Journal of Cellular Biochemistry 97:109–121 (2006)

DDR1 Signaling is Essential to Sustain Stat5 Function During Lactogenesis

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Abstract Postnatal development of the mammary gland is achieved by an interplay of endocrine and extracellular matrix-derived signals. Despite intense research, a comprehensive understanding of the temporal and spatial coordination of these hormonal and basement membrane stimuli is still lacking. Here, we address the role of the collagen-receptor DDR1 in integrating extracellular matrix-derived signaling with the lactogenic pathway initiated by the prolactin receptor. We found that stimulation of DDR1-overexpressing mammary epithelial HC11 cells with collagen and prolactin resulted in stronger and more sustained induction of Stat5 phosphorylation as compared to control cells. Enhanced Stat5 activity in HC11-DDR1 cells correlated with increased beta-casein gene expression. In contrast, cells derived from DDR1-null mice showed reduced Stat5 activation upon lactogenic stimulation and completely failed to induce beta-casein expression. The cell-autonomous role of DDR1 in controlling ductal branching and alveologenesis prior to the onset of lactogenesis was corroborated by mammary tissue transplantation experiments. Our results show that aside from hormone- and cytokine receptors, DDR1 signaling establishes a third matrix-derived pathway vital to maintain mammary gland function. J. Cell. Biochem. 97: 109–121, 2006.

Key words: mammary gland; collagen; tyrosine kinase; lactation; prolactin

At puberty, a mix of steroid hormones, cytokines, and cell-matrix contacts guide the arborization of ductal epithelial cells within the mammary fat pad. Upon pregnancy, altered combinations of these factors induce further differentiation and are essential to maintain lactation. The pituitary gland hormone, prolactin, is a prime initiator of these processes reaching peak expression level in late pregnant mice. Knockout studies confirmed that prolactin, the prolactin-receptor (PrlR) as well as their downstream signal transducers Jak2 and Stat5a/b are essential for mammary gland function [Ormandy et al., 1997; Udy et al., 1997; Teglund et al., 1998; Shillingford et al., 2002; Cui et al., 2004; Wagner et al., 2004]. Upon activation of the PrlR, the tyrosine kinase Jak2 is recruited to the membrane, where it facilitates phosphorylation of the receptor as well as downstream substrates, such as Stat5 [Clevenger and Kline, 2001]. In a second step, tyrosine phosphorylated Stat5a and Stat5b form homo- or hetero-dimers that translocate to the nucleus, where they become part of a larger complex and execute the transcription of milk proteins [Ali and Ali, 1998]. Apart from the PrlR pathway, local signals emanating from the stromal microenvironment surrounding the ductal epithelium play an equally important role in lactogenesis, particularly signals derived from the collagenous extracellular matrix [Silberstein, 2001; Fata et al., 2003]. Experiments in tissue culture showed that mammary gland epithelial cells require collagen and other basement membrane proteins to execute prolactin-induced milk protein transcription [Emerman and Pitelka, 1977; Li et al., 1987; Streuli et al., 1995; Klinowska et al., 1999] Although collagens are a critical component of all basement membranes and evidence from mice with defects in collagen degradation or neo-synthesis

Grant sponsor: Association for International Cancer Research (AICR); Grant sponsor: National Cancer Institute of Canada (NCIC).

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Received 22 June 2005; Accepted 18 July 2005

DOI 10.1002/jcb.20618

support their role in mammary gland development, the precise nature of the collagen-receptor pathway responsible for transducing these fundamentally important signals during pregnancy and lactation remains largely undefined.

Currently, three different types of collagen-receptors are known: the tyrosine kinases discoidin domain receptor 1 and 2 (DDR1 and DDR2), integrin heterodimers containing the β1 subunit, and glycoprotein VI [Vogel, 2001]. While glycoprotein VI is only found on platelets, integrins α1β1 and α2β1 as well as DDR1 are expressed in the mammary gland [Vogel et al., 1997]. Knockout mice lacking α2 integrin showed minor alterations in epithelial duct branching that did not affect lactation, while mammary glands from α1-null animals were normal [Gardner et al., 1996; Chen et al., 2002]. In contrast, deletion of DDR1 in mice resulted in severe alterations in tissue architecture and a complete failure of lactation, supporting its pivotal involvement in mammary gland function [Vogel et al., 2001].

The objectives of this study are to first analyze the mammary gland phenotype of DDR1-null mice in vivo by tissue transplantation experiments. Secondly, we use cultures of primary cells and a non-transformed mouse mammary epithelial cell line to study DDR1-dependent signaling. Our data provide strong evidence that the DDR1 pathway synergizes with the PrlR pathway in maintaining the activation level of Stat5 during mammary gland function.

MATERIALS AND METHODS

Plasmids and Cell Lines

The cDNAs coding for full-length DDR1 (b-isoform, tagged with Flag and cloned into pLXSN vector) and the generation of stable cell lines were described previously [Alves et al., 1995; Alves et al., 2001]. Over-expression of DDR1 was confirmed by anti-Flag Western blotting. Differentiation of non-transformed mouse mammary epithelial HC11 cells in cultures containing DIP (1 μM dexamethasone, 5 μg/ml insulin, and 5 μg/ml prolactin) medium was detailed earlier [Chammas et al., 1994]. Cell differentiation was assessed by Western blotting using anti-beta-casein antibody and real-time PCR (see below). Primary mouse mammary epithelial cells were isolated as previously described [Imagawa et al., 1995]. Briefly, mouse mammary glands were dissected from DDR1-null and control mice and digested with 3 mg/ml collagenase (Roche Diagnostics, Laval, Canada) in DMEM-F12 medium at 37°C for 30 min. Digested cells were recovered by centrifugation at 100*g* for 3 min. Pellets were washed four times with DMEM-F12 to remove collagenase. Further purification was performed by Percoll (Amersham Biosciences, Baie d'Urfe, Canada) gradient centrifugation. Cells were placed on top of the gradient and centrifuged 20 min at 800*g*. Purified cells were washed 3 times with DMEM-F12 to remove Percoll and plated on tissue culture plates coated with collagen.

Mammary Gland Transplantation

All procedures were carried out in accordance with the guidelines of the Canadian Council for Animal Care. For mammary gland transplantation, a protocol originally established by DeOme at al. was used [DeOme et al., 1959]. Small pieces of mammary gland tissue with 1.5–2.0 mm in diameter were isolated from adult virgin control and DDR1-null mice. Control mice were littermates of DDR1-null animals in a 129/Sv background. The 4th mammary gland anlage of a wild type, 3.5-week old mouse was removed on both sides and the transplant grafted into the cleared fat on one side, while the contra-lateral side remained empty. Four weeks after transplantation, mice were euthanized and mammary glands harvested. Alternatively, the recipients were bred 2 weeks following transplantation and tissue was harvested on the day E14.5 of pregnancy. For whole mount analysis, mammary glands were removed, fixed in acetone overnight, and stained in Harris' haematoxylin (Sigma-Aldrich Ltd., Oakville, Canada). They were destained using a solution of 1% HCl in 70% ethanol for 3–4 h, cleared in xylene overnight, and mounted with Permount (Invitrogen, Burlington, Canada). Following a published protocol, branch point analysis was performed on several transplanted mammary glands by counting ductal bifurcations in 8 randomly selected areas of 1.5×1.5 mm size [Wiseman et al., 2003]. Student's *t*-test was used for statistical analysis.

Electromobility Shift Assay (EMSA)

Stat5 binding to a double-stranded, beta-casein promoter-derived oligonucleotide was performed as described earlier [Kabotyanski and Rosen, 2003]. Briefly, 9 μl of nuclear lysate

were mixed with 1 μl pdI/C (Sigma, 2 mg/ml) and incubated on ice for 15 min. For supershift analysis, 1 μl anti-Stat5a or-Stat5b antibody (200 μg/ml, Zymed Laboratories, now Invitrogen) was added. Samples were mixed with 5 μl of binding buffer (50 μg/ml $p(dN)_6$, 2.5 mg/ml BSA, 4% Ficoll 400, 10% glycerol), and 5 μl of ^{32}P-labeled oligonucleotide (app. 20,000 cpm) and left on ice for additional 15 min. Samples were run on a 4% polyacrylamide gel and DNA complexes detected by autoradiography. EMSA was also performed with a ^{32}P-labeled probe resembling the iron-responsive element (IRE) in the absence or presence of a 20-fold excess of cold IRE-probe [Popovic and Templeton, 2004].

Immunoprecipitation and Western Blotting

Protein gel and Western blot analysis were performed as previously published [Vogel et al., 1997]. For nuclear extracts, cells were lysed in hypotonic buffer (0.1% Triton X-100, 20 mM HEPES, pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 20% glycerol, and protease inhibitors). After low-speed centrifugation, the pellet was washed once with hypotonic buffer and then re-solubilized in extraction buffer (hypotonic buffer plus 420 mM NaCl) at 4°C for 20 min. Nuclear extracts were obtained after centrifugation at 14,000*g* for 10 min. Absence of contaminating cytoplasmic proteins was confirmed by anti-vinculin Western blotting. The following antibodies were used in a 1:1,000 dilution: 4G10 anti-phosphotyrosine and phospho-Stat5a/b antibodies (Upstate Biotechnology, Lake Placid), anti-Stat5a and Stat5b antibodies (Zymed), anti-IRS-1 (Santa Cruz, California) or antibodies against beta-casein or mouse milk (1:500 dilution, kindly provided by I. Barash, Bet-Dagan, Israel and N. Hynes, Basel, Switzerland). Blots were re-probed with anti-DDR1 (Santa Cruz), anti-Flag (Sigma-Aldrich, Oakville, Canada), anti-Stat5a/b (N-20, Santa Cruz) or anti-actin (JLA20, Developmental Studies Hybridoma Bank, Iowa City) antibodies. X-ray films were digitalized and quantified using the TINA 2.0 software (Raytest, Straubenhardt, Germany).

Real-Time PCR Assay

Total RNA was extracted from HC11 cells using the RNeasy Kit (Qiagen, Mississauga, Canada). Ten micrograms of total RNA were transcribed into cDNA using Superscript II and AncT Primer T_{20}VN (Invitrogen) in a total volume of 20 μl at 42°C for 1 h. Real-time PCR was performed in triplicate with an iCycler (BioRad, Hercules, California) and SYBR Green Mastermix (Molecular Probes, now Invitrogen). The cDNA was diluted 1:10 with H_2O and 1 μl used per PCR reaction in a total volume of 50 μl and a concentration of 1 pM each primer. PCR primers for beta-casein were: TCA CTC CAG CAT CCA GTC ACA (forward), GGC CCA AGA GAT GGC ACC A (reverse) and for GAPDH: GAT GAC ATC AAG AAG GTG GTG (forward), GCT GTA GCC AAA TTC GTT GTC (reverse). The iCycler software was used to calculate threshold values (Ct). The specificity of primers was confirmed by melting curve analysis. Results for beta-casein were normalized for GAPDH expression and ΔCt values calculated.

Luciferase Assay

HC11-control and HC11-DDR1 cells were transiently transfected with a glucocorticoid receptor-luciferase-reporter plasmid together with a galactosidase expression vector. Cells were kept in medium without hormones overnight and induced with DIP for 24 h. Luciferase and galactosidase activity was quantified 48 h after transfection. Data were normalized for galactosidase expression.

RESULTS

To address whether the lactational failure in DDR1-null mice is caused by defects intrinsic to the epithelial cells or by paracrine or endocrine malfunction, we transplanted small pieces of mammary gland tissue from adult DDR1-null and control mice into the 4th inguinal mammary gland of 3.5-week-old wild-type recipients, from which the endogenous epithelial anlage had been removed. The contra-lateral mammary gland was cleared in the same manner, but did not receive a transplant [DeOme et al., 1959]. Four weeks after transplantation, we assessed mammary gland development by whole-mount analysis (Fig. 1A,D). Although cells from both knockout and wild type transplants completely repopulated the fat pad, knockout ducts showed a significantly reduced number of branch points (13.0/field in the wild type, 7.5/field in the knockout, $P < 0.05$). Furthermore, the terminal end buds in DDR1-null transplants remained much larger than those observed in wild type transplants (Fig. 1B,E). A second set of mice was mated with control males 2 weeks post transplantation and

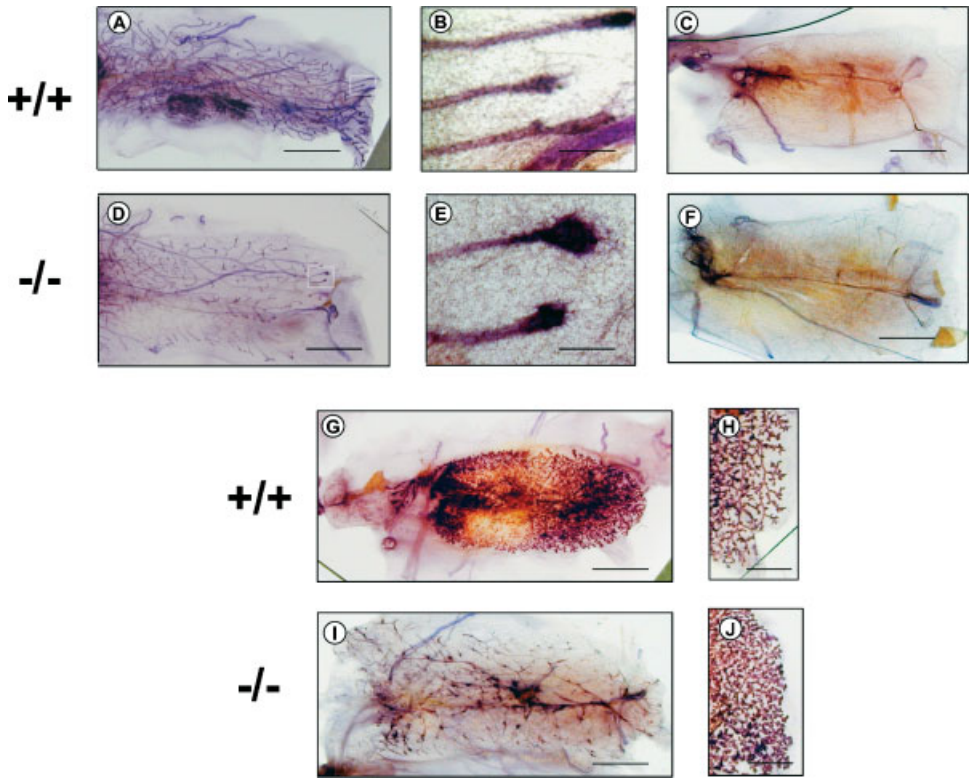
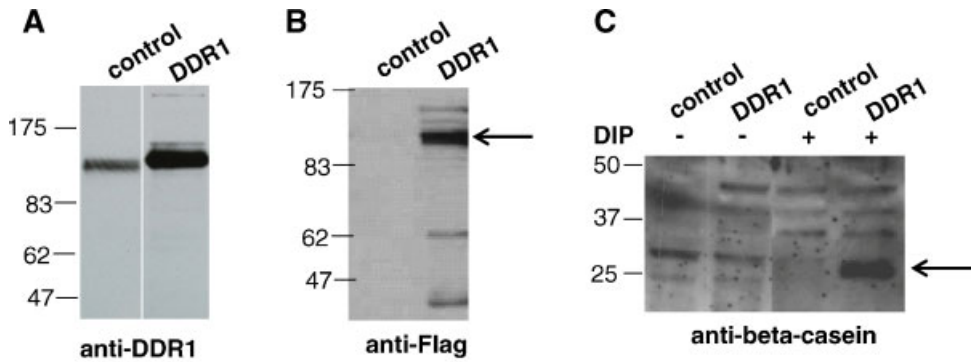


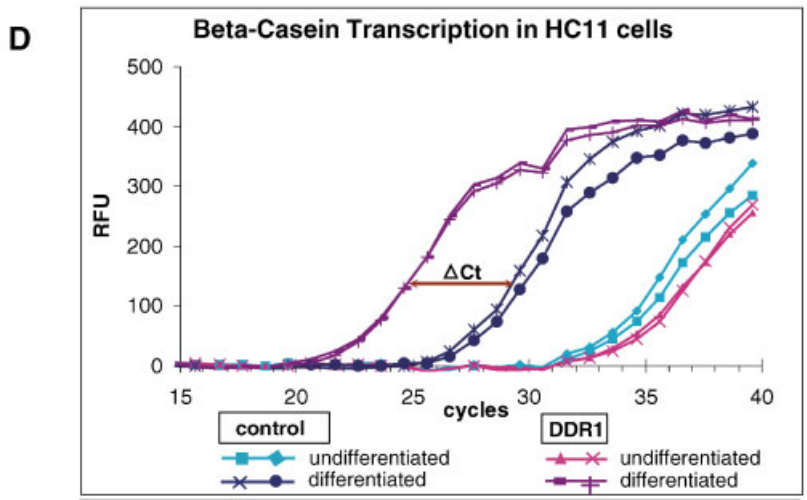
Fig. 1. Mammary glands transplantation of DDR1-null tissue. The 4th mammary gland anlage of 3.5-week-old wild type mice was removed and small pieces of mammary gland tissue from adult wild type (**A**, **B**, **G**) or DDR1-null (**D**, **E**, **I**) were transplanted into the cleared fat pad. Mice were sacrificed after 4 weeks and mammary gland whole mounts prepared. A second set of transplanted mice was mated 2 weeks post transplantation, sacrificed at day E 14.5 of pregnancy, and whole mounts prepared (G, I). As controls, whole mount analyses of the contralateral mammary gland, which was cleared but not transplanted (**C**, **F**), and the 2nd mammary gland were performed (**H**, **J**). Note the enlarged terminal end buds in virgin DDR1-null transplants (A and D; higher magnification in B and E) and the absence of alveolar outgrowth in pregnant transplanted mice (G, I). Scale bars are 4 mm (A, C, D, F, G, I), 500 μm (B, E), and 1 mm (H, J).

whole mount analysis was performed at day 14.5 of pregnancy. Tissue transplanted from DDR1-null mice failed to undergo lobulo-alveolar outgrowth upon induction of pregnancy, while control transplants developed normally and reached a stage comparable to the endogenous epithelium of the 2nd mammary gland (Fig. 1G–J). The alterations in morphology observed with DDR1-null tissue transplanted into wild type fat pads closely resemble the ones seen in DDR1 knockout mice at various stages of mammary gland development. Hence, the defects in DDR1-null mice are cell-autonomous and confined to the epithelial compartment of the mammary gland.

Previously, transplantation of mouse mammary epithelium from PrlR-null and Stat5-null mice followed by induction of pregnancy has been performed [Brisken et al., 1999; Miyoshi et al., 2001]. While the epithelium from both of these knockout mice failed to undergo proper alveolar differentiation, the phenotypic alterations were distinctly different on the cellular level. In contrast to the single-sheeted luminal epithelium in PrlR-null transplants, Stat5-null cells formed multiple layers of disorganized cell aggregates. These observations suggest that the PrlR-Stat5 signaling pathway is intersected by additional stimuli, one of it possibly being activated DDR1.

To test this, we over-expressed DDR1 in non-transformed mouse mammary epithelial HC11 cells and evaluated differentiation and lactogenic responses. HC11 cells endogenously express DDR1, and the expression level was enhanced by introducing Flag-tagged, full-length DDR1 (b isoform) through retroviral transfection (Fig. 2A,B). Cells were treated with the lactogenic hormones dexamethasone, insulin, and prolactin, (DIP) for 2 days and the induction of beta-casein expression was determined by Western blot analysis. While beta-casein was not detected in control HC11 cells at this time point, HC11-DDR1 cells showed high





Ct-values	beta-casein		GAPDH	
	control	DDR1	control	DDR1
- DIP	34.7	35.7	23.1	24.2
+ DIP	28.6	23.8	23.2	24.0

Fig. 2. Increased Stat5 activity and beta-casein expression in HC11 cells over-expressing DDR1. **A**: Concanavalin A lectin matrix was used to purify DDR1 from HC11 cells transfected with DDR1 or a control plasmid. Lysates were analyzed by anti-DDR1 Western blotting. **B**: Over-expression of DDR1 in HC11 cells was confirmed by anti-Flag Western blotting. **C**: Differentiation of HC11 cells was induced with lactogenic hormones (DIP: dexamethasone, insulin, and prolactin). Total cells lysates from untreated or differentiated HC11 and HC11-DDR1 cells were analyzed by Western blotting using an antibody to beta-casein. **D**: Real-time PCR analysis of beta-casein mRNA expression in untreated and differentiated cells. Threshold cycles are given in the table, indicating an approximately 15 higher level of beta-casein in differentiated HC11-DDR1 than control cells. GAPDH mRNA levels were used to normalize the data. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

expression upon DIP stimulation (Fig. 2C). We verified DDR1's function in enhancing lactogenic responses using real-time PCR and found an approximately 15-fold increased beta-casein expression in the presence of DDR1 (Fig. 2D).

Upon lactogenic hormone stimulation, the transcriptional activation of milk genes such as beta-casein is mediated by tyrosine-phosphorylated Stat5a and Stat5b. Therefore, we tested the activation of Stat5a/b in HC11 cells by Western blot using a phospho-Stat5-specific antibody (Y^{694} and Y^{699} in Stat5a and Stat5b, respectively) and found an approximately two-fold higher level of Stat5a/b phosphorylation in DDR1 transfected versus control cells (Fig. 3A). This suggests that the enhanced lactogenic response is mediated through DDR1 by sustaining Stat5a/b activation.

Activation of the Stat5 pathway has been found to not only take place after prolonged lactogenic hormone treatment but also within minutes of stimulation [Teglund et al.,

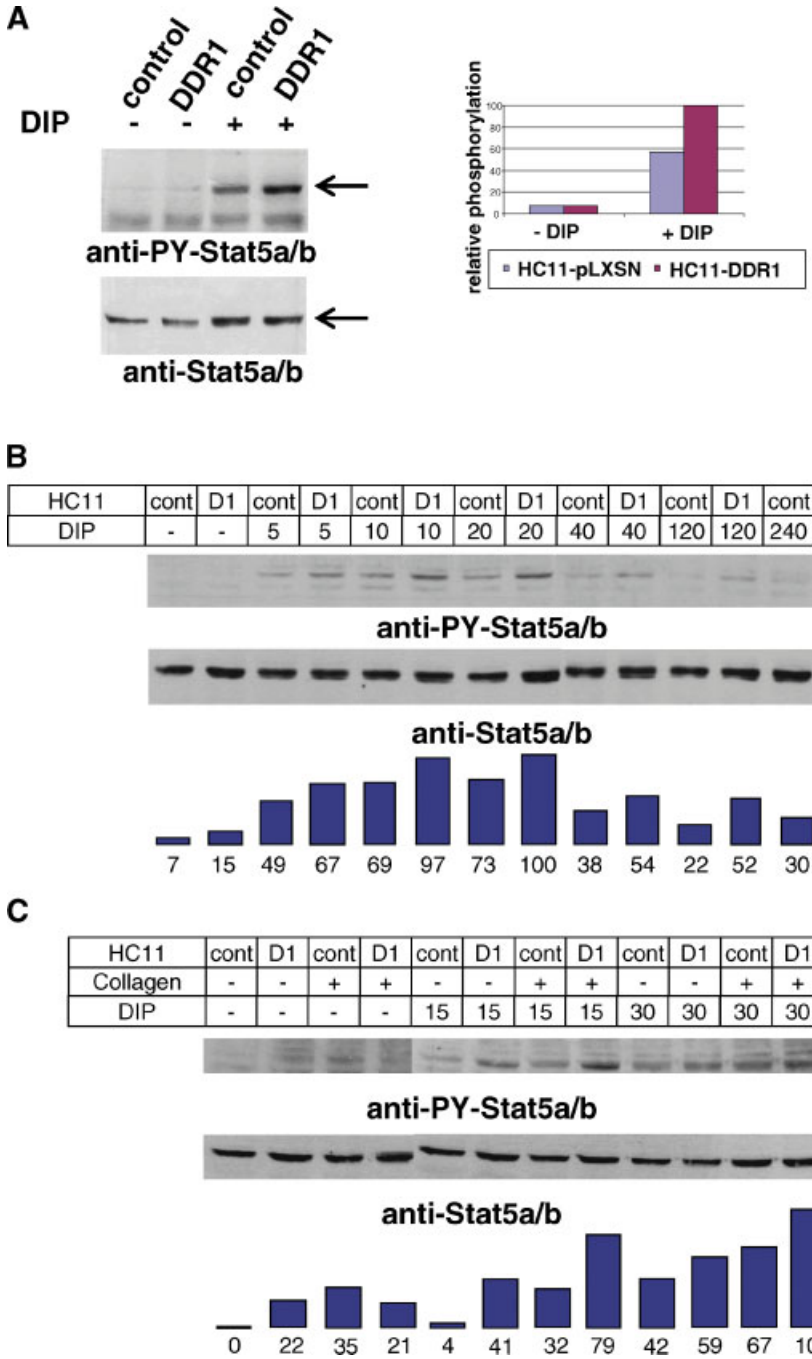


Fig. 3. Enhanced Stat5b phosphorylation in DDR1 over-expressing cells. **A**: HC11 and HC11-DDR1 cells were stimulated with DIP for 2 days or left untreated. Total cellular lysates were probed with phospho-Stat5a/b antibody followed by reprobing with an antibody for Stat5a/b. Signals were quantified and normalized for total Stat5a/b. This experiment was repeated three times and the approximately twofold increase of DDR1-mediated Stat5a/b phosphorylation was consistently found. **B** and **C**: DDR1-over-expressing and control HC11 cells were stimulated with DIP (B) or with DIP and collagen (C) for the indicated periods of time and Western blot analysis of total cell lysates was performed as in (A). Signals were quantified by densitometry and normalized for total Stat5a/b expression. Values are given in percentage of maximal activity. Experiments were repeated twice and essentially identical kinetics of activation observed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

1998; Hennighausen and Robinson, 2001]. We found maximal Stat5a/b phosphorylation after 10–20 min of DIP exposure in both cell types, however control cells reached only 75% of Stat5 activity compared to HC11-DDR1 cells (Fig. 3B). Furthermore, after 2 h of DIP stimulation, Stat5 phosphorylation remained twice as strong in DDR1-transfected as in control cells. Next, we wanted to test whether activation of DDR1 by exogenous collagen can further enhance the Stat5 pathway. Activation of DDR1 with type I collagen (10 μg/ml) increased DIP-induced Stat5a/b phosphorylation to a much higher degree in DDR1-overexpressing than in control cells (Fig. 3C). These results indicate that simultaneous stimulation of the hormone-receptor and DDR1 pathways are necessary to achieve maximal Stat5 activation. In order to dissect the effects of the three different lactogenic hormones, we analyzed DIP-treated cells for insulin-receptor-substrate-1 phosphorylation, which is the prime signaling mediator downstream of the insulin-receptor, or for glucocorticoid receptor activity. Importantly, we found that DDR1 overexpression did not affect either of these two pathways (Fig. 4A,B). In other words, DDR1 signaling impacts solely on the PrlR pathway.

While both Stat5a and Stat5b are downstream of activated PrlR, recent work indicated that the two Stat5 forms display different kinetics of phosphorylation and nuclear import and retention upon receptor engagement [Kazansky et al., 1999]. To test whether this is relevant within the context of the DDR1 signaling cascade, we separately measured the amounts of Stat5a or Stat5b in the nucleus of prolactin- and collagen-stimulated HC11-DDR1 cells. While Stat5a had higher overall nuclear levels than Stat5b after 20 and 120 min of prolactin exposure, only the level of Stat5b and not of Stat5a increased upon collagen-stimulation (Fig. 5A). To assess the relevance of the endogenous collagenous matrix laid down by HC11 cells, we treated cells simultaneously with prolactin and bacterial collagenase, an enzyme that efficiently degrades all types of collagen, but not affects cell morphology or viability (Fig. 5B). We found complete abrogation of Stat5a and Stat5b nuclear translocation upon collagenase treatment supporting the notion that the presence of an intact collagenous extracellular matrix is essential for PrlR signal transduction. Next, DNA-binding activity of

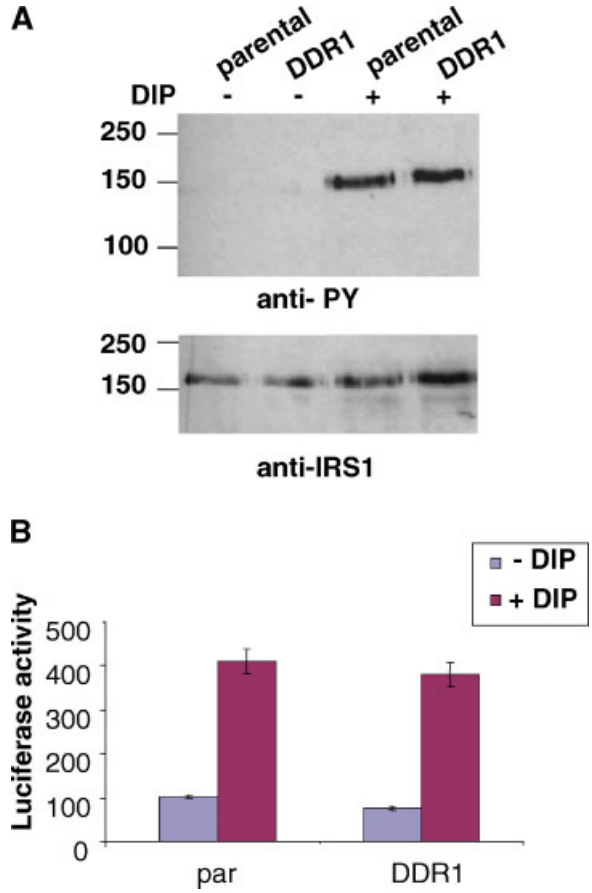


Fig. 4. Insulin or dexamethasone-induced activation of HC11 cells is independent of DDR1. **A**: IRS-1 was immuoprecipitated from cell lysates of untreated or DIP medium-differentiated HC11 and HC11-DDR1 cells and analyzed by Western blotting using a phosphotyrosine antibody (upper panel). The membrane was striped and reprobed with anti-IRS-1 to monitor total IRS-1 levels (lower panel). **B**: Glucocorticoid receptor activity levels in DIP-treated HC11 and HC11-DDR1 cells were measured by luciferase assay. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Stat5 to the beta-casein promoter was determined by EMSA using nuclear lysates from HC11-DDR1 cells. We found a weak signal in cells stimulated with collagen only, which was drastically enhanced in prolactin-stimulated cells (Fig. 5C). Additionally, we confirmed the identity of the Stat5-DNA complex by supershift analysis. Pretreatment of cells with bacterial collagenase abolished Stat5 activity, but did not affect the RNA-binding activity of the iron-response-element, a factor unrelated to the prolactin pathway [Fig. 5D, Popovic and Templeton, 2004]. Taken together, these data indicate that overexpression and activation of DDR1 in HC11 cells enhances Stat5 activity upon hormone stimulation and results in

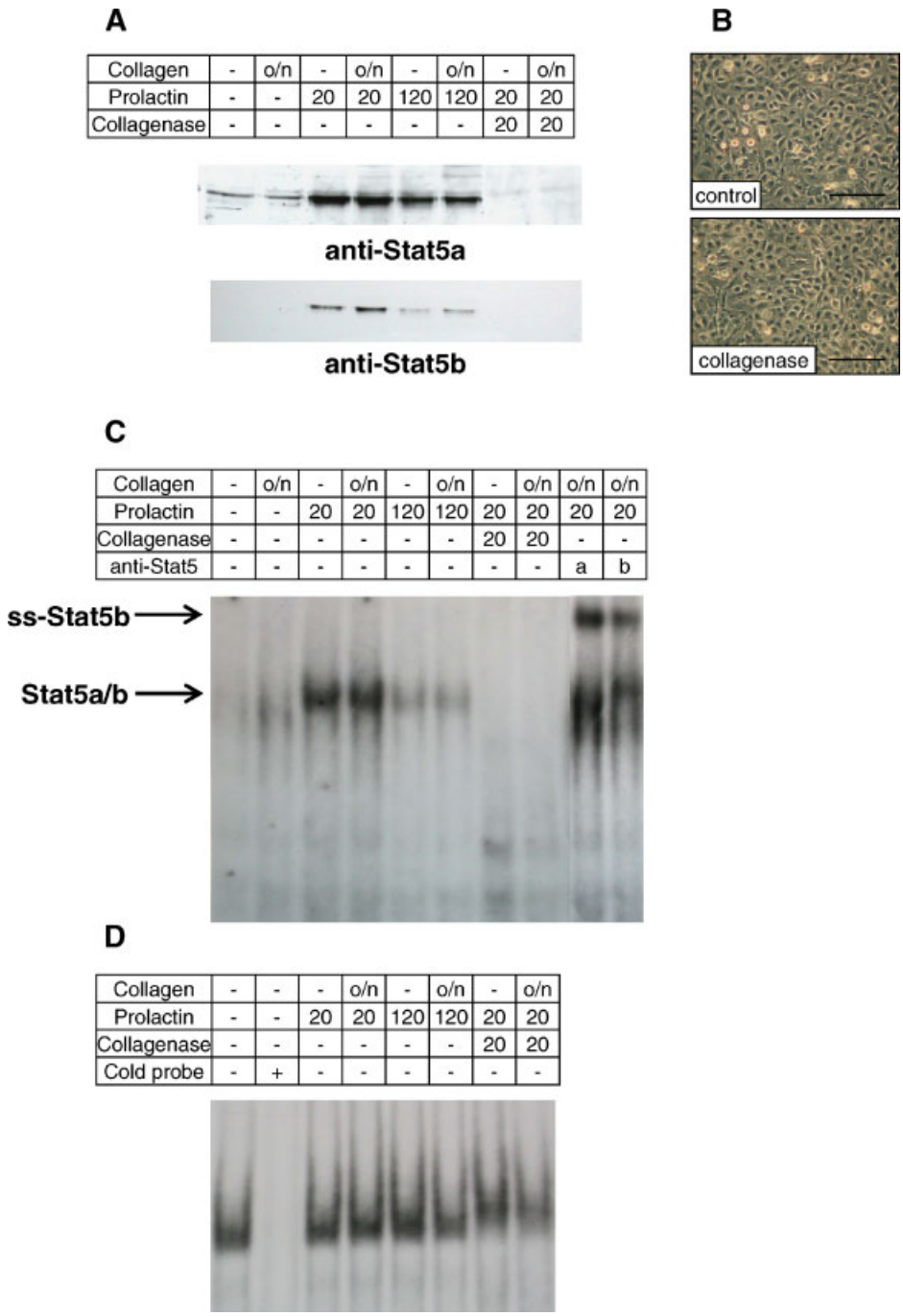


Fig. 5. DDR1 enhances nuclear localization of Stat5a and the DNA-binding capacity of Stat5. **A**: HC11-DDR1 cells were stimulated with collagen, prolactin, and bacterial collagenase, as indicated. Nuclear lysates were prepared and analyzed by Western blotting using antibodies specific to either Stat5a (top panel) or Stat5b (lower panel). **B**: Digital images show HC11-DDR1 cells treated with bacterial collagenase for 20 min or left untreated. Scale bar is 50 μm. **C**: Electromobility shift assay with a radiolabeled DNA probe for the beta-casein promoter was done using nuclear lysates prepared in (A). Supershift analysis was performed using antibodies specific to Stat5a or Stat5b. Arrows indicate the Stat5-DNA-shift or-supershift complex, respectively. **D**: As a control for the collagenase-treatment, an electromobility shift assay with a radiolabeled RNA probe for iron-regulatory element was performed. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

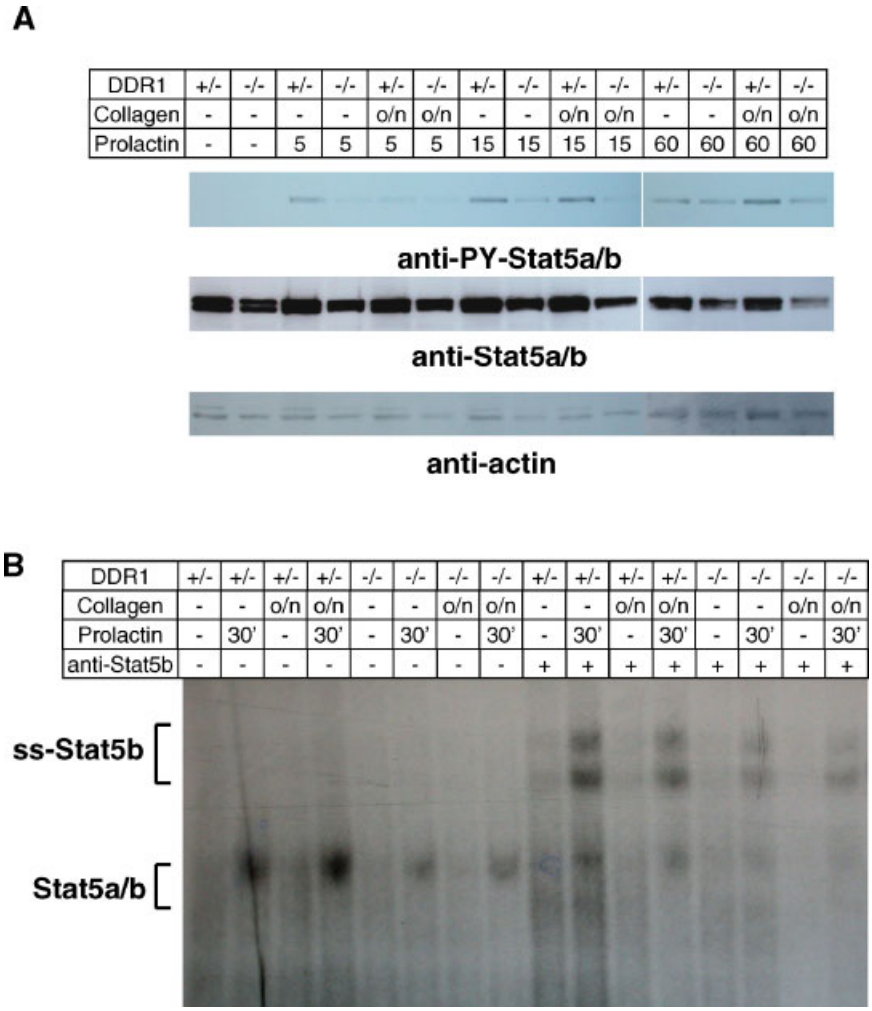


Fig. 6. Reduced Stat5a/b activity in primary DDR1-null mammary epithelial cells. (**A** and **B**) Primary mouse mammary epithelial cells isolated from DDR1-null or control mice were stimulated with collagen and prolactin as indicated. A: Total cellular lysates were probed with a phospho-Stat5a/b antibody (upper panel), reprobed with a Stat5a/b antibody (middle panel) and anti-actin antibody (lower panel). B: Electromobility shift assay with a radiolabeled DNA probe for the beta-casein promoter using nuclear lysates prepared from DDR1-null and control cells. Supershift analysis was performed using antibodies specific to Stat5b. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

significantly higher levels of beta-casein expression.

The last set of experiments was performed to confirm the interplay of the PrlR and DDR1 signaling pathways in primary mouse mammary epithelial cells isolated from knockout mice. We found that upon collagen stimulation, DDR1-null cells showed reduced levels of Stat5a/b phosphorylation following stimulation with prolactin for 5–60 min (Fig. 6A). EMSA analysis of primary cells confirmed that DDR1-null cells had reduced Stat5 DNA-binding activity compared to control cells (Fig. 6B). Most of the band-shift complex could be super-shifted with an anti-Stat5b antibody suggesting that in primary cells Stat5b is a more predominant player than Stat5a. Finally, we tested beta-casein expression in cells grown for 5 days in a 3D collagen gel supplemented with lactogenic hormones and found that knockout cells failed to express milk proteins, including beta-casein (Fig. 7A,B). Hence, our results with primary cells from DDR1 knockout mice are in agreement with our findings in HC11 cells and are in strong support of an essential role of DDR1 in maintaining Stat5 activity.

DISCUSSION

The results presented here provide a molecular explanation for the notion that DDR1 regulates normal mammary gland function.

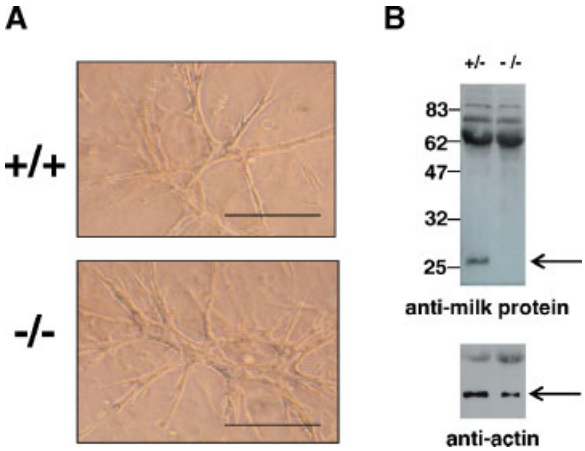


Fig. 7. Absence of beta-casein expression in primary DDR1-null mammary epithelial cells. **A**: Primary mammary epithelial cells from DDR1-null or control mice were cultivated in 3D collagen gels in the presence of lactogenic hormones and images taken after 5 days of culture. Scale bar is 50 μm. **B**: Cells were lysed and the resulting lysates analyzed by Western blotting using an antibody against mouse milk (upper panel). The blot was reprobed with an actin antibody (lower panel). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Previously, we found that DDR1-null females are unable to nourish their litter because the mammary gland alveoli fail to secrete milk [Vogel et al., 2001]. During puberty, the mammary gland epithelium in DDR1-null mice shows reduced branching and migration leading to enlarged terminal end buds. Mammary glands of adult knockout animals exhibit increased ECM deposition in the stroma as well as hyper-proliferative epithelial cells suggesting that DDR1 is as key regulator of cell motility, differentiation, and collagen synthesis. Since the DDR1 phenotype is highly related to knockouts of some members of the PrlR pathway, in particular Stat5a/b, we were prompted to investigate a potential connection between the PrlR as a cytokine receptor and DDR1 as a receptor for triple-helical collagen [Teglund et al., 1998].

We demonstrate here that DDR1-expressing mammary epithelial cells, when contacted by collagen-rich matrix, participate in the PrlR signaling cascade by sustaining Stat5 phosphorylation and transcriptional activity. This new role of DDR1 in the context of collagen signaling is of particular relevance, because previous work only found the involvement of laminin and its respective integrin receptors in Stat5 activation, but not of collagen [Streuli et al., 1995; Zoubiane et al., 2004].

The transplantation experiments shown here demonstrate that the function of DDR1 in the mammary gland is localized to the epithelium and that the absence of DDR1 in the surrounding stroma and in endocrine glands does not contribute to the observed knockout phenotype. Importantly, we found a highly similar morphology of the ductal tree in the transplanted DDR1-null tissue compared to the knockout, including enlarged terminal end buds [Vogel et al., 2001]. During pregnancy, transplanted DDR1-null tissue failed to undergo alveolar development, a phenotype highly related to transplanted Stat5a/b-null tissue, which provides further evidence of their cross talk on a molecular level [Miyoshi et al., 2001]. Recently, conditional knockouts for both Stat5 genes under the control of different promoters showed that Stat5 is not only a trigger of differentiation during pregnancy but also a survival factor for fully differentiated alveoli [Cui et al., 2004]. These events at late pregnancy and parturition appear to constitute a pathway distinct from prolactin, since the tyrosine kinase receptor ErbB4 was identified as mediator of Stat5 activation, independent of PrlR activation [Long et al., 2003]. Potentially, several factors, including DDR1 and ErbB4 are essential to maintain the integrity of the lactogenic tissue for an extended period of time, which is necessary to guarantee a thriving litter. To further address the role of collagen-receptors during different stages of mammary gland development, a line of animals transgenic for DDR1 is currently being generated in our laboratory.

Our present model proposes that Stat5 transiently interacts with DDR1 at the cell membrane and raises the tyrosine phosphorylation level of Stat5 in combination with PrlR-triggered Jak2 activity. We assume that DDR1 transiently acts on Stat5, because we did not detect binding of DDR1 to Stat5 using co-immunoprecipitation (data not shown). This hypothesis is supported by the fact that we found no increase of Jak2 activity upon DDR1 activation, excluding the possibility that DDR1 signals to Stat5 only via the PrlR or Jak2. Potentially, the SH2 domain of Stat5 is more suited to allow the dimerization of two Stat5 molecules rather than binding permanently to transmembrane receptors. This is supported by previous work showing that another tyrosine kinase receptor, the platelet-derived growth

factor receptor, is capable of activating Stat5 in the absence of Jak- and Src-family kinases [Paukku et al., 2000].

We found that DDR1 selectively enhances the nuclear localization of Stat5b and not Stat5a. Previous work suggested that the two Stat5 forms are differentially activated by outside stimuli and take unique routes within the cells. Specifically, tyrosine phosphorylated Stat5b (but not Stat5a) has been found to translocate to the nucleus upon c-src or c-abl activation in a manner independent of the PrlR/Jak2 pathway [Kazansky et al., 1999; Olayioye et al., 1999]. Under these conditions, nuclear Stat5b was found to not only be phosphorylated at Y^{699}, but also on at least three other C-terminal tyrosines (Y^{725}, Y^{740}, and Y^{743}), suggesting a more intricate pattern of regulation. The epidermal growth factor receptors ErbB1 and ErbB4 were found to target these non-canonical sites in a similar way, while the stimulation with cytokines, such as prolactin or growth hormone, were ineffective [Jones et al., 1999; Kloth et al., 2003]. Potentially, DDR1 is capable of directly phosphorylating Stat5b on some of these non-canonical sites at the C-terminus, thereby ensuring prolonged nuclear retention of Stat5b. In several human tumors, constitutive phosphorylation of Stat5b was found to correlate with increased carcinogensis, possibly linking Stat5b activation with DDR1's established role in tumor progression [Xi et al., 2003]. Also, it is of interest to note that recent work by others on a cohort of patients with metastatic ovarian cancer showed that expression of DDR1 is specifically increased, compared to control tissue, in the apical membrane, where cytokine signaling pathways, including PrlR activity, reside [Heinzelmann-Schwarz et al., 2004].

Interestingly, the involvement of SOCS, a family of eight related proteins, acting on Stat5 in response to tyrosine kinases, has not been explored. This is of particular importance since recent work showed that SOCS-7 negatively regulates Stat5 activity upon prolactin stimulation [Martens et al., 2004]. Additional experiments are necessary to test whether DDR1 can sequester and/or inhibit SOCS proteins.

Our data using primary mammary epithelial cells from DDR1 knockout mice, are in strong support of an essential role of DDR1 in maintaining Stat5b activity. We found that female DDR1-null mice had reduced milk protein expression despite the fact that the prolactin levels in the serum of wild type and knockout animals were comparable [Vogel et al., 2001].

Taken together, our data show that activated DDR1 is an essential link between the collagenous extracellular matrix and the secretion of milk proteins. In conjunction with the prolactin and steroid hormone pathways, activated DDR1 constitutes a third matrix-derived pathway essential to maintain lactogenesis. Future research will address the role of individual types of collagen in triggering distinct responses during mammary gland development. It will be of particular importance to study the role of collagen fragments generated by cellular proteases and the function of cryptic epitopes within the collagen triple helix.

ACKNOWLEDGMENTS

We thank Dr. V. Jassal for assistance with mammary gland transplantation and Dr. J. Shillingford and Dr.C. Shemanko for help with manuscript preparation. We acknowledge the help of Dr. Z. Popovic and Dr.D. Templeton with the IRE-assay. This work was supported in part by grants from the Association for International Cancer Research (AICR) and the National Cancer Institute of Canada (NCIC) to W.F.V.

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