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Pim-1 kinase expression during murine mammary development ☆

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Abstract

Pim-1 kinase phosphorylates substrates whose activities are linked to proliferation, survival, differentiation, and apoptosis. Although *pim-1* is induced by hormones and cytokines, the hormonal control and contribution of Pim-1 to mammary gland development have not been evaluated. We examined Pim-1 expression in mammary cell lines, investigated whether Pim-1 levels could be altered in breast epithelia by mammogenic hormones, and evaluated Pim-1 expression during mammary development. We found that Pim-1 was elevated in most mammary carcinoma cell lines and progesterone increased Pim-1 protein to some extent in non-tumorigenic mammary epithelia. Pim-1 expression in situ was consistent with the documented profile of progesterone activity in mouse mammary glands. Pim-1 nuclear localization correlated with cytoplasmic distribution for its substrate, $p21^{CIP/Waf1}$, and we found that Pim-1 and p21 associate in vitro. Our results suggest that Pim-1 expression may be regulated by progesterone during mammary development and Pim-1 associates with p21 in mammary epithelial cells.
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It is commonly accepted that morphogenesis and differentiation of the mammary gland depend on the orchestration of steroid and polypeptide hormones [1]. The mammary gland rudiment is established during embryogenesis and undergoes different stages of development postnatally. The murine pubertal stage starts at 3–4 weeks of age and in response to ovarian estrogen and progesterone, the mammary gland experiences rapid proliferation of epithelial cells in the terminal end buds (TEB). Elongation of the TEB into the fat pad yields a ductal tree that will later serve as a scaffold for milk secreting lobules. Increased progesterone secretion from the placenta in pregnant mammals promotes additional proliferation of epithelial branches and the lobuloalveoli located at the tips of these branches differentiate and secrete milk in response to pituitary prolactin secretion and decreased progesterone at parturition [2]. The rise or fall of hormone levels causes coordinated changes in gene expression and protein activity in the breast, which leads to alterations in cellular maintenance. Identifying hormonally controlled genes/proteins and analyzing their roles in mammary gland development may help our understanding of how the altered activity and/or levels of these hormones contribute to breast cell maintenance.

Pim-1 is a strong candidate to be one of the genes regulated by hormones that govern mammary development for the following reasons: (1) its substrates are involved with a number of cell cycle regulatory processes (reviewed in [3]), (2) Pim-1 expression correlates with differentiation or maturation of hematopoietic cells [4,5] and epithelial cells [6], and (3) *pim-1* gene and protein expression can be altered by a number of hormones and cytokines [7–10]. In hematopoietic cells, *pim-1* is induced by prolactin [11,12], NF-κB [13], and Signal Transducers and Activators of Transcription (STAT) [14]; these molecules are known to promote breast development [15–17].

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Pim-1 influences cellular activities by phosphorylating substrates such as pro-apoptotic Bad [18], Nuclear Mitotic Apparatus protein (NuMA) [19], a protein that is directly involved in chromosome separation during mitosis, the phosphatases Cdc25A and Cdc25C which promote G_1 and G_2/M cell cycle progression, respectively [20,21], and Cdc25C-associated kinase 1 (C-TAK1), a kinase tied to G_2/M cell cycle progression [22]. One of the more interesting substrates of Pim-1 is $p21^{CIP/WAF1}$, the Cdc2 kinase inhibitor. Phosphorylation of p21 on threonine 145 by Pim-1 results in p21 cytoplasmic localization [23]; this localization leads to less growth inhibition due to reduced p21 activity upon molecules such as Cdc2 kinase [24].

Although Pim-1 is ubiquitously expressed [25], the induction and function of Pim-1 protein during mammary gland development have not been evaluated. We hypothesized that Pim-1 expression might be regulated during breast development, and breast tumor cells may have the capacity for altered Pim-1 expression. We first assessed the expression of Pim-1 in mammary cell lines and during murine mammary gland development. Next, we determined whether Pim-1 expression could be modulated in vitro using mammogenic hormone treatment of MCF10A non-tumorigenic mammary epithelial cells, and lastly, analyzed whether Pim-1 associates with p21 in breast epithelia.

Materials and methods

Cell culture. Cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in a 5% CO_2 humidified atmosphere at 37 °C. K562 chronic myelogenous leukemia cells were grown in RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (Hyclone Labs Inc., Logan, UT). Non-tumorigenic MCF10A cells have characteristics of normal breast epithelium such as a normal karyotype and a requirement for hormones and growth factors [26]. MCF10A cells were grown in Ham F12 (Gibco) supplemented with 2% (v/v) fetal calf serum, 0.01% (v/v) bovine serum albumin, 1 μg/mL hydrocortisone, 5 μg/mL insulin, 10 ng/mL epidermal growth factor, 1 μg/mL selenium, and 10 ng/mL transferrin. MDA MB231, MDA MB453, MCF7, SKBR3, BT474, and Hs578T were grown in Dulbecco modified Eagle's medium containing 10% (v/v) fetal calf serum.

Antibodies. 19F7 made to murine Pim-1 but recognizing both human and murine Pim-1 (Santa Cruz, CA) was used for the breast development immunoblot. Antibody raised against recombinant human Pim-1-GST fusion protein [27] was also used for the breast development immunoblot and for immunohistochemistry. A similar antibody made to recombinant human Pim-1 with the GST-tag removed was used for MCF10A immunoblots. 19F7 Pim-1 mouse monoclonal antibody (kindly provided by Dr. Michael Lilly, Loma Linda University) was used for immunoprecipitation, and p21 was localized using C19 (Santa Cruz) for immunoprecipitation and immunoblot. As an internal protein loading control in immunoblots, actin antibody (Sigma, St. Louis, MO) was used to detect actin. Anti-digoxygenin (DIG) antibody (Boehringer Mannheim, Germany) was used to detect *pim-1* in Northern blots.

Northern blotting. Total RNA was extracted using TriZol (Invitrogen Corp., Carlsbad, CA) and quantified at $OD_{260/280}$. Sample RNA's were resolved through a 1% (w/v) formaldehyde agarose gel and transferred to positively charged membrane (Ambion, Austin, TX) in 10X SSC (1.5 mol/L sodium chloride, 150 mmol/L sodium acetate). *Pim-1* mRNA was detected with 20 ng/mL of random primed DIG-labeled human *pim-1* open reading frame probe in hybridization solution (7% {w/v} SDS, 50% {v/v} formamide, 750 mmol/L NaCl, 75 mmol/L sodium acetate, 50 mmol/L monobasic sodium phosphate, 0.1% {w/v} *N*-lauryl sarcosine, and 2% {w/v} block {Boehringer Mannheim}) at 62 °C. Probe hybridization was detected by CDP Star Chemiluminescence substrate (New England BioLabs, Beverly, MA) and exposure to X-ray film. *Pim-1* mRNA expression was quantified by densitometric analysis of the film with Quantity One software (Bio-Rad, Hercules, CA). K562 human erythroleukemia cells, which constitutively express *pim-1* mRNA [28], were used as a positive control.

SDS–PAGE and immunoblotting. Hormone-treated MCF10A mammary cells were lysed directly in 100 mm^2 culture dishes. All other mammary cell lines were washed with PBS and released from 100 mm^2 culture dishes using 0.05% trypsin (w/v), 0.025% (w/v) EDTA prior to cell lysis using 10 mmol/L Tris–HCl, pH 7.6, 5 mmol/L EDTA, 50 mmol/L NaCl, 30 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1% (v/v) Triton X-100, and protease inhibitor cocktail (10 μg/mL leupeptin, 10 μg/mL aprotinin, 5 μg/mL pepstatin A, and 0.5 mmol/L phenylmethylsulfonylfluoride). Both right and left side mammary glands were removed from euthanized mice and placed in protein extraction solution (20 mmol/L Hepes, 1 mmol/L EDTA, and 1 mmol/L magnesium chloride) containing protease inhibitors. For each stage of development, glands from three mice were combined and homogenized on ice using a Tissue Tearor (model 985370, Biospec Products, Inc.). Protein samples were stored at −80 °C until quantified using the Bradford assay (Bio-Rad). Cell extracts were subjected to 12% SDS–PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF Plus, 0.45 μm, ISC BioExpress, Kaysville, UT). For immunoblotting with antibody raised to human Pim-1-GST fusion protein, incubations were performed in 5% (w/v) non-fat milk in 0.1 mol/L Tris–base, pH 7.5, 0.15 mol/L NaCl, and 0.05% (v/v) Tween-20 (TTBS). Immunoblots detected with anti-Pim-1 19F7 (Santa Cruz), actin, or antibody raised to human Pim-1-GST fusion protein with the GST tag removed were incubated in 1% (w/v) bovine serum albumin diluted in TTBS. Positive antibody reactivity was detected using the appropriate secondary antibody and the Pierce Super Signal West Pico System (Pierce, Rockford, IL) followed by exposure to X-ray film (CL-X Posure, Pierce). Proteins were quantified by densitometric analysis of the film with Quantity One software (Bio-Rad).

Hormone induction of MCF10A mammary epithelial cells. Cells were grown to 80% confluence in 100 mm^2 diameter tissue culture plates, rinsed with PBS, and synchronized by culturing an additional 24 h in fresh medium lacking serum or growth factors [26]. Progesterone (P6149, Sigma) and glucocorticoid (dexamethasone, D4902, Sigma) were dissolved in ethanol. Ovine prolactin (NIDDK, AFP-9221A) was obtained through the NIH Pituitary Hormone and Anti-sera Program (Rockville, MD) and was dissolved to 2 mg/mL in 0.01 mol/L sodium bicarbonate. Hormones were further diluted to the desired concentration (progesterone, 10^{-7} mol/L; glucocorticoid, 0.5 μg/mL; and prolactin, 5 μg/mL) in culture medium lacking serum or growth factors. Cells were treated with hormone or vehicle for 2, 4, and 6 h before proteins were extracted as described.

Immunohistochemistry. Mammary glands were removed from euthanized mice and fixed in Bouin solution (75% {v/v} saturated picric acid, 9% {v/v} formaldehyde, and 5% {v/v} glacial acetic acid) overnight at 4 °C. Within 24 h fixed glands were placed in 70% (v/v) ethanol and stored at 4 °C. Fixed glands were dehydrated, embedded in paraffin wax, sectioned, and 0.4 μm sections placed on poly L-lysine-coated slides (Reproductive Biology Center Histology Core, Washington State University, Pullman, WA). After de-waxing and rehydration through xylene, ethanol, and water, tissue epitopes were further exposed by boiling the slides for 10 min in 0.01 mol/L sodium citrate. Pim-1 and $p21^{CIP/WAF1}$ was localized with the appropriate antibody followed by color detection with a Zymed Histostain SP kit (Zymed, San Francisco, CA). Nuclei were counterstained blue using hematoxylin. Digital images were captured using a Leica M-series stereo-microscope (Heerbrugg, Switzerland) equipped with Leitz DMRB with epifluorescence and a Magnafire digital camera (Optronics, Goleta, CA).

Apoptosis assay. Glass slides carrying paraffin-embedded tissue sections were analyzed for apoptosis using the DeadEnd Fluorometric TUNEL system as described by the manufacturer's protocol (Promega,

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