Differential activation of STATs 3 and 5 during mammary gland development

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Abstract We have investigated the activity of STAT family members throughout a mammary developmental cycle. Transcripts for Stat 5 were upregulated during pregnancy whilst STAT1 and STAT3 mRNAs were expressed at constant levels. DNA binding complexes containing both STAT5a and 5b showed differing affinities for two naturally occurring STAT5 binding sites. In the involuting mammary gland STAT5 activity decreased whereas STAT3 was specifically activated. These observations reveal a complex pattern of activation of STAT factors during mammary growth, differentiation and remodelling and provide the first evidence for the involvement of STAT3 in development of the mammary gland.

Key words: STAT; Mammary gland; Apoptosis; Signal transduction

1. Introduction

Signal transducer and activator of transcription (STAT) factors are latent cytoplasmic transcriptional regulators activated by cytokines and growth factors. Janus kinases (JAKs) associate with cytokine receptors and phosphorylate STAT factors on a particular tyrosine residue. Activated STATs then form either homo of heterodimers and translocate to the nucleus where they bind to variations of a consensus palindromic 9 bp recognition sequence [1]. To date six STAT family members have been identified.

The cytokine prolactin (PRL) promotes the growth and differentiation of the mammary gland. During lactation, PRL stimulates the sustained activation of STAT5, a factor that is essential for high level transcription of milk protein genes [2,3]. STAT5 is encoded by two closely related genes, STAT5a and 5b [4,5]. We have shown previously that the proximal 400 bp promoter of the milk protein gene β-lactoglobulin (BLG) has three STAT factor binding sites and that these sites have different affinities for STAT5 [6]. Additional factors that modulate growth of the mammary gland such as growth hormone, epidermal growth factor (EGF) and fibroblast growth factor (FGF) have been shown to activate STATs 1, 3 and 5 in tissue culture cells. Therefore, the STAT factor binding sites in the promoters of milk protein genes, such as BLG, may not only act as PRL response elements but also serve to integrate the array of signals that control differentiation of the mammary gland.

We investigated the expression and binding activity of STATs 1, 3, 5a and 5b throughout the mammary gland developmental cycle. STAT3 and STAT5 are activated in a reciprocal fashion, suggesting different roles for these factors during mammary development. Complexes containing STATs 5a and 5b are found from early pregnancy through lactation and have different relative affinities for the two highest affinity sites in the BLG promoter. Interestingly, STAT3 is most strongly activated during early involution when apoptosis is initiated.

2. Materials and methods

2.1. RNA extraction and Northern analysis

RNA was isolated from HC11 cells and mouse mammary gland tissue at various stages of development by the acid-phenol-guanidine thiocyanate single-step method [7]. Following electrophoresis on a 1.5% agarose gel containing 3.8% formaldehyde, RNA was transferred to the Zetaprobe nylon membrane (Bio-Rad, Richmond, CA) and fixed by baking for 1 h at 65°C. Hybridisations were performed overnight at 65°C in buffer containing 0.25 M Na_2HPO_4 and 7% sodium dodecyl sulfate. Filters were washed extensively in 20 mM Na_2HPO_4, 5% sodium dodecyl sulfate at 65°C before autoradiography or quantitation. Equal loading of RNA on gels was confirmed by hybridisation with an oligonucleotide specific for the mouse 28S ribosomal RNA.

2.2. Western blot analysis

Cell lysates were prepared from mouse mammary tissues by grinding in liquid nitrogen and then resuspending in lysis buffer (50 mM HEPES, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium pervanadate, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin). The resulting solution was passed through a 20 gauge needle and syringe and insoluble material removed by centrifugation at 15000×*g* at 4°C for 15 min. Protein estimations were performed using the Pierce Kit. Mammary tissue samples (40 μg per lane) were heated to 95°C for 5 min with an equal volume of loading buffer and loaded onto polyacrylamide gels, using the Biorad protean II system, with a 3% stacking gel and a 10% resolving gel. The gel was run overnight at 40 V.

Protein was transferred to nitrocellulose using an LKB Multiphor system run at 4°C, 8 mA/cm^2 for 1 h. Loading was checked by Coomassie staining of the blotted gel. Blocking was achieved with 5% BSA in PBS/Tween (0.1%) for 1 h at room temperature. Primary antibodies were added at a dilution of 1/1000 in 5% BSA in PBS/Tween. Specifically bound antibody was detected using the ECL System (Amersham) and recorded using X-ray film.

2.3. Nuclear extracts and EMSA

Nuclear extracts were prepared from mammary tissue using the method of Dignam with minor modifications [6]. Daudi cells were treated with 1000 units IFN-α/ml for 20 min and YHHH cells (derivatives of MOLT4) with 1000 units IFN-α/ml for 20 min before the preparation of nuclear extracts, which were kindly provided by Dr. John Girdlestone, (University of Birmingham, UK). EMSA were carried out as previously described [6]. Binding sites were created by annealing the following oligonucleotide pairs: STM 5′ GATTCCGGGAACCGCT 3′ and 5′ ACGCGGTTCCCGGAATC 3′ and A3S 5′

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GATCTCTACCAGGAACCGTCT 3′ and 5′ GATCAGACGGTTC-CTGGTAGA 3′. Antibody supershift analysis was carried out by the addition of antibody to the binding reaction for 2 h at 4°C before the addition of radiolabelled probe. The STAT1 antibody was purchased from Transduction Laboratories (G16930/L6) and STAT3 (sc-482), STAT5a (sc-1081) and STAT5b (sc-835) were purchased from Santa Cruz. The phospho-specific STAT3 (Tyr-705) antibody kit was from NEB.

2.4. Quantitation

Data were quantitated with a Molecular Dynamics Phosphorimager system (Sunnyvale, CA).

3. Results

3.1. Differential transcriptional regulation of STAT genes during mammary gland development

To determine which STAT factors are expressed in the mammary gland, RNA from mouse mammary glands at various stages of pregnancy and lactation was analysed on Northern blot hybridised with partial cDNA clones for STATs 1, 3, 4 and 5 (Fig. 1). The sizes of the STAT transcripts found in the mammary gland corresponded with those previously reported for other tissues [5,8,9]. Low steady-state levels of STAT4 RNA were detected during early development (virgin and days 5 and 10). In contrast, STATs 1 and 3 were expressed at fairly constant levels whilst STAT5 (a and b) was dramatically upregulated during late gestation to reach a peak just before birth. STAT5 was detected in virgin mammary tissue and was expressed at low levels until day 13 of gestation, when differentiation of the epithelial cells commences. Because of the heterogeneity of mammary tissue, RNA from HC11 mammary epithelial cells, originally derived from a mid-pregnant mouse, was also included in this analysis. STATs 1, 3 and 5 were all expressed suggesting that these factors are expressed in the epithelial compartment. In contrast, STAT4 was not detected suggesting that this STAT is expressed in the non-epithelial cells. The downregulation of STAT5 steady-state RNA levels during lactation, when the milk protein β-casein is maximally expressed (lane 6), was surprising. We therefore examined the levels of these STAT proteins.

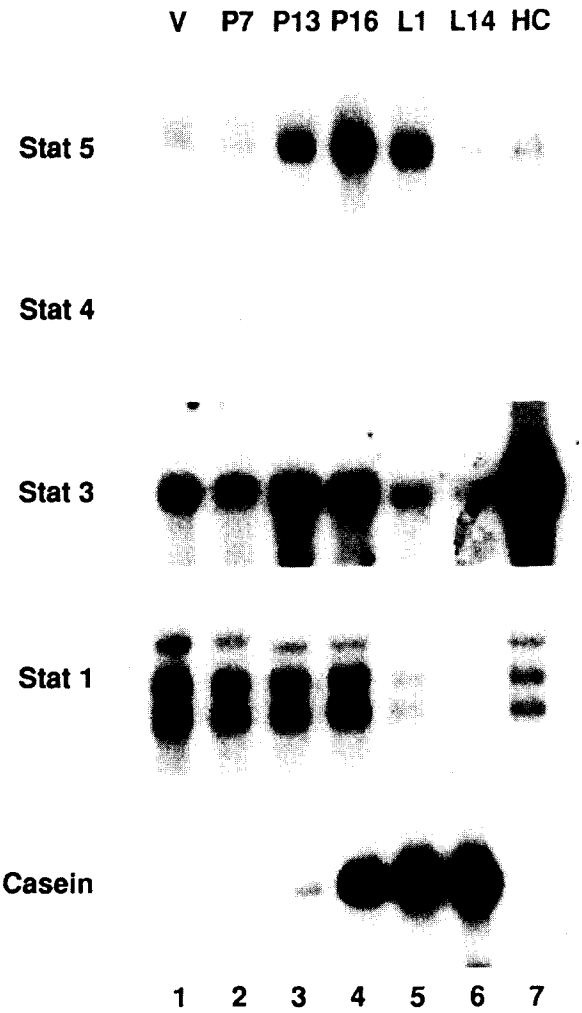

Fig. 1. Northern blot analysis of mRNA levels of 4 members of the STAT family of transcription factors during mammary gland development. Total RNA (10 μg) was fractionated, blotted and hybridised with ^{32}P-labelled STAT-specific cDNA probes spanning the SH2 domain and a cDNA for β-casein. Autoradiography was for 2–7 days following STAT hybridisation and approx. 2 h for the β-casein hybridisation.

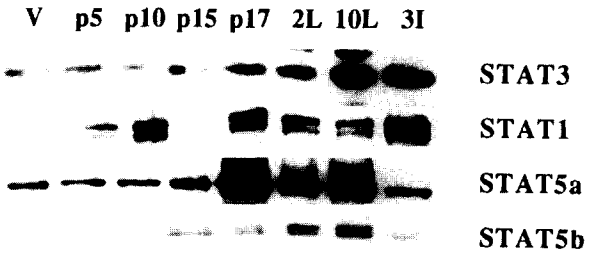

Fig. 2. Western blot analysis of STAT protein levels during mammary development. Whole cell protein extracts (40 μg) of mammary tissue were run on SDS-PAGE gels, blotted, probed with antibodies specific for STATs 1, 3, 5a and 5b (Santa Cruz and Transduction Labs) and detected with the ECL system (Amersham). p, pregnancy; L, lactation; I, involution.

3.2. STAT5a protein is strongly developmentally regulated compared to STAT5b, STAT1 and STAT3

Total protein was analysed by Western blot analysis with antibodies specific for STATs 1, 3, 5a and 5b. Two bands were observed for STAT1 which probably correspond to the p91 and p84 alternatively spliced isoforms. Both proteins were expressed at a fairly constant level from mid-gestation onwards, with a small increase in abundance in involution. The pattern for STAT3 was similar, although in this case a single species was observed. The STAT5b-specific antibody reacted with two closely spaced bands. The upper band appears to be developmentally regulated since it is present from day 15 onwards and throughout lactation, disappearing during involution. This band may be an additional isoform of STAT5b or a differently phosphorylated form. STAT5a shows a dramatic increase from days 15 to 17 of pregnancy whilst STAT5b does not. This does not correlate with the RNA expression profiles since STAT5a and STAT5b are expressed at similar levels [10] and the peak mRNA level occurs before lactation.

3.3. STATs 5a and 5b form heterodimers throughout mammary gland development and have different affinities for the two sites A3S and STM

STAT factors have been shown to form heterodimers. Stim-

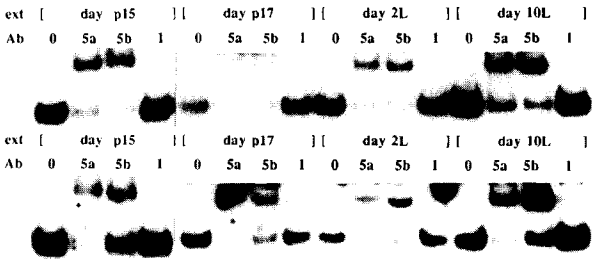

Fig. 3. Analysis of STAT5 heterodimerisation. Antibody supershift analysis was carried out with mammary nuclear extracts from days 15 and 17 of pregnancy and days 2 and 10 of lactation using antibodies which specifically recognise either STAT 5a or 5b or STAT1. In (A), the STM site was used as probe whereas in (B), the A3S site was the probe.

ulation of cells with IL-6 induces the formation of both homodimers of STAT1 and STAT3 and heterodimers of STATs1 and 3 [1]. Given the high degree of similarity between both STAT5 sequences, it seemed likely that STAT5a and STAT5b could form heterodimers. To investigate this possibility and to determine whether the ratio of homo- to heterodimers changes during mammary gland development, we carried out antibody supershift EMSA. The results, shown in Fig. 3 (upper panel), show that similar proportions of STAT5a and 5b bind to the STM site (77% 5a compared to 81% 5b). This indicates that at least 59% of the bound complexes are heterodimers of STATs 5a and 5b, since addition of both antibodies abolished binding (data not shown). Similar results were obtained for the other developmental time points examined (days 15 and 17 of pregnancy and day 2 lactation) and virgin and days 5 and 10 of pregnancy (data not shown).

We have previously shown that the three STAT sites in the BLG promoter have different affinities for STAT5 in the lactating mammary gland [6]. In Fig. 3 (lower panel), we show that this different affinity is reflected in the different relative ratios of STAT5a and 5b dimers which bind to the sites STM and A3S. Antibody supershift analysis with the A3S probe shows that most (89%) of the STAT binding activity in the 0-day lactation sample contains STAT5a. However, a STAT5b-specific antibody reduced binding by only 75%. This result could suggest that heterodimers of 5a and 5b bind preferentially to the STM site whilst homodimers of 5a have a higher affinity for the A3S site.

3.4. STATs 1 and 3 are activated during early apoptosis

The expression of milk protein genes is downregulated during the involution and remodelling of the mammary gland. We showed in Fig. 2 that the levels of STAT5a and 5b protein are decreased during early involution (day 3 following forced weaning at day 6 of lactation). This decrease was not observed for STAT1 and STAT3. It was of interest therefore to determine whether the relatively higher levels of these STATs in the involuting mammary gland has functional significance. Fig. 4 shows an EMSA analysis of nuclear extracts from day 10 lactating and day 3 involuting mammary glands with the A3S STAT binding site. Two complexes were formed with the involuting sample (Fig. 4, lane 6) compared to a single band for the day 10 lactation sample which appears to consist almost entirely of STAT5 (Fig. 4, lane 2). Addition of antibodies to STAT3 practically abolished both bands whilst antibodies to STAT1 abolished the upper band (Fig. 4, lanes 9,10, respectively). The remaining complex is likely to be STAT5. Similar results were obtained with the lowest affinity BLG site A1 (data not shown).

The activation of STAT3 during involution was confirmed using an antibody which specifically recognises tyrosine phosphorylated STAT3 (on residue Tyr 705). In Fig. 5A shows a Western blot analysis of mammary developmental time points with the STAT3 phospho-specific antibody. Highest levels are observed in the involution sample whilst phosphorylated STAT3 is also present from day 5 of pregnancy, a period of high epithelial cell proliferation, decreasing through to day 15 of pregnancy. Fig. 5B is the same blot re-probed to show the total STAT3 protein. By day 3 of involution, apoptosis (programmed cell death) has been initiated in the mammary epithelial cells. It is possible that this STAT activation is occurring in the fat cells or in invading macrophages although, at this early time in remodelling of the gland, the abundance of macrophages is just beginning to rise [11]. In order to investigate the time course of STAT3 activation, samples from days 1, 3, 4 and 6 of involution were examined for phosphorylated STAT3. The results, which are shown in Fig. 5C, show that STAT3 is activated within a day following forced weaning and levels remain constant until day 6.

These results demonstrate that activation of STAT3 is a feature of involution in the mammary gland and suggest that STAT3 activation in the secretory epithelial cells is an early event in the apoptotic pathway.

4. Discussion

The importance of STAT5 as a mediator of PRL induced transcription of milk protein genes is well established [12]. However the roles of the two STAT5 genes and other STAT family members in the development of the mammary gland has not been determined.

Transcripts encoding STATs 1, 3, 4 and 5 were found in the mammary gland. STAT4 mRNA was detected only in undifferentiated mammary tissue but not in a mammary cell line, indicating that its expression is limited to non-epithelial cells or a subset of undifferentiated epithelial cells. STAT4 has a restricted pattern of expression in the mouse and is only detected at high levels in the testes, thymus, spleen and myeloid cells [9]. The mammary gland does contain lymphatic tissue and this may be the source of STAT4 expression. In contrast to STATs 1 and 3, STAT5 mRNA increased during pregnancy and paralleled the rise in casein mRNA, indicating

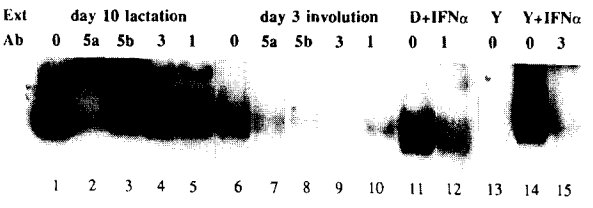

Fig. 4. Activation of STAT1 and STAT3 during mammary gland involution. Antibody interference analysis with extracts from day 10 lactation (lanes 1–5) and day 3 involution (lanes 6–10) and A3S probe. Extracts were pre-incubated with the antibodies indicated above each lane. Control extracts are Daudi cells treated with IFN-α for 20 min (lanes 11,12) which induces primarily STAT1 and YHHH cells (derivatives of MOLT4) also treated with IFN-α for 20 min which induces predominantly STAT3 (lanes 13–15). These extracts were kindly provided by Dr. John Girdlestone (University of Birmingham).

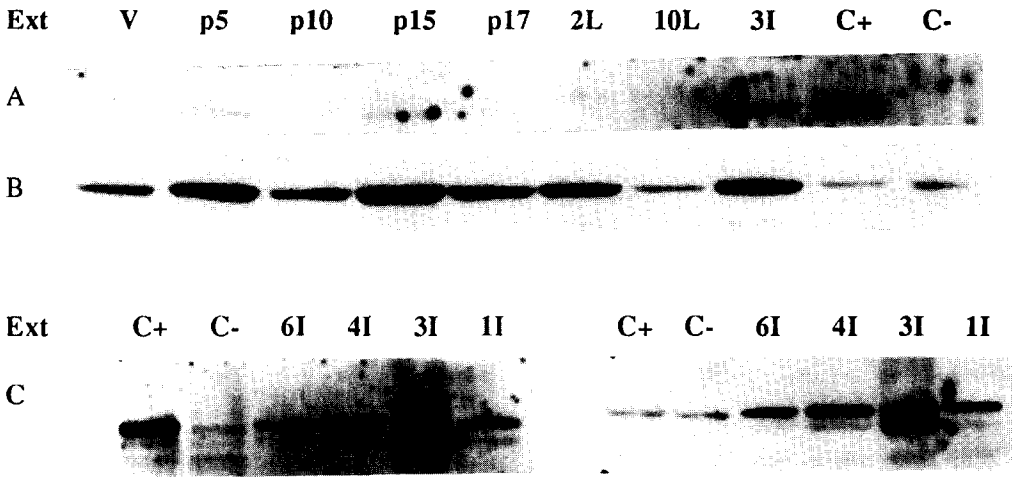

Fig. 5. Detection of tyrosine phosphorylated STAT3 during mammary gland development using a phospho-specific antibody. Whole cell protein extracts (20 μg) of mammary tissue were run on SDS-PAGE gels, blotted, and probed with antibodies to detect either tyrosine phosphorylated STAT3 (A) or total STAT3 (B) using the NEB PhosphoPlus STAT3 (Tyr-705) antibody kit. In (C), involution mammary samples were Western blotted and probed with antibodies to detect either tyrosine phosphorylated STAT3 (left) or total STAT3 (right).

that STAT5 might regulate its own transcription. Such an autoregulatory loop has been described in rat hepatoma cells where STAT3 is induced 3–5-fold by IL-6 [13], a cytokine that activates STAT3.

The decrease in STAT mRNAs during lactation is a phenomenon that affects many genes including housekeeping genes [14] but did not suppress levels of STAT5a and 5b protein. Complexes containing STAT5 heterodimers and homodimers were detected throughout pregnancy and lactation. However, the complexes exhibited preferential binding to different binding sites. How this selective binding affects milk protein gene expression is not known. Nevertheless, this observation together with the marked increase in STAT5a relative to 5b during late pregnancy, provides an additional mechanism for explaining the unique temporal expression patterns of different milk protein genes during gestation. Since the STAT binding sites in the promoters of these genes have different sequences, it is possible that a genes expression reflects both the sequence preferences of STAT5a and STAT5b homo and heterodimers and their relative abundance.

We have detected phosphorylated STAT3 during early pregnancy and involution in the mammary gland suggesting that STAT3 may play a role in proliferation and apoptosis. This correlates with our earlier observations that STATs 1 and 3 are highly activated in breast carcinomas [15]. In this context, it is interesting that overexpression of STAT3 results in apoptosis in M1 cells [16]. Recently, a dominant negative form of STAT5 was shown to inhibit partially the growth of Ba/F3 cells implicating STAT proteins in cell cycle regulation [17]. Furthermore, it has been demonstrated that STAT1 mediates the suppression of cell growth in response to IFN-γ by stimulating transcription of the cell cycle regulator $p21^{WAF1/CIP1}$ [18]. The reciprocal pattern of activation of STAT5 and STAT3 suggests that these STAT factors have distinct roles in the growth and remodelling of the mammary gland. Although it is known that PRL activates STAT5, it remains to be determined which factors activate STAT3 in the mammary gland and whether this is important for growth and apoptosis.

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