A role for the cytoskeleton in prolactin-dependent mammary epithelial cell differentiation

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Summary

The function of exocrine glands depends on signals within the extracellular environment. In the mammary gland, integrin-mediated adhesion to the extracellular matrix protein laminin co-operates with soluble factors such as prolactin to regulate tissue-specific gene expression. The mechanism of matrix and prolactin crosstalk and the activation of downstream signals are not fully understood. Because integrins organize the cytoskeleton, we analysed the contribution of the cytoskeleton to prolactin receptor activation and the resultant stimulation of milk protein gene expression. We show that the proximal signalling events initiated by prolactin (i.e. tyrosine phosphorylation of receptor and the associated kinase Jak2) do not depend on an intact actin cytoskeleton. However, actin networks and microtubules are both necessary for continued mammary cell differentiation, because cytoskeletal integrity is required to transduce the signals between prolactin receptor and Stat5, a transcription factor necessary for milk protein gene transcription. The two different cytoskeletal scaffolds regulate prolactin signalling through separate mechanisms that are specific to cellular differentiation but do not affect the general profile of protein synthesis.

Key words: Cytochalasin D, Colchicine, Cytoskeleton, Microtubules, Prolactin, Mammary epithelial differentiation

Introduction

Many aspects of cell physiology, including cellular differentiation, cell cycle progression, suppression of apoptosis and migration, depend on signals being received through adhesion complexes (Aplin et al., 1999; Assoian and Schwartz, 2001; Schwartz and Baron, 1999; Streuli, 1999). Adhesion complexes are sites of physical interaction between most eukaryotic cells and their extracellular environment. Transmembrane integrin receptors link the extracellular matrix (ECM) to internal cytoskeletal structures in macromolecular assemblies that contain both structural molecules and signalling enzymes.

Adhesion to the ECM has a crucial role in regulating the differentiated state of lactational epithelial cells in the mammary gland (Roskelley et al., 1995; Streuli and Edwards, 1998). Differentiation is maintained by circulating lactogenic hormones such as prolactin, which influence the transcription of mammary-tissue-specific genes. Prolactin mediates transcriptional control of the milk protein β-casein through a pathway involving dimerization of its receptor (PrlR), activation of the associated protein tyrosine kinase Jak2 and phosphorylation and translocation to the nucleus of the signal transducer and activator of transcription Stat5, which binds specific sequences within the β-casein promoter (Groner and Gouilleux, 1995). However, soluble differentiation factors are not sufficient to maintain differentiation – cellular interaction with ECM is also required.

Mammary epithelial cells in vivo contact a specialized ECM, the basement membrane (Prince et al., 2002). Using culture models, it has been established that adhesion to basement membrane proteins (especially laminin-1) is required for the expression of milk protein genes (Aggeler et al., 1991; Barcellos-Hoff et al., 1989; Li et al., 1987; Roskelley et al., 1994; Schmidhauser et al., 1990; Streuli and Bissell, 1990; Streuli et al., 1995b). Differentiation does not occur when cells contact stromal ECM proteins such as collagen 1 and fibronectin.

The laminin signal for mammary differentiation acts in part by controlling the ability of prolactin to activate its receptor and thereby the DNA binding activity of Stat5 (Edwards et al., 1998; Myers et al., 1998; Streuli et al., 1995a). Prolactin cannot activate this pathway in cells cultured on collagen I or fibronectin (Edwards et al., 1998). Thus, adhesion to specific ECM molecules modulates the enzyme-signalling pathway driven by prolactin and thereby regulates milk protein gene transcription and differentiation. The mechanism of crosstalk between the adhesion and prolactin response is not well understood but involves integrins.

Integrins are heterodimeric plasma membrane receptors that transduce signals from the ECM. Evidence of β1 integrin playing a role in mammary differentiation comes from antibody inhibition studies. By using single cells embedded within basement membrane and studying casein expression, it was discovered that β1 integrins regulate the capacity of prolactin to drive differentiation (Streuli et al., 1991). Moreover, transgenic mice expressing a dominant negative form of β1 integrin within mammary epithelial cells show reduced differentiation in vivo (Faraldo et al., 1998). Although

interactions between mammary cells and ECM are mediated by integrins (Edwards and Streuli, 1999), the heterodimers involved in binding stromal ECM proteins are different to those mediating adhesion to laminin, suggesting that specific integrin subunits are required for laminin-induced differentiation signals.

Integrins influence cellular processes directly through signalling enzymes and indirectly via the actin-based cytoskeleton (Aplin et al., 1999; Assoian and Schwartz, 2001; Giancotti and Ruoslahti, 1999; van der Flier and Sonnenberg, 2001). Thus, several possibilities can be considered to explain integrin crosstalk with signalling pathways triggered by soluble factors. Integrin-containing adhesion complexes are focal sites for the accumulation of structural elements including talin, vinculin, α-actinin and actin into multiprotein assemblies (Critchley, 2000; Sastry and Burridge, 2000). Onto these proteins are assembled a range of signalling intermediates, including adaptor proteins and kinases, as well as growth-factor receptors (Howe et al., 1998; Petit and Thiery, 2000; Plopper et al., 1995; Turner, 2000; Yamada and Miyamoto, 1995). Cell-ECM adhesions might therefore control pathways triggered by soluble factors through the accumulation or spatial organization of specific signalling molecules in supramolecular complexes. Alternatively, the integrin-dependent activation of signalling receptors might involve long-range kinase or GTPase signals activated within adhesion complexes (Sastry and Burridge, 2000; Schwartz and Shattil, 2000).

Sites of integrin-mediated adhesion also provide focal points for the assembly of both stress fibres and the cortical actin cytoskeleton. As an alternative to controlling cell phenotype via enzyme pathways, adhesion to the ECM might therefore regulate receptor-mediated events indirectly through the cytoskeleton (Aplin et al., 1999). Indeed, it has been argued that the maintenance of epithelial cell differentiation is dependent on the actin cytoskeleton (Hay, 1993). In mammary cells cultured on basement membrane, we find that the actomyosin filaments are chiefly present in a cortical actin network, whereas, in cells cultured on collagen, they are present as stress fibres. Thus, the cytoskeletal architecture is organized differently in mammary cells that are capable of milk protein gene expression compared with those that are unable to respond to prolactin. This suggests that the cytoskeleton might contribute to prolactin signalling and therefore to cellular differentiation.

The aim of this study was to determine whether an intact cytoskeleton is required for prolactin-mediated signalling, thus providing a mechanistic basis for the crosstalk between ECM receptors and the PrlR.

Materials and Methods

Cell culture

In all of this work, we used first-passage mammary epithelial cells derived from late pregnant mice. Mammary epithelial cells were isolated from 14.5-18.5 day pregnant ICR mice (Pullan and Streuli, 1996). Cells were plated on dishes coated with either collagen I (8 μg cm^{-2}) or basement membrane (laminin-rich Matrigel 14 mg ml^{-1}; Becton and Dickinson) and cultured for 48 hours as described (Streuli et al., 1995a). Cultures were washed and serum starved in Dulbecco's modified Eagle's medium (DMEM)-Ham's F12 medium (Life Technologies, Paisley, UK) containing insulin and hydrocortisone for a further 24-72 hours before stimulating with 150 nM ovine prolactin (Sigma). Cytochalasin D and colchicine (Calbiochem-Novabiochem, Nottingham, UK) were used at final concentrations of 2.0 μM and 2.5 μM, respectively. Control experiments were performed with the vehicle alone; DMSO for cytochalasin D and ethanol for colchicine. In some experiments, cells were plated on dishes precoated with the non-adhesive substrate polyhydroxyethylmethacrylate (polyHEMA) (50 mg ml^{-1} in 95% ethanol).

Immunohistochemistry

Primary cells plated on collagen were passaged onto either basement-membrane- or collagen-coated coverslips. Cells were grown in DMEM-F12 medium and treated with inhibitors for different times. Cells were then fixed in 2% paraformaldehyde, permeabilized for 20 minutes in 0.1% Triton X-100 and incubated with either TRITC-conjugated phalloidin for 1 hour at room temperature (RT) or anti-tubulin antibody (generous gift from K. Gull, University of Manchester) for 1 hour at RT, followed by FITC-labelled anti-mouse secondary antibody for 30 minutes at RT. Cells were viewed using either a Zeiss Axiovert 100M confocal microscope for cells on Matrigel or Zeiss Axioplan microscope for cells on collagen.

Analysis of milk protein expression

First-passage mammary epithelial cells were plated onto basement membrane in DMEM-F12 supplemented with insulin and hydrocortisone. Cytoskeletal inhibitors and prolactin were added for various amount of time. Pulse-labelled cells were starved of methionine for 20 minutes and metabolically labelled with 200 μCi ml^{-1} ^{35}S-methionine for 1 hour in a methionine-free DMEM-F12 base medium containing hormones and inhibitors. The cells were then harvested and aliquots representing equal numbers of total counts were used for immune precipitation with rabbit anti-mouse milk antiserum as described (Streuli et al., 1995a).

RNA extraction and northern blotting

Total RNA was prepared, electrophoresed, transferred to a Zetaprobe membrane (BioRad) and probed with ^{32}P-labelled cDNA fragments of gel-purified cDNA sequences corresponding to a 540 bp *Pst*I fragment of mouse β-casein and 18S cDNA probe, as described previously (Streuli and Bissell, 1990).

Electrophoretic mobility shift assay

Primary mammary epithelial cultures were harvested by rapid trypsinization. Cell pellets were immediately snap frozen in liquid N_2 and nuclear extracts were prepared and analysed as previously described (Edwards et al., 1998; Streuli et al., 1995a; Watson et al., 1991). In supershift assays, nuclear extracts were incubated for 30 minutes at RT with antibodies to Stat 1, Stat 3, Stat 5a or Stat 5b (Santa Cruz Biotechnology, Santa Cruz, CA) before addition of the radiolabelled probe. Gels were exposed to Kodak XAR film or to Fujix Bas 2000 storage phosphorimaging plates for quantitative analysis.

Immunoprecipitation and immunoblotting

Cells were scraped into NET buffer (100 mM Tris, pH 7.4, 300 mM NaCl, 10 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride containing freshly added 0.5 mM PMSF, 13 μM aprotinin and 20 μM leupeptin) and then lysed in NET buffer containing 2% Nonidet P-40. Lysates for pp125 focal adhesion kinase (FAK) immune precipitations were prepared using RIPA buffer (NET buffer plus 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS) Cell lysates were homogenized, rotated for 1 hour at 4°C before centrifugation at 21,000 ***g*** for 30 minutes to clear the detergent-insoluble proteins.

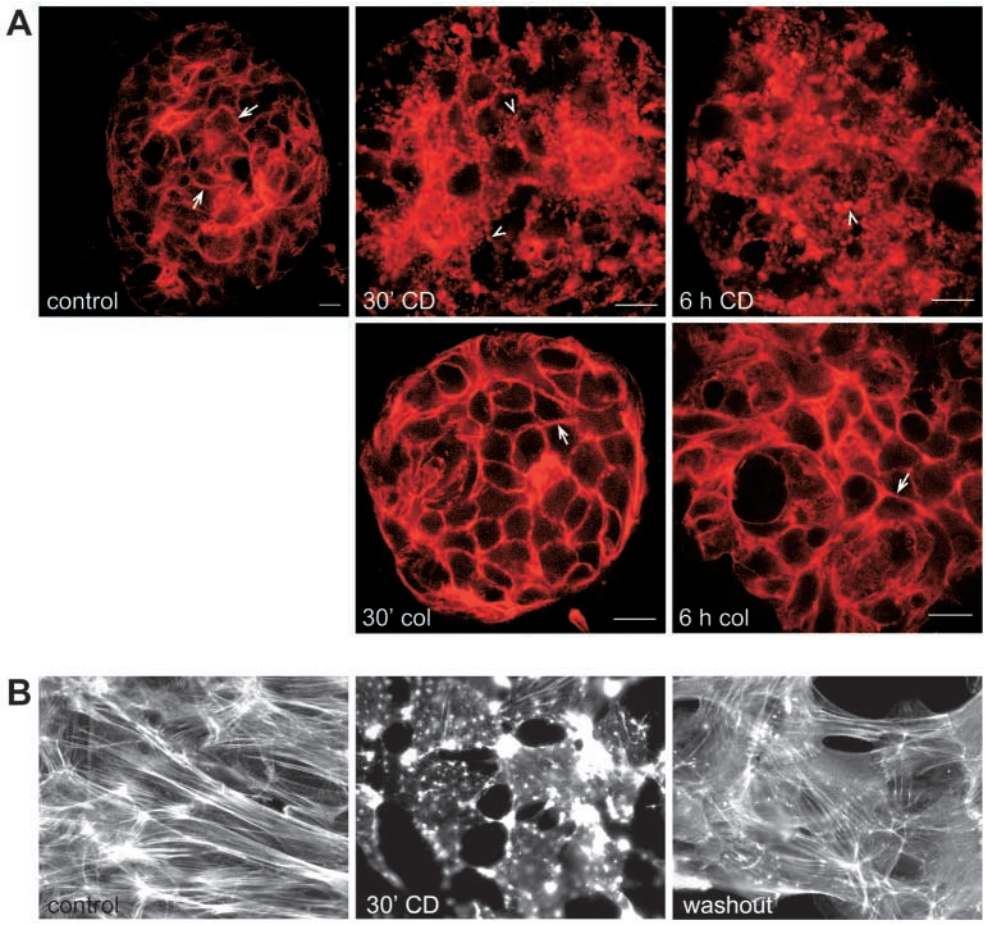

Fig. 1. Cytochalasin D disrupts microfilament networks in primary mammary epithelial cells. (A) First-passage mammary epithelial cells were plated on basement-membrane-coated coverslips in DMEM-F12 supplemented with insulin and hydrocortisone for 24 hours. Under these conditions, the cells form aggregates and become completely surrounded by basement membrane, developing into hollow structures resembling alveoli (Aggeler et al., 1991). Most epithelial cells interact directly with basement membrane (Streuli et al., 1991). The micrographs depict sections at the edges of hollow 'alveoli', where more cells are available for inspection, but the lumens are not visible. Confocal micrographs of control cultures that were fixed and stained for the presence of actin using TRITC-conjugated phalloidin revealed a cortical actin network (arrows) that was not disrupted after 30 minutes or 6 hours of treatment with colchicine (col). By contrast, the cortical actin network was completely disrupted within 30 minutes of cytochalasin-D treatment (CD), and the staining became punctate (arrowheads). Scale bar, 10 μm. (B) Mammary cells were plated on collagen-I-coated coverslips and were either left untreated as controls or treated with 2 μM cytochalasin D (CD) for 30 minutes, then rinsed and harvested at 4 hours following cytochalasin-D removal (washout). Cells were stained for microfilaments using FITC-conjugated phalloidin. Similar experiments using cells plated on basement membrane indicate that the cortical actin organization can be restored after washing out cytochalasin D for several hours (data not shown).

Lysates from protein-normalized samples, using SDS-PAGE followed by Coomassie Blue staining, were immunoprecipitated with rabbit polyclonal anti-Stat5a and anti-Stat5b antibodies (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-Jak2 antibody (1:5000; Upstate Biotechnology, Lake Placid, NY), rabbit polyclonal anti-prolactin receptor antibody or rabbit polyclonal anti-FAK antibody (1 μg ml^{-1}, generous gift of Andy Ziemieki, Laboratory of Clinical Research, Berne, Switzerland), followed by protein-A/Sepharose (Zymed Laboratories, South San Francisco, CA) overnight at 4°C before separation by 6.25% SDS-PAGE. The anti-prolactin-receptor antibody was prepared in our lab against a cytoplasmic peptide corresponding to amino acid residues 466-478 within the mouse prolactin receptor; the IgG fraction from immune serum was antigen purified before use. After transfer to Immobilon P membrane (Millipore, Watford, UK), phosphorylated proteins were revealed with mouse monoclonal anti-phosphotyrosine antibodies 4G10 (1 μg ml^{-1}, Upstate Biotechnology) or PY-20 for the FAK blots (1:500; Transduction Laboratories), followed by enhanced chemiluminescence using an ECL kit (Amersham International, Little Chalfont, UK). Blots were stripped according to Amersham protocol and reprobed with precipitating antibody.

Results

Cytochalasin D and colchicine rapidly disrupt the cytoskeleton in mammary epithelial cells

Previous studies have indicated that the cytoskeleton is necessary to maintain steady-state levels of milk proteins in mammary cells, but they did not examine whether it is required for the prolactin signalling pathway, which is essential for milk protein gene transcription (Blum and Wicha, 1988; Seely and Aggeler, 1991). To understand the role played by actin microfilaments and microtubules in mammary epithelial cell differentiation, we compromised these cytoskeletal networks in cells cultured on basement membrane using pharmacological agents and then determined the ability of prolactin to drive its signalling pathway and milk protein gene expression.

First, we determined the distribution of the actin cytoskeleton. The microfilament networks are mostly present as subcortical actin filaments in primary mammary epithelial cells cultured as multicellular 'alveoli' on basement membrane, where they can undergo lactational differentiation (Fig. 1A). By contrast, stress fibres predominate in monolayer cultures, in which cells cannot express milk proteins (Fig. 1B). Thus, at the morphological level, microfilaments are distributed in different arrays in cells cultured on differentiation-permissive or -non-permissive ECM.

The kinetics of disappearance of the microfilament and microtubule networks were characterized after treatment with cytochalasin D or colchicine. Cells were plated either on basement membrane or collagen I for 24 hours before the addition of 2 μM cytochalasin D to cause actin filament

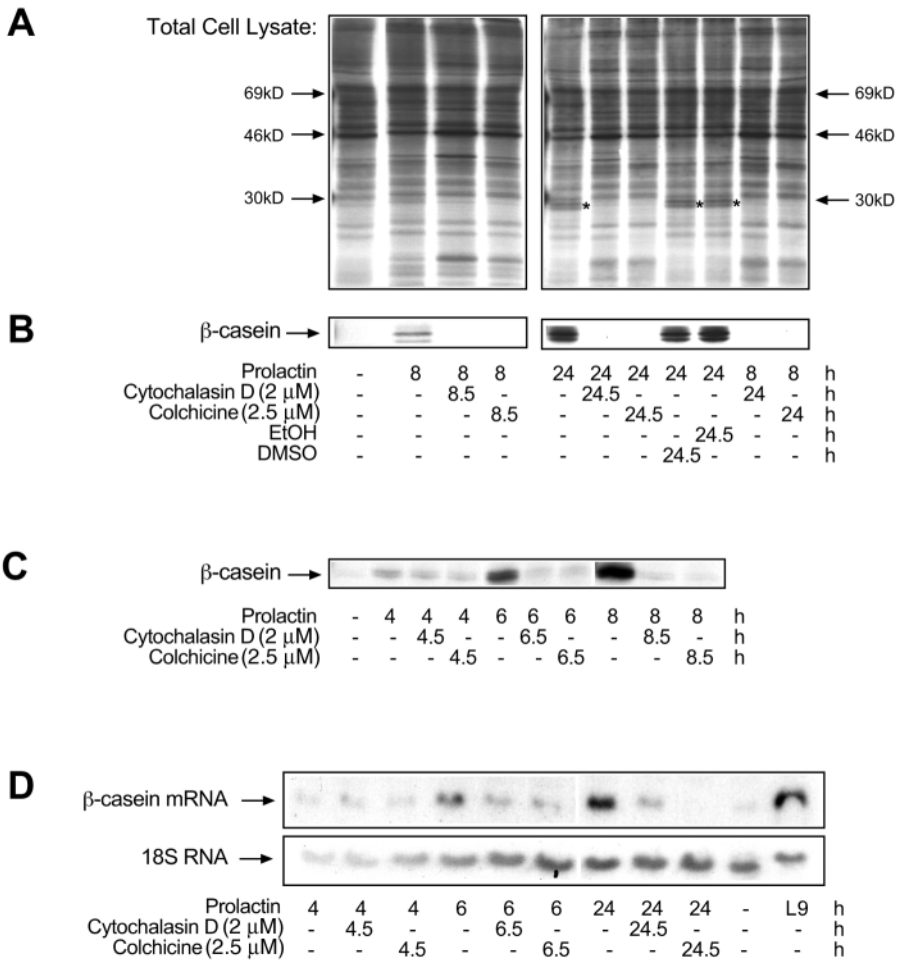

Fig. 2. Cytoskeletal inhibitors affect β-casein expression in primary mammary epithelial cells First-passage mammary epithelial cells were plated on basement membrane in DMEM-F12 supplemented with insulin and hydrocortisone. Cytoskeletal inhibitors and prolactin were added for the indicated times and cells were extracted for protein and RNA analysis. (A-C) Cells were ^{35}S-methionine labelled for 1 hour. Equal amounts of trichloroacetic-acid-precipitable counts were either directly analysed by gel electrophoresis to detect newly synthesized proteins (A) or immunoprecipitated with rabbit anti-mouse milk antiserum before SDS-PAGE, for β-casein detection (B,C). Notice that the overall spectrums of newly synthesized proteins in (A) are not significantly affected by drug treatment. The asterisk in (A) corresponds to β-casein, the levels of which are sufficient to be visualized within the total cell proteins 24 hours after inducing differentiation. Notice also that the exposure time for the gel in (C) is considerably longer than for that in (B). (D) RNA was extracted from cells and 5 μg total RNA was separated by agarose gel electrophoresis before northern blotting for β-casein mRNA levels. The blot was reprobed with an 18S cDNA probe. L9 represents mammary tissue extract from day 9 of lactation, used as a control.

destabilization (Schliwa, 1982). Treatment with the drug for 30 minutes results in disruption of the subcortical actin filaments in cells cultured on basement membrane (Fig. 1A) and in disruption of actin stress fibres in cells on collagen I (Fig. 1B). This inhibition is specific to actin because cytochalasin D has no effect on the microtubule network (data not shown). Actin structures remain retracted over 24 hours treatment but are fully and rapidly reformed after removing cytochalasin D (Fig. 1B). In parallel experiments, cells were treated with 2.5 μM colchicine, which inhibits microtubule polymerization (Deery and Weisenberg, 1981). 30 minutes of drug treatment results in disappearance of microtubules in cells plated on collagen I (data not shown). Colchicine inhibits the microtubule network specifically and does not result in microfilament destabilization either in multicellular ‘alveoli’ or in monolayers.

Together, these results demonstrate that cytochalasin D rapidly and specifically disrupts the organization of stress fibres and subcortical actin networks in primary mammary epithelial cells, whereas colchicine results in the disappearance of microtubules.

Intact cytoskeleton is required for β-casein production

To determine whether subcortical actin networks or microtubules are necessary for milk protein synthesis, mammary cells were plated onto basement membrane and treated with prolactin and cytoskeletal inhibitors for different lengths of time. Cells were then pulse-labelled with ^{35}S-methionine for 1 hour and harvested into RIPA buffer on ice, and newly synthesized proteins were detected by gel electrophoresis. To ensure that the cytoskeleton was disrupted before the induction of differentiation, inhibitors were added 30 minutes before prolactin. Because cytochalasin-D-induced microfilament disruption is rapidly reversible (Fig. 1A), experiments to examine the effect of long-term cytoskeletal inhibition required the continuous presence of drug.

Pulse-labelling cells for 1 hour allowed us to determine whether pharmacological inhibition of cytoskeletal proteins compromises the levels and profile of protein synthesis. The amount of ^{35}S incorporation into protein is not altered even after 24 hours of treatment with cytochalasin D or during the first 8 hours of treatment with colchicine. In the case of colchicine treatment, there is some reduction in protein synthesis at 24 hours, correlating with reduced cell viability. However, importantly, the overall spectrum of newly synthesized proteins is not notably affected by the addition of either of the cytoskeletal inhibitors (Fig. 2A).

In contrast to the lack of an effect on the total profile of protein synthesis, disrupting the cytoskeleton profoundly inhibits synthesis of the milk protein β-casein. This is particularly noticeable after inducing differentiation with prolactin for 24 hours (Fig. 2A,B) but inhibition is also seen

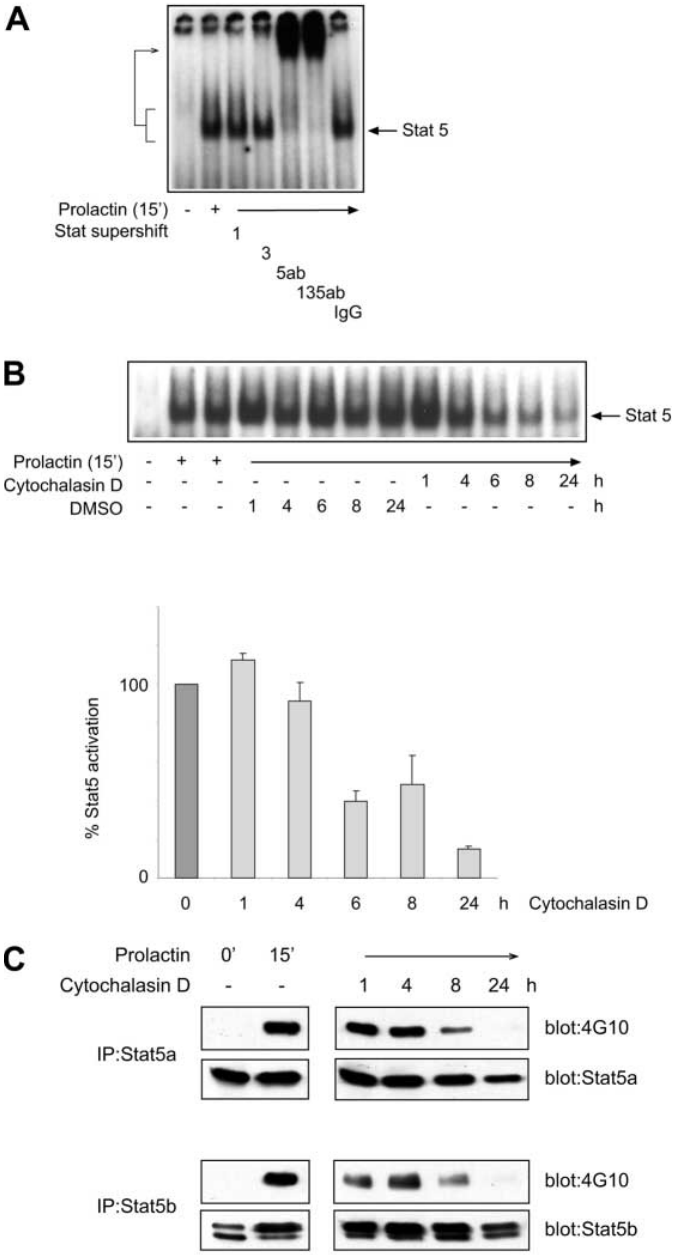

Fig. 3. Blockade of Stat5 activation by cytochalasin D. Primary mammary epithelial cells were cultured on basement membrane and exposed to cytochalasin D for the indicated times. Prolactin was added to the cultures 15 minutes before preparing nuclear extracts for electrophoretic mobility shift assay (EMSA) (A,B) or detergent-soluble lysates for immunoprecipitation of prolactin signalling components (C). (A) Control cultures were either left untreated or were incubated with prolactin 15 minutes before harvesting. Extracts were subjected to EMSA with Stat5 oligodeoxynucleotides, in either the absence or the presence of 2 μg antibodies to Stat1, Stat3, Stat5 or control IgG. Notice that the mobility of the Stat5 band becomes almost completely supershifted in the presence of Stat5 antibodies. (B) Nuclear extracts of cells cultured with cytochalasin D or its carrier DMSO were assessed by EMSA for their ability to recognize Stat5 probe. Protein-DNA complexes were visualized by autoradiography (top) and quantified following storage phosphor image analysis (bottom). In the quantitative analysis, the levels of radioactivity in the samples of cytochalasin-treated cells are compared with those in extracts of cells treated with vehicle alone for the equivalent time and are plotted relative to the signal in control cultures treated with prolactin only. The data were obtained from three independent experiments. (C) Cell lysates were immunoprecipitated (IP) with antibodies to Stat5a or Stat5b. After separation by 6.25% SDS-PAGE, precipitated proteins were analysed by immunoblotting with antibodies for phosphotyrosine (4G10) or the appropriate precipitating antibody. The complete absence of Stat5 phosphorylation after 24 hours of cytochalasin-D treatment, in comparison to its residual DNA binding activity in (B), might reflect differences in the sensitivity of the assays.

over the first 6-8 hours of prolactin stimulation when relatively modest levels of β-casein are synthesized (Fig. 2C). A similar inhibition of β-casein expression to that observed with cytochalasin D also occurs when cells are treated with Latrunculin B, an agent that interacts with actin monomers to prevent polymerization (Morton et al., 2000) (data not shown). The decrease in milk protein expression resulting from cytoskeleton disruption is due to a decrease in mRNA accumulation, because β-casein mRNA levels are reduced after 6 hours of treatment with cytochalasin D or colchicine and are almost zero after 24 hours (Fig. 2D). Together, these results demonstrate that an intact cytoskeleton is necessary for sustained β-casein mRNA and protein expression, and therefore for full mammary differentiation.

However, we observed a different result after short-term cytoskeletal perturbation. The induced level of milk protein synthesis is very low after stimulation with prolactin for only 4 hours, but this does not appear to be compromised by cytochalasin D or colchicine (Fig. 2C). Thus, even though the cytoskeleton is disrupted within 30 minutes of drug treatment, prolactin-induced β-casein synthesis can continue in its absence, at least for 4 hours.

To support these two conclusions, we performed subsequent studies on the signalling pathway regulated by prolactin, that involves Jak2 and Stat5. We also examined this pathway to ask, first, whether cytokine signalling per se can be regulated by the cytoskeleton and, second, whether there are distinct mechanisms by which microfilament and microtubule disruption inhibit differentiation.

Requirement of the actin-based cytoskeleton for Stat5 activation

We asked whether the subcortical actin cytoskeleton is necessary for activation of Stat5, a Prl-induced transcription factor that is essential for β-casein transcription. Primary mammary epithelial cells were plated onto basement membrane and cytochalasin D was added for up to 24 hours before stimulation of the cells with prolactin for 15 minutes. To measure Stat5 DNA-binding ability, cells were harvested and lysates were subjected to an electromobility shift assay, which specifically detects Stat5 DNA binding (Fig. 3A). To determine the tyrosine-phosphorylation status of Stat5, cells were lysed directly on the dish and extracts were analysed for the presence of tyrosine-phosphorylated components within the Prl signalling pathway. Our data led to two conclusions.

First, a brief treatment with prolactin can induce Stat5 DNA binding and phosphorylation even after 4 hours microfilament disruption (Fig. 3B,C). The result of the previous experiments

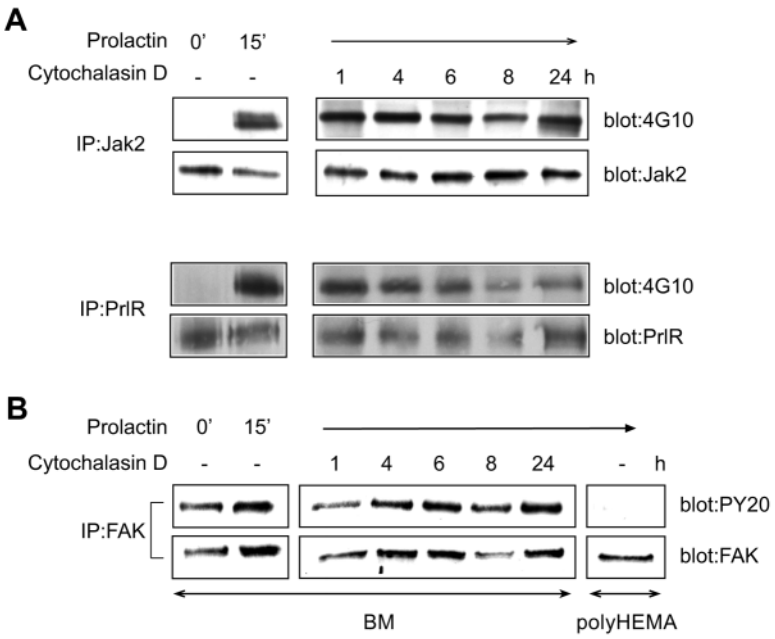

Fig. 4. Cytochalasin D does not compromise proximal events in prolactin signalling or tyrosine phosphorylation of FAK. Cells were cultured as in Fig. 3. (A) Lysates were immunoprecipitated with antibodies to Jak2 or PrlR and then blotted with antibodies for phosphotyrosine (4G10) or the appropriate precipitating antibody. (B) Lysates were immunoprecipitated with antibodies to FAK and then blotted with antibodies for phosphotyrosine (PY20) or the appropriate precipitating antibody. In control experiments, primary mammary epithelial cells were removed to the non-adhesive substratum polyHEMA for 1 hour. Under these conditions, FAK becomes dephosphorylated, in contrast to the cells on basement membrane, in which FAK phosphorylation remains even after cytochalasin-D treatment.

suggested that short-term cytochalasin D treatment does not compromise β-casein expression (Fig. 2C). The inability of cytochalasin D to affect Stat5 function over short time frames supports this conclusion and indicates that cortical actin filaments are not required per se for prolactin to deliver intracellular signals. Second, there is a pronounced reduction in the ability of Stat5 to bind DNA after a 6-24 hour cytochalasin D treatment (Fig. 3B). In addition, the tyrosine phosphorylation of Stat5 declines following drug treatment for 8-24 hours, but this is not due to a notable reduction in the levels of Stat5 protein (Fig. 3C). These results correlate with the earlier data in which cytochalasin-D treatment for 6-24 hours leads to low steady-state levels of β-casein mRNA and protein (Fig. 2). Together, they indicate that the absence of milk protein gene expression following prolonged filamentous actin disruption is caused by an inhibition of the activity of Stat5, a factor essential for transcription of the β-casein gene. This implicates the cytoskeleton in the control of ligand-dependent transcription factor activation.

Cortical microfilament network is not necessary for proximal prolactin signal transduction

Stat5 is controlled through a prolactin-mediated signalling pathway that is regulated by the kinase Jak2, so we next asked whether the actin cytoskeleton is required for upstream signalling at the level of PrlR and Jak2. In striking contrast to the data on Stat5 (Fig. 3), microfilament disruption, even for 24 hours, has no significant effect on the activity of either PrlR or Jak2 as measured by tyrosine phosphorylation (Fig. 4A). Because adhesion signalling downstream of cell interactions with basement membrane proteins might be necessary for efficient prolactin signalling, we examined whether small adhesion complexes are disrupted by cytochalasin D. Surprisingly, and in contrast to studies with other cell types such as fibroblasts (Seufferlein and Rozengurt, 1994), cytochalasin D is unable to inhibit tyrosine phosphorylation of FAK in mammary cells (Fig. 4B).

These results demonstrate, first, that the proximal signalling events initiated by prolactin binding to its receptor (i.e. Jak2 activation and PrlR tyrosine phosphorylation) do not depend on the actin cytoskeleton. We have not been able to dissociate Jak/PrlR activation from FAK phosphorylation and it therefore remains possible that adhesion signalling is necessary for proximal prolactin signals. Second, our data suggest that the failure of Stat5 activation and milk protein gene expression after prolonged cytoskeletal disruption is due to an inhibition of prolactin signal transduction downstream of Jak2.

Role for microtubules in mammary differentiation

Two components of the cytoskeleton, actin microfilaments and microtubules, co-operate to provide the dynamic architectural basis for controlling cell shape and mediating organelle positioning, cell polarity, cytokinesis etc. (Small et al., 1999). To determine whether there is any contribution by the microtubule network to differentiation, we performed additional experiments with the microtubule-destabilizing agent colchicine. We unexpectedly discovered that microtubules are also required for β-casein expression and therefore for mammary epithelial cell differentiation, as described above (Fig. 2). Moreover, this requirement for microfilaments is also manifest at the level of Stat5 phosphorylation and DNA binding (Fig. 5A-C).

We therefore determined whether the mechanism for inhibiting Stat5 activation following microtubule disruption was similar or different to that after compromising the microfilament network. We examined the ability of prolactin to activate proximal elements in its signalling pathway and found that colchicine inhibits tyrosine phosphorylation of Jak2 in a time course that coincides with Stat5 phosphorylation and activation (Fig. 5D). This contrasts with the effect of cytochalasin D, which does not inhibit prolactin-induced Jak2 phosphorylation (Fig. 4A). Further analysis indicates that the levels of PrlR fall in response to colchicine, suggesting that the role played by microtubules in the prolactin signalling pathway is at the level of the plasma membrane through a control on receptor levels (Fig. 5E).

Our experiments reveal an important difference in the mechanism though which microtubules and microfilaments are required for Prl signalling and Stat5 activation. Whereas

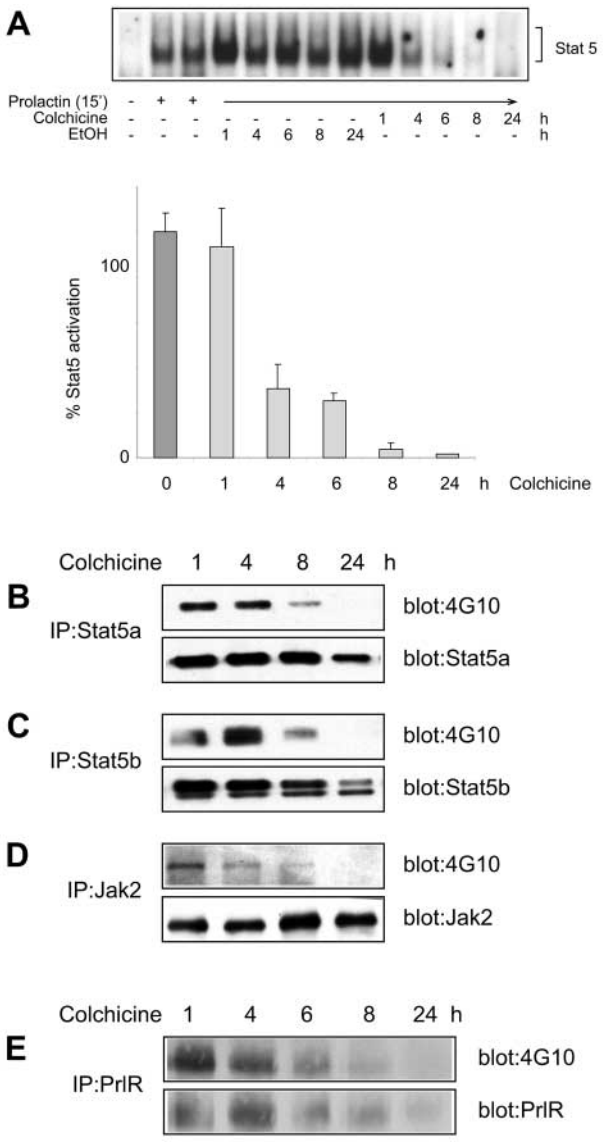

Fig. 5. Inhibition of the prolactin signalling pathway by colchicine. Primary mammary epithelial cells were cultured on basement membrane and exposed to colchicine for the indicated times. Prolactin was added to the cultures 15 minutes before harvesting the cells as in Fig. 3. (A) Nuclear extracts were analysed by electrophoretic mobility shift assay for Stat5-DNA interactions. (B-E) Detergent lysates were immunoprecipitated for Stat5 (B,C), Jak2 (D) or PrlR (E) and immunoblotted with antibodies for phosphotyrosine (4G10) or the appropriate precipitating antibody.

cytochalasin D prevents signal transduction downstream of PrlR, colchicine leads to a downregulation of PrlR itself, leading to the prevention of signal propagation.

Discussion

We have previously demonstrated that there is a crosstalk between ECM receptors and PrlR in mammary epithelial cells that occurs at the level of PrlR activation and influences cellular differentiation (Edwards et al., 1998). In this study, we addressed the hypothesis that the cytoskeleton mediates this crosstalk, thereby allowing prolactin signal transduction and milk protein gene expression to occur. We demonstrate that the absence of an actin microfilamentous network does not compromise proximal events in prolactin signalling. Thus, although the cytoskeleton might be important for coordinating crosstalk between integrin and growth factor receptors in the control of proliferation in certain cell types, it is not required for proximal prolactin signalling in mammary differentiation (Aplin et al., 1999).

We have also discovered a novel role for cytoskeletal integrity in prolactin signalling. The actin-based cytoskeleton appears to be involved with a specific signalling pathway that regulates milk protein gene expression and mammary differentiation, because microfilaments are required to transduce signals between PrlR/Jak2 and its cognate transcription factor, Stat5. Furthermore, microtubules are necessary for mammary differentiation but the mechanism is distinct from the requirement for microfilaments, because microtubules are needed to maintain PrlR levels rather than to communicate between the receptor and its downstream effectors.

ECM dependence of mammary differentiation and the cytoskeleton

Two lines of argument suggest that the ECM might organize the cytoskeleton to provide a permissive environment for mammary differentiation. First, integrins are necessary (Streuli et al., 1991). Integrins are transmembrane receptors that link ECM proteins with microfilament networks and directly regulate cytoskeletal organization in epithelial cells (Geiger et al., 2001; Schoenwaelder and Burridge, 1999; Wang et al., 1999). Second, only specific ECM signals (i.e. those provided by laminin) can co-operate with cytokines to drive tissue-specific gene expression, and mammary cells only form cortical microfilament networks when they are cultured on a laminin-rich matrix (Streuli et al., 1995b). Because the configuration of actin microfilaments adopted by mammary cells varies in response to different ECM proteins, integrins might control mammary differentiation by coordinating the cytoskeleton (Fig. 6A). In other cell types (e.g. 3T3 fibroblasts), there is a link between the cortical actin cytoskeleton and epidermal-growth-factor-mediated signalling to mitogen-activated-protein kinase (Aplin and Juliano, 1999).

We therefore examined mammary differentiation in response to cytoskeletal disruption with pharmacological agents but found no detrimental effect on prolactin signalling for at least 3-4 hours after microfilament disassembly (Fig. 6B). Lack of differentiation in monolayer-cultured mammary cells results from the inability of prolactin to phosphorylate Jak2 and thereby activate Stat5 DNA binding and casein expression (Edwards et al., 1998; Streuli et al., 1995a). Our present data argue that the different cytoskeletal configuration observed in monolayer-cultured cells in comparison to those on basement membrane is not responsible for this inability of prolactin to signal, because microfilament absence following cytochalasin D treatment still allows ligand-induced tyrosine phosphorylation of both Jak2 and PrlR. In support of this, we have found that monolayer-cultured mammary cells induced to differentiate by adding diluted basement membrane proteins to the medium retain abundant stress fibres (N.A. and C.H.S., unpublished).

Communication between adhesion receptors and PrlR is

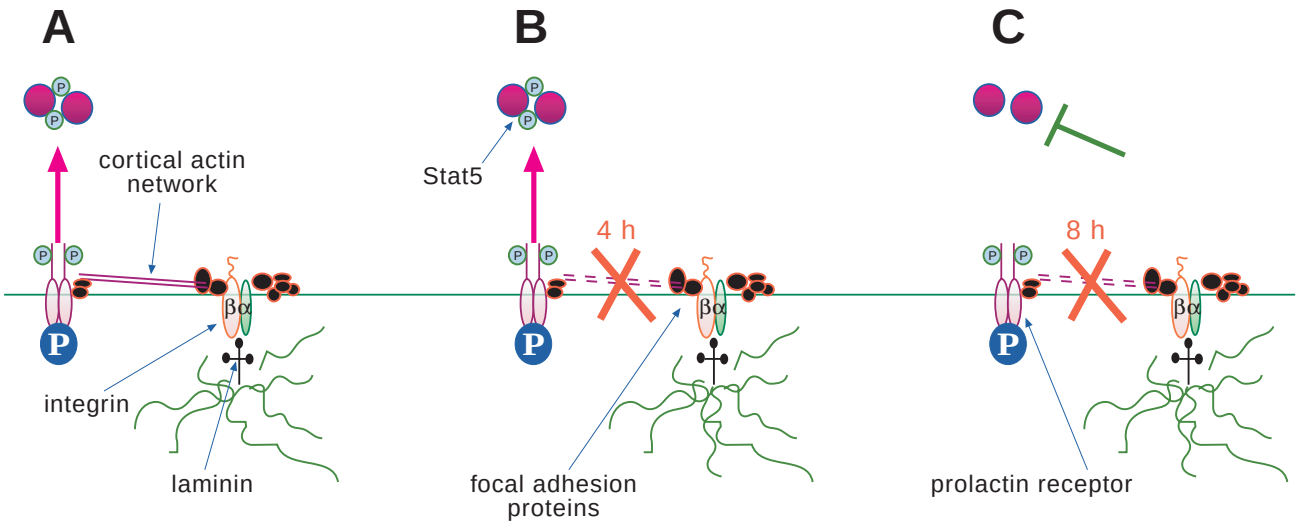

Fig. 6. Modelling a requirement for the cytoskeleton in prolactin signalling. (A) Our initial hypothesis suggested that the actin cytoskeleton is necessary for crosstalk between integrin-containing adhesion complexes and prolactin (P)-mediated signal transduction (magenta arrow), but the data in this paper suggest that this model is not correct. (B) Disruption of the microfilaments for up to 4 hours has no effect on prolactin signalling and activation of Stat5. This indicates that the signals downstream of PrlR are independent of the cortical actin network. Because Prl signalling is ECM dependent (Edwards et al., 1998), ligand-activated prolactin receptors might accumulate within multiprotein clusters of proteins localized to focal adhesions or, alternatively, that integrin-regulated adhesion complexes activate PrlR through long-range signals. (C) Disruption of the microfilaments for more than 4 hours leads to delayed restriction in Stat5 activation. Phosphorylation of PrlR, but not Stat5, can still be induced by ligand. Loss of microfilaments might lead to a delayed activation or synthesis of phosphatases or other inhibitors of cytokine signalling (green inhibitory arrow).

therefore dependent on an alternative mechanism of crosstalk. One possibility is that adhesion receptors directly regulate the distribution of PrlR or its efficiency of signalling, as occurs for the interferon-γ receptor (Ivaska et al., 2003). Another is that crosstalk between ECM and prolactin receptors is mediated by long-range signals, for example small GTPases (Almeida et al., 2000; Berrier et al., 2000; Gilmore et al., 2000; Schwartz and Shattil, 2000). A further long-range integrin signalling kinase, FAK, is involved with adhesion control of survival regulation in mammary cells (Gilmore et al., 2000), but we have not yet been able to dissociate its phosphorylation from Jak/PrlR activation. Interestingly, FAK binds Jak2 after stimulation by the related cytokine, growth hormone; however, in this case, it has been ruled out as a requirement for Stat-mediated transcription (Zhu et al., 1998). These possible crosstalk mechanisms are currently under investigation in our laboratory.

Microfilaments are essential for propagating cytokine signals downstream of the Jak2-PrlR complex

Microfilaments have been shown to be required for the maintenance of milk protein gene expression but the mechanism has not previously been addressed (Blum and Wicha, 1988; Seely and Aggeler, 1991). Our study now demonstrates that microfilaments are involved in PrlR/Jak2-mediated signalling to Stat5, and it therefore provides novel insights into both the mechanisms of signal transduction and those controlling epithelial cell differentiation.

A model to explain how signals generated at the plasma membrane reach the nucleus is that the kinase-mediated reactions occur within the vicinity of the plasma membrane, whereas the final activated component in the pathway, in this case Stat5, is delivered to the nucleus via the cytoskeletal scaffold. Mechanical networks are important for other signalling pathways, because p53 is transported to the nucleus on microtubules, whereas activated ERK requires the actin cytoskeleton for transport into the nucleus, where it can phosphorylate Elk-1 (Aplin et al., 2001; Forgacs, 1995; Giannakakou et al., 2000). Similarly, factors waiting to become phosphorylated by plasma membrane receptors might be delivered by a microfilament-controlled mechanism (Fincham et al., 1996). However, because of the delayed response of mammary cells to cytochalasin D in terms of reduced Stat5 activation and cellular differentiation, our data argue that microfilaments are not actually required for either the arrival of Stat5 at the Jak2-PrlR complex or Stat5 translocation to the nucleus. Similarly, the related transcription factor Stat1 has been shown to translocate from a Jak/interferon-γ-receptor complex to the nucleus by random diffusion (Lillemeier et al., 2001).

We therefore hypothesize that, after prolonged microfilament disruption, inhibitory factors become activated. Thus, although microfilaments are necessary for the prolactin signalling pathway, this requirement might be indirect (Fig. 6C). Protein tyrosine phosphatases, cytokine-inducible SH2-containing proteins and suppressor-of-cytokine-signalling proteins all negatively regulate Jak2-Stat5 signalling, and some of these inhibitory proteins might be activated slowly by microfilament disruption (Barkai et al., 2000; Matsumoto et al., 1999; Ram and Waxman, 1999; Tomic et al., 1999; Yasukawa et al., 2000). This is a new hypothesis that now needs further exploration. However, our preliminary analysis by immunoblotting cell extracts with antibodies to SOCS-3 (which binds PrlR and thereby inhibits downstream signals) and CIS (which inhibits Stat5) (Dif et al., 2001; Levy and

Darnell, 2002) indicates that neither of these proteins are increased after cytochalasin treatment. In addition, we have attempted to examine the contribution of newly synthesized, possibly inhibitory, factors that might affect Stat5 phosphorylation by treating cells with cycloheximide for 8 hours. However, we find that this treatment completely blocks Stat5 activation in mammary cells without affecting its levels (G.S.Z. et al., unpublished). Therefore to understand further why several hours of cytoskeleton disruption disrupts PrlR signalling, we now plan future experiments to examine trafficking of GFP-Stat5 and also to measure changes in the levels and activity of Stat5 inhibitory proteins. Many changes in cell phenotype occur slowly over a period of hours to days, even though the signalling pathways that are frequently measured in biochemical analyses are extremely rapid. It might be that the cytoskeleton has a more important role to play in the orchestration of cellular responses than has previously been envisaged, and that it contributes to some of the delayed responses that are commonly seen in normal cell behaviour.

Microtubule network is required for prolactin signalling and mammary differentiation

This study also shows that the requirement for cytoskeleton in the control of Prl signal transduction and epithelial-cell differentiation is dependent on microtubules. The microtubule network is emerging as a regulator of gene expression. Microtubule disruption alters the transcription of several genes including, for example, NFκB-dependent genes and those encoding interleukin-1 and cyclooxygenase-2 (Manie et al., 1993; Rosette and Karin, 1995; Subbaramaiah et al., 2000). Microtubules are known to have a role in regulating the expression of milk protein genes (Blum and Wicha, 1988; Houdebine and Djiane, 1980) and our results now reveal a mechanism, because the phosphorylation and DNA-binding activity of Stat5 disappear within a few hours of microtubule disruption. Interestingly, there is a gap between the loss of Stat5 DNA binding and the loss of the phosphorylation status of Stat5 after colchicine treatment, indicating that disruption of the microtubule network might have more than one inhibitory effect on the prolactin signalling pathway. We have explored further the mechanism for Stat5 inactivation after microtubule disruption and discovered that the PrlR protein itself disappears in response to colchicine. This suggests that microtubules are required for receptor availability at the plasma membrane, and thereby a signalling response.

In the current model for PrlR turnover at the cell surface, there is a dynamic balance between receptor endocytosis and receptor insertion into the plasma membrane (Genty et al., 1994). Studies using transfected PrlR in CHO cells indicate that PrlR is constitutively internalized with a half-life of 80 minutes, and is subsequently replenished with newly synthesized PrlR. Very little is known about the mechanism of PrlR internalization, but the receptor might be degraded through the proteasome (as is the case for its relative, growth hormone receptor) (van Kerkhof et al., 2000). Our experiments indicate that endogenous PrlR in mammary cells might have a longer half-life at the plasma membrane than transfected PrlR in CHO cells. Nevertheless, because PrlR disappears following colchicine treatment, we propose that microtubules are involved in its trafficking. If so, this would provide a new mechanism for the cytoskeleton in receptor signalling, by organizing the exocytic pathway and therefore providing sufficient receptor to initiate signal transduction.

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