the mice (Fig. 4J and K).

Delayed involution in MMTV-infected mammary glands is accompanied by altered gene expression. We next sought to determine whether the expression of specific genes that are induced during mammary gland involution was altered by MMTV infection. Lactating mammary gland differentiation, as measured by the β -casein milk protein gene mRNA levels, decreases from the very high levels during lactation to about one-fourth these levels by day 3 after weaning, a level similar to that at the end of pregnancy; this then decreases to even lower levels over the next 2 days (33). Indeed, we found that β -casein RNA levels declined dramatically with the progression of involution (Fig. 5A). There was no difference in β -casein gene expression among the groups at invo-

lution day 2 or 5, indicating that MMTV infection did not affect mammary gland functional differentiation. At later involution stages, there was a large variation in the β -casein RNA levels that was not dependent on infection status (not shown).

The initial phase of apoptosis in the mammary gland is characterized by the induction of apoptosis-associated genes such as sulfated glycoprotein-2 (SGP-2) (33). SGP-2 expression was increased 2-fold in MMTV-HP mammary gland compared to controls or HP^{YY}-infected animals at involution day 2, indicating that apoptosis was delayed with MMTV infection (Fig. 5A). SGP-2 expression was greatly decreased in all animals in the later stages of involution.

Interactions between the mesenchyme and epithelium are important for normal development, as well as involution of the mammary gland (42). The matrix metalloproteinases (MMPs) stromelysin-1, stromelysin-3, and gelatinase A (MMP2) increase

FIG 5 Delayed involution in MMTV-infected mammary glands is accompanied by altered gene expression. (A) Expression of casein, MMP2, and SGP2 were measured using reverse-transcribed RT-qPCR. Mammary glands from control, MMTV HP, and HP^{YY} animals during involution days 2 and 5 are represented ($n = 3$). (B) MMTV and Src kinase activities were evaluated using anti-pSrc (Tyr416) and anti-MMTV gp52 antibodies. **, $P \leq 0.01$.

during involution (7, 14, 31, 32, 45) and are responsible along with the serine proteinase urokinase-type plasminogen activator (uPA) for the degradation of the ECM and basement membranes. MMP2 RNA levels were increased 3- to 4-fold at involution day 5 (the tissue remodeling phase) compared to early involution (Fig. 5A). Interestingly, MMTV-HP mammary gland showed significantly lower expression of MMP2 compared to the control group or HP^{YY}-infected mice at involution day 2. These data suggest that matrix degradation is delayed in MMTV-infected mammary glands.

Next, we sought to determine whether Src kinase was involved in the apoptosis resistance observed in the MMTV-HP-infected mammary gland. There was no difference in total Src kinase among the different animals at the same involution stage, and the total Src kinase was significantly decreased with the progression of involution (Fig. 5B). Although activated p-Src was not detected in most of the mammary tissue samples, it has shown to be elevated in two HP-infected mammary glands, one at day 2 and the other at day 5 of involution. Interestingly, these two samples also expressed the highest level of MMTV Env protein (Fig. 5B).

DISCUSSION

Many receptors that function in hematopoietic cells contain highly conserved ITAMs which affect cell proliferation, differentiation, and survival through triggering Src kinase signaling pathways. We previously described that expression of both receptor- and virus-encoded ITAMs in nonhematopoietic systems induces transformation of normal mammary epithelial cells and that Src kinases were important mediators in this transformation (20, 27). We further investigated here how ITAM signaling affects mammary cell transformation and survival. NMuMG cells transduced with two different ITAM-containing genes (MMTV provirus and MAHB) consistently showed increased viability in serum-free conditions and ITAM-induced apoptosis resistance was com-

pletely restored by a Src inhibitor, indicating that Src kinase is a key mediator of this effect. A number of studies have demonstrated that Src inhibits apoptosis by suppressing proapoptotic genes (40) or positively regulating antiapoptotic genes (6, 19, 26, 51). Src is a potent activator of the PI3K/Akt pathway, which protects against proapoptotic stimuli via the phosphorylation and inactivation of death accelerators, such as Bad, Bax, and caspase-9 (9, 53, 54). Src-dependent activation of p38-MAPK similarly leads to the phosphorylation of caspases-8 and -3 (2). In addition, Src activation triggers caspase-8 phosphorylation on Tyr380 and impairs Fas-induced apoptosis (12).

In addition to determining the role of ITAM-mediated signaling in resistance to serum-starvation-induced apoptosis, we tested whether MMTV expression conferred resistance to apoptosis-inducing chemotherapeutic drugs. Four different anticancer drugs, all of which are in use clinically, were tested: cisplatin, etoposide, paclitaxel, and vinorelbine. These agents act via different mechanisms. Cisplatin binds DNA causing DNA adducts, thereby preventing DNA replication and elongation, inducing cell cycle arrest, and apoptosis (11, 15). Etoposide forms a ternary complex with DNA and topoisomerase II and causes DNA strand breakage by preventing religation of the DNA strands (21). Paclitaxel and vinorelbine bind tubulin, enhancing tubulin polymerization and inhibiting microtubule disassembly, thus preventing completion of mitosis and producing a G₂-M block (43). NMuMG-HP cells were resistant to all tested drugs regardless of their targets. This suggests that Src may act as a master regulator of apoptosis resistance conferred by ITAMs. Our results also suggest that addition of Src inhibitors could increase the therapeutic efficacy of chemotherapeutic drugs that induce apoptosis. Indeed, it has recently been reported that saracatinib, an orally available specific inhibitor of Src family kinases, when used together with the aromatase inhibitor anastrozole, reduced drug resistance and showed greater

antitumor efficacy than either of the drugs alone in a xenograft model (10).

In vivo, MMTV infection delayed involution-induced apoptosis in the mammary gland. MMTV-infected mammary glands exhibited distended alveoli with significantly reduced epithelial apoptosis and shedding in the lumen compared to the mammary tissues from uninfected and MMTV-HP^{YY}-infected mice. In addition, expression of the apoptosis-associated gene SGP-2/clusterin was increased in MMTV-infected mammary glands compared to uninfected or HP^{YY}-infected animals at involution day 2. SGP-2/clusterin is a multifunctional glycoprotein complex found on the surfaces of cells lining body cavities and in virtually all body fluids, including milk (25, 47). SGP-2/clusterin has diverse functions, including lipid transport, complement inhibition, and regulation of apoptosis (47). During mouse mammary gland development, SGP-2/clusterin is upregulated twice, at the end of pregnancy and at the beginning of the involution, when dramatic phenotypic and functional changes occur in the mammary gland (24). Although SGP-2/clusterin is associated with apoptosis during involution, there is accumulating evidence suggesting that it is an antiapoptotic protein associated with both cancer drug therapy resistance and cellular protection against exogenous stresses (36, 48, 49). Intracellular SGP-2/clusterin inhibits apoptosis by interfering with Bax activation in mitochondria. Intriguingly, in contrast to other inhibitors of Bax, clusterin specifically interacts with conformation-altered Bax in response to chemotherapeutic drugs. This interaction impedes Bax oligomerization, which leads to the release of cytochrome *c* from mitochondria and caspase activation (55).

At involution day 2, MMTV-HP-infected mammary glands showed significantly less expression of MMP2 compared to control or HP^{YY}-infected mice, suggesting that matrix degradation is delayed in MMTV-infected mammary glands. Expression of the matrix metalloproteinases, which are low during lactation, is strongly upregulated at later stages of mammary apoptosis, coinciding with start of the collapse of the lobuloalveolar structures and the intensive tissue remodeling that occurs in involution (33). Indeed, there was no difference in MMP2 expression among the mice at later stages of involution. Although MMP2 mRNA level was 4-fold lower at the early compared to the later stage of involution, MMP2 was expressed in the early involution stage, showing matrix degradation and tissue remodeling is possibly initiated from the early stage of involution before the visible collapse of lobuloalveolar structures. Interestingly, MMP2 expression was clearly reduced in the MMTV-HP-infected mammary gland compared to that of wild-type and MMTV-HP^{YY}-infected mice at the early involution stage. The major sites of mRNA synthesis for these proteinases are fibroblast-like cells in the periductal stroma and stromal cells surrounding the collapsed alveoli, suggesting that the degradative phase of involution is due to a specialized mesenchymal-epithelial interaction (33). Altered MMP2 expression in the MMTV-infected mammary gland suggests that ITAM-mediated signaling in the epithelial cell may ultimately affect communication between infected epithelial cells and the periductal stromal cells during mammary involution in a paracrine manner.

We found that Src activity was increased in the mammary tissues of mice expressing high levels of MMTV. We also found that the total Src protein level was higher in the early stages compared to later stages of involution. Src is an essential signaling modulator in mammary gland development. Src-deficient mice show defects

in ductal development as well as in uterine and ovarian development (28). Src-deficient mammary epithelial cells also revealed defects in the ability of mammary epithelial cells to activate a number of signaling pathways in response to exogenous estrogen stimulation, demonstrating that c-Src plays a role in estrogen receptor α signaling *in vivo* (28). Src^{-/-} mice also exhibit a block in secretory activation that results in lactation failure and precocious involution (50). Further studies are needed on the role of Src kinase, especially virally activated Src pathway, during mammary gland involution.

Our results show that MMTV suppresses apoptosis through ITAM-mediated Src tyrosine kinase signaling. MMTV may have evolved this function to prolong the period when mammary epithelial cells are susceptible to virus infection; if mammary epithelial cells proliferate for longer periods, then virus infection levels and hence transmission to the next generation would be more likely.

Our previous study showed that mice infected with HP^{YY} had decreased and delayed mammary tumorigenesis (41), and the results presented here suggest that apoptosis suppression may thus play a role in the ability of MMTV to transform mammary epithelial cells. The studies in signaling pathways and apoptosis resistance induced by ITAM-containing proteins in nonhematopoietic cells could lead to the development of effective treatment of cancers in which ITAM-mediated signaling plays a role.

ACKNOWLEDGMENTS

This research was supported by PHS grants R01 CA73746 and R01 CA114273.

REFERENCES

- Alexander CM, Selvarajan S, Mudgett J, Werb Z. 2001. Stromelysin-1 regulates adipogenesis during mammary gland involution. *J. Cell Biol.* 152:693–703.
- Alvarado-Kristensson M, et al. 2004. p38-MAPK signals survival by phosphorylation of caspase-8 and caspase-3 in human neutrophils. *J. Exp. Med.* 199:449–458.
- Aranda V, et al. 2006. Par6-aPKC uncouples ErbB2 induced disruption of polarized epithelial organization from proliferation control. *Nat. Cell Biol.* 8:1235–1245.
- Bannish G, Fuentes-Panana EM, Cambier JC, Pear WS, Monroe JG. 2001. Ligand-independent signaling functions for the B lymphocyte antigen receptor and their role in positive selection during B lymphopoiesis. *J. Exp. Med.* 194:1583–1596.
- Beaufils P, Choquet D, Mamoun R, Malissen B. 1993. The (XXL/I)2 motif found in the cytoplasmic segments of the bovine leukemia virus envelope protein and Epstein-Barr virus latent membrane protein 2A can elicit early and late lymphocyte activation events. *EMBO J.* 12:5105–5112.
- Bonni A, et al. 1999. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* 286:1358–1362.
- Busso N, Huarte J, Vassalli JD, Sappino AP, Belin D. 1989. Plasminogen activators in the mouse mammary gland. Decreased expression during lactation. *J. Biol. Chem.* 264:7455–7457.
- Cantor GH. 1996. A potential proline-rich motif upstream of the immunoreceptor tyrosine-based activation motif in bovine leukemia virus gp30, Epstein-Barr virus LMP2A, herpesvirus papio LMP2A, and African horseshoe virus VP7. *Virology* 220:265–266.
- Cardone MH, et al. 1998. Regulation of cell death protease caspase-9 by phosphorylation. *Science* 282:1318–1321.
- Chen Y, et al. 2009. Combined Src and aromatase inhibition impairs human breast cancer growth *in vivo* and bypass pathways are activated in AZD0530-resistant tumors. *Clin. Cancer Res.* 15:3396–3405.
- Chu G. 1994. Cellular responses to cisplatin. The roles of DNA-binding proteins and DNA repair. *J. Biol. Chem.* 269:787–790.
- Cursi S, et al. 2006. Src kinase phosphorylates caspase-8 on Tyr380: a novel mechanism of apoptosis suppression. *EMBO J.* 25:1895–1905.

13. Debnath J, Muthuswamy SK, Brugge JS. 2003. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods* 30:256–268.
14. Dickson SR, Warburton MJ. 1992. Enhanced synthesis of gelatinase and stromelysin by myoepithelial cells during involution of the rat mammary gland. *J. Histochem Cytochem.* 40:697–703.
15. Eastman A. 1990. Activation of programmed cell death by anticancer agents: cisplatin as a model system. *Cancer Cells.* 2:275–280.
16. Finn RS. 2008. Targeting Src in breast cancer. *Ann. Oncol.* 19:1379–1386.
17. Flaswinkel H, Barner M, Reth M. 1995. The tyrosine activation motif as a target of protein tyrosine kinases and SH2 domains. *Semin. Immunol.* 7:21–27.
18. Fruehling S, Longnecker R. 1997. The immunoreceptor tyrosine-based activation motif of Epstein-Barr virus LMP2A is essential for blocking BGC-mediated signal transduction. *Viol.* 235:241–251.
19. Gillet G, Guerin M, Trembleau A, Brun G. 1995. A Bcl-2-related gene is activated in avian cells transformed by the Rous sarcoma virus. *EMBO J.* 14:1372–1381.
20. Grande SM, et al. 2006. Cellular ITAM-containing proteins are oncoproteins in nonhematopoietic cells. *Oncogene* 25:2748–2757.
21. Hande KR. 1998. Etoposide: four decades of development of a topoisomerase II inhibitor. *Eur. J. Cancer* 34:1514–1521.
22. Hunter T. 1987. A tail of two src's: mutatis mutandis. *Cell* 49:1–4.
23. Isakov N. 1998. ITAMs: immunoregulatory scaffolds that link immunoreceptors to their intracellular signaling pathways. *Receptors Channels* 5:243–253.
24. Itahana Y, et al. 2007. Regulation of clusterin expression in mammary epithelial cells. *Exp. Cell Res.* 313:943–951.
25. Jenne DE, Tschopp J. 1992. Clusterin: the intriguing guises of a widely expressed glycoprotein. *Trends Biochem. Sci.* 17:154–159.
26. Johnson D, et al. 2000. Regulation of both apoptosis and cell survival by the v-Src oncoprotein. *Cell Death Differ.* 7:685–696.
27. Katz E, et al. 2005. MMTV Env encodes an ITAM responsible for trans-formation of mammary epithelial cells in three-dimensional culture. *J. Exp. Med.* 201:431–439.
28. Kim H, Laing M, Muller W. 2005. c-Src-null mice exhibit defects in normal mammary gland development and ER α signaling. *Oncogene* 24:5629–5636.
29. Lagunoff M, Majeti R, Weiss A, Ganem D. 1999. Deregulated signal transduction by the K1 gene product of Kaposi's sarcoma-associated herpesvirus. *Proc. Natl. Acad. Sci. U. S. A.* 96:5704–5709.
30. Lee H, et al. 1998. Identification of an immunoreceptor tyrosine-based activation motif of K1 transforming protein of Kaposi's sarcoma-associated herpesvirus. *Mol. Cell. Biol.* 18:5219–5228.
31. Lefebvre O, et al. 1992. The breast cancer-associated stromelysin-3 gene is expressed during mouse mammary gland apoptosis. *J. Cell Biol.* 119:997–1002.
32. Li F, et al. 1994. Expression of stromelysin-1 and TIMP-1 in the involuting mammary gland and in early invasive tumors of the mouse. *Int. J. Cancer* 59:560–568.
33. Lund LR, et al. 1996. Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development* 122:181–193.
34. Martin GS. 2001. The hunting of the Src. *Nat. Rev. Mol. Cell. Biol.* 2:467–475.
35. Merchant M, Caldwell RG, Longnecker R. 2000. The LMP2A ITAM is essential for providing B cells with development and survival signals in vivo. *J. Virol.* 74:9115–9124.
36. Miyake H, Hara I, Kamidono S, Gleave ME, Eto H. 2003. Resistance to cytotoxic chemotherapy-induced apoptosis in human prostate cancer cells is associated with intracellular clusterin expression. *Oncol. Rep.* 10:469–473.
37. Monroe JG. 2006. ITAM-mediated tonic signaling through Pre-BCR and BCR complexes. *Nat. Immunol.* 6:283–294.
38. Niirio H, Clark EA. 2002. Regulation of B-cell fate by antigen-receptor signals. *Nat. Rev. Immunol.* 2:945–956.
39. Prakash O, et al. 2005. Activation of src kinase lyn by Kaposi sarcoma-associated herpesvirus K1 protein: implications for lymphomagenesis. *Blood* 105:3987–3994.
40. Reginato MJ, et al. 2005. Bim regulation of lumen formation in cultured mammary epithelial acini is targeted by oncogenes. *Mol. Cell. Biol.* 25:4591–4601.
41. Ross SR, et al. 2006. An immunoreceptor tyrosine activation motif in the mouse mammary tumor virus envelope protein plays a role in virus-induced mammary tumors. *J. Virol.* 80:9000–9008.
42. Sakakura T. 1991. New aspects of stroma-parenchyma relations in mammary gland differentiation. *Int. Rev. Cytol.* 125:165–202.
43. Schiff PB, Horwitz SB. 1980. Taxol stabilizes microtubules in mouse fibroblast cells. *Proc. Natl. Acad. Sci. U. S. A.* 77:1561–1565.
44. Schlessinger J. 2000. New roles for Src kinases in control of cell survival and angiogenesis. *Cell* 100:293–296.
45. Talhouk RS, Bissell MJ, Werb Z. 1992. Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. *J. Cell Biol.* 118:1271–1282.
46. Thomas SM, Brugge JS. 1997. Cellular functions regulated by Src family kinases. *Annu. Rev. Cell Dev. Biol.* 13:513–609.
47. Trougakos IP, Gonos ES. 2002. Clusterin/apolipoprotein J in human aging and cancer. *Int. J. Biochem. Cell Biol.* 34:1430–1448.
48. Trougakos IP, So A, Jansen B, Gleave ME, Gonos ES. 2004. Silencing expression of the clusterin/apolipoprotein j gene in human cancer cells using small interfering RNA induces spontaneous apoptosis, reduced growth ability, and cell sensitization to genotoxic and oxidative stress. *Cancer Res.* 64:1834–1842.
49. Viard I, et al. 1999. Clusterin gene expression mediates resistance to apoptotic cell death induced by heat shock and oxidative stress. *J. Invest. Dermatol.* 112:290–296.
50. Watkin H, et al. 2008. Lactation failure in Src knockout mice is due to impaired secretory activation. *BMC Dev. Biol.* 8:6. doi:10.1186/1471-213X-8-6.
51. Webb BL, Jimenez E, Martin GS. 2000. v-Src generates a p53-independent apoptotic signal. *Mol. Cell. Biol.* 20:9271–9280.
52. Willems L, et al. 1995. The YXXL signaling motifs of the bovine leukemia virus transmembrane protein are required for in vivo infection and maintenance of high viral loads. *J. Virol.* 69:4137–4141.
53. Yamaguchi H, Wang HG. 2001. The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. *Oncogene* 20:7779–7786.
54. Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. 1996. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* 87:619–628.
55. Zhang H, et al. 2005. Clusterin inhibits apoptosis by interacting with activated Bax. *Nat. Cell Biol.* 7:909–915.