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MAP kinase pathway signalling is essential for extracellular matrix determined mammary epithelial cell survival

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Abstract

Mammary epithelial cells in primary cell culture require both growth factors and specific extracellular matrix (ECM)-attachment for survival. Here we demonstrate for the first time that inhibition of the ECM-induced Erk 1/Erk 2 (p42/44 MAPK) pathway, by PD 98059, leads to apoptosis in these cells. Associated with this cell death is a possible compensatory signalling through the p38 MAP kinase pathway the inhibition of which, by SB 203580, leads to a more rapid onset of apoptosis. This provides evidence for a hitherto undescribed Erk 1/Erk 2 to p38 MAP kinase pathway 'cross-talk' that is essential for the survival of these cells. The cell death associated with inhibition of these two MAP kinase pathways however, occurred in the presence of insulin that activates the classical PI-3 kinase-dependent Akt/PKB survival signals and Akt phosphorylation. Cell death induced by inhibition of the MAP kinase pathways did not affect Akt phosphorylation and may, thus, be independent of PI-3 kinase signalling. *Cell Death and Differentiation* (2000) **7,** 302–313.

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Abbreviations: EHS, Engelbreth-Holm-Swarm tumour; FACS, fluorescence assisted cell sorting; Erk, extracellular regulated kinase; MAP, mitogen activated protein (kinase); MEKK, mitogen activated protein kinase kinase kinase; PKB, protein kinase B; DAPI, 4,6-diamido-2-phenylindole

Introduction

Pregnancy is associated with an extensive expansion of mammary epithelial cell numbers as the milk secreting alveoli are generated. In mid-to-late pregnancy these alveolar epithelial cells differentiate in response to lactogenic hormones, particularly prolactin. It is hypothesised that the alveolar epithelial cells, once differentiated, are programmed to die and require survival factors for their functional maintenance throughout the remainder of pregnancy and during lactation, until they die by apoptosis in the post-weaning involution period.[1,2] A range of studies have characterised insulin/IGF-1,[3–7] hydrocortisone or progesterone,[8,9] Stat 5a[10,11] and the association of the mammary epithelial cells with a laminin-rich extracellular matrix (ECM)[12–16] either alone or in combination, as potential survival signals for differentiated alveolar mammary epithelial cells, *in vivo*, and in tissue culture.

The transition of the alveolar mammary epithelial cell from the proliferating to the differentiated state, to apoptosis, seen *in vivo*, can be reiterated *in vitro*, in primary cultures of mid-pregnant mammary epithelial cells:[13,17–20] The cells will proliferate in the presence of serum and growth factors (EGF); survival in the absence of growth factors can be achieved by maintaining the cells in the presence of insulin (in high concentrations) and in association with an extracellular matrix, rich in laminin (e.g.[14,16]); and differentiation responses (expression of milk protein genes) are observed under these latter culturing conditions in response to prolactin stimulation.[13,20,21] Apoptosis ensues if either of the survival factors (insulin or ECM) is omitted from the culture. The laminin-rich ECM is proposed to act through $\alpha 6\beta 1$-integrin receptors to effect cell survival, at least in part, by stabilising PI-3 kinase-dependent survival responses to insulin (or IGF-1).[12,14,16] Insulin, in higher doses, and IGF-1 at physiological concentrations protects mammary epithelial cells from apoptosis by a PI-3 kinase-dependent mechanism, which is associated with the maintained phosphorylation of Akt.[16] It is quite possible that this latter signalling pathway causes phosphorylation of the Bcl-2 family protein Bad, and thus, causes Bad neutralisation and, hence, the anti-apoptotic influence.[22–28] While these central functional requirements for cell survival responses of ECM-integrin receptor interactions are firmly established for the mammary epithelial cell, a full picture of the intracellular signalling responses to these stimuli are not.

Faraldo *et al*[29] disrupted $\beta 1$-integrin signalling in mouse mammary gland by expression of a chimeric integrin, which uncoupled $\beta 1$-integrin adhesion from intracellular signalling. This was associated with significant developmental restriction and increased apoptosis during pregnancy, reduced milk protein gene expression and apparent defects in cell polarisation during lactation, reflecting the importance of ECM-integrin signalling in the functioning gland. ECM-integrin receptor interactions in mammary epithelial cells most likely trigger formation of focal adhesion complexes with recruitment of actin-cytoskeletal anchoring components and a range of intracellular signalling molecules (reviewed

in[30,31]). The regulatory/signalling components recruited, will for instance, include Rho which would play a key role in regulating the assembly of the adhesion complexes and associated stress fibres; Ras, Src and associated proteins, possibly MAP kinases and PI-3 kinase but with an anchoring contribution from recruited, activated focal adhesion kinase (FAK). For instance, Miyamoto *et al*[32] have elegantly demonstrated how integrin clustering in single fibroblasts by multivalent ligands leads to the 'hierarchical' recruitment of a wide range of structural, signalling and regulatory components to focal adhesions, including Src, FAK and MAP kinase components. Integrins also recruit a range of growth factor receptors e.g. epidermal growth factor (EGF), platelet derived growth factor and basic fibroblast growth factor receptors to focal adhesions. This recruitment increases phosphorylation and activation of the growth factor receptors (GFR) implying integrin-GFR 'cross-talk'. Recent reports suggest potential functional 'cross-talk' in mammary epithelial cells between integrins and: the prolactin receptor signalling system;[33] the insulin receptor PI-3 kinase signalling system;[16] and, the EGF receptor signalling system.[34] While FAK phosphorylation most likely follows laminin-integrin receptor interaction in mammary epithelial cells,[35] the recruitment of other signalling components, for instance, MAP kinases and their subsequent contribution to cell signalling cell survival and differentiation responses remains undescribed.

We now report that inhibition of the MAP kinase, Erk 1/Erk 2, signalling pathway impairs mammary epithelial cell survival in the presence of cell-ECM association and insulin. Inhibition of both the Erk 1/Erk 2 and p38 MAP kinase pathways led to a more rapid apoptotic response in the ECM-overlaid cells. We show that ECM overlay leads to a rapid short-term phosphorylation (and subsequent pulsatile phosphorylation) of Erk 1/Erk 2 but when the Erk 1/Erk 2 pathway is inhibited a slower, perhaps compensatory, activation of the p38 MAP kinase pathway in response to ECM overlay is detected. Omission of insulin from the culture medium, or selective inhibition of the PI-3 kinase signalling pathway, precipitates apoptosis of the ECM-overlaid cells and causes a loss of phosphorylation of Akt/PKB, a known cell survival pathway that links extracellular signals to the cell death machinery.[23,24] In contrast, apoptosis triggered by selective inhibition of the Erk 1/Erk 2 and p38 MAP kinase pathways does not affect Akt/PKB phosphorylation. This suggests the existence of an ECM-determined MAP kinase-dependent mammary epithelial cell survival pathway that may not signal through the PI-3 kinase- Akt- associated intracellular signalling pathway.

Results

Mammary epithelial cells from mid-pregnant mice maintained in primary culture can reiterate the phases of proliferation, differentiation, survival and apoptosis characteristic of lobuloalveolar epithelial cells, *in vivo*.

Using nucleosomal laddering analysis to detect apoptosis, we have shown (Figure 1A) that such primary cultures maintained on plastic in differentiation medium (containing hydrocortisone, insulin and prolactin (see Materials and Methods)) (lane 4) display abundant nucleosomal laddering but when, in addition, are overlaid with a laminin-rich ECM (Matrigel/EHS matrix) (lane 3) are protected from cell death. When proliferating in the presence of serum, EGF and lactogenic hormones there was also no evidence of nuclear fragmentation (Figure 1A, lane 2). As with cells cultured in the presence of EHS ECM which showed no detectable DNA laddering (Figure 1B, lane 2), cells cultured in the absence of ECM but in the presence of soluble laminin also showed no significant cell death (lane 4). In contrast, cells cultured on a collagen matrix showed extensive apoptosis (Figure 1B, lane 5) comparable to that detected in cells cultured in the absence of ECM-association (lane 3). This confirms, as suggested by the work of others,[12,16,36] that laminin is the component of the EHS matrix that is sufficient to determine mammary epithelial cell survival. When we assessed apoptosis by FACS cell cycle analysis using the appearance of a sub-G1/G0 peak as a cell death marker[37] (Figure 1C) we could again see a suppression of the apoptotic response in cells cultured in the presence of ECM overlay when compared to cells cultured in the absence of this laminin-rich matrix (compare right hand- to left hand-panel). Quantitative evaluation of seven independent experiments showed that ECM overlay reduced sub-G1/G0 fluorescence by 78% (mean value). That is, ECM overlay reduced the number of sub-G1/G0 cells from 40.3% ($\pm$5.1% S.E.M.) to 9.0% ($\pm$2.1% S.E.M.) (Figure 1C, bottom panel). Furthermore, Figure 1D shows analysis of apoptotic death in these cells adjudged by nuclear morphology after DAPI staining (see Materials and Methods). Culturing of the cells in the presence of ECM and the lactogenic hormones, hydrocortisone, insulin and prolactin for 48 h shows only trace amounts of apoptosis (lane 1). However, omission of an ECM overlay induces a significant amount of death ($P<0.001$) (lane 2). As will be discussed below, when the cells are cultured with both hormones and ECM overlay but in the presence of the MAP kinase inhibitors, PD 98059+SB 203580 (lane 3), or the PI 3 kinase inhibitor, LY 294002 (lane 4), we again see significant levels of apoptosis ($P<0.001$). Thus, we show by three different techniques that in the presence of insulin a laminin-rich ECM is a sufficient survival factor for cultured primary mammary epithelial cells. Also, in the presence of ECM-overlay, and on prolactin stimulation, these cells show potential for differentiation[13] (and results not shown).

The analysis of the nucleosomal DNA laddering presented in Figures 1, 2 and 5 was carried out on small molecular weight DNA cellular isolates, Hirt supernatants.[38] This technique usually results in only low or negligible amounts of 'carried over' high molecular weight DNA to the supernatant extract in the absence of apoptosis (e.g. Figure 1A, lane 3 and Figure 2A lanes 4 and 6) although there can be considerable variation (e.g. Figure 1B, lanes 2 and 4 and Figure 5A, upper panel, lane 2). We emphasise that the absence of a high molecular weight DNA band does not reflect a DNA sample loading error; analysis of five times the sample amount shown in Figure 1A, lane 3 still showed no evidence of nucleosomal DNA laddering (data not shown). We propose that this technique can be used to generate valid DNA laddering profiles as three different,

quantitative analyses, FACS analysis (Figure 1C), nuclear morphology assays (Figure 1D) and death effector activity measurements (Figure 2C), all generated consistent data.

A wide range of studies (e.g.[32,39]) would suggest that occupation of integrin receptors by ECM ligands, with subsequent formation of focal adhesions, triggers activation of intracellular signalling cascades which modulate cellular behaviour and that MAP kinase dependent cascades are often among those activated. We investigated the dependence of ECM-determined mammary epithelial cell survival on MAP kinases using the MAPK kinase (MEK1) specific inhibitor, PD 98059, which blocks activation of the Erk 1/Erk 2 (p42/p44 MAPK) pathway.[40]

Figure 2A (top panel) shows that inhibition of the Erk 1/Erk 2 pathway by PD 98059 (10 μM) led to an abrogation of cell survival (lane 5) as did the lack of an ECM overlay (lane 3). Inhibition of the p38 MAP kinase pathway by incubating the ECM-overlaid cells with the specific p38 MAP kinase inhibitor, SB 203580 (1.0 μM),[41] had no effect on the ECM induced survival (lane 6), indicating the selectivity of the PD 98059 effect. A time-course nucleosomal laddering analysis (Figure 2A, middle panel) showed

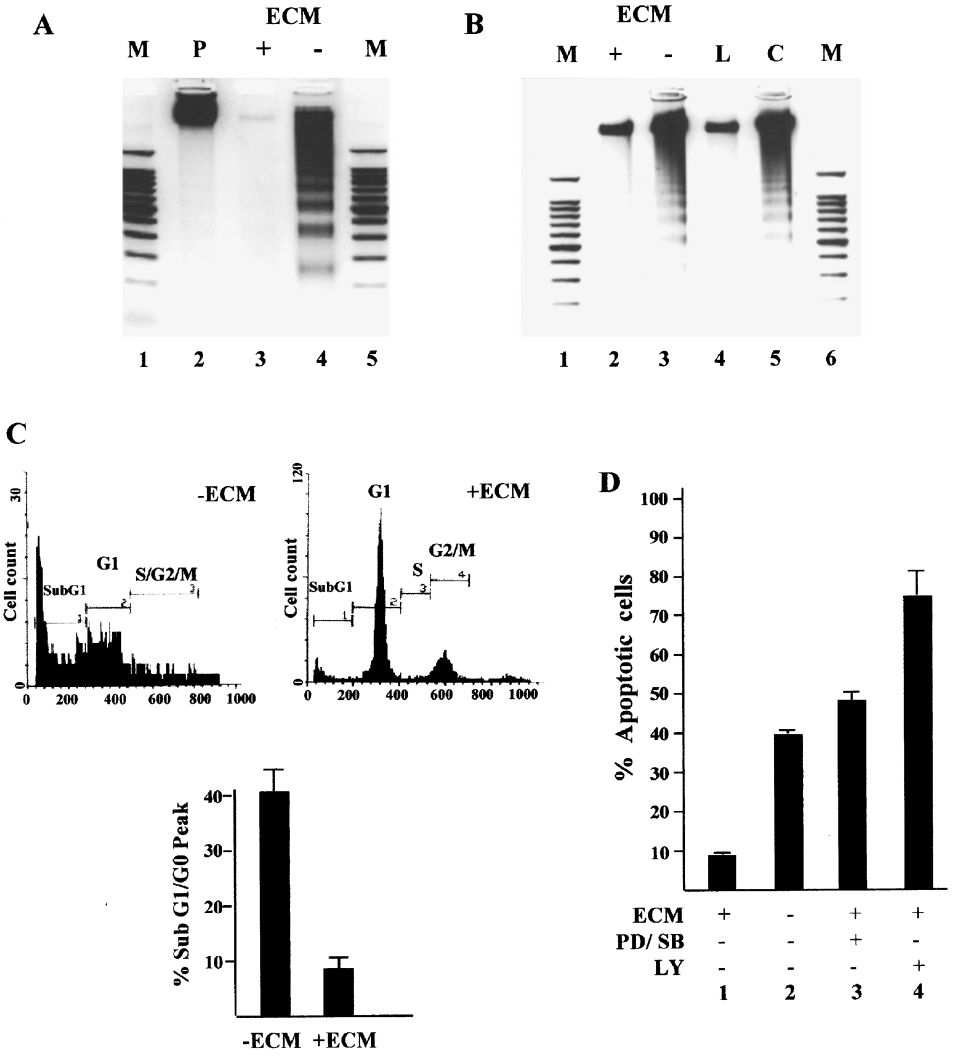

Figure 1 Extracellular matrix promotes survival of mammary epithelial cells in primary culture. (**A**) Nucleosomal laddering as a marker of apoptosis. Shown is an image of an ethidium bromide stained agarose gel electrophoresis analysis of low molecular weight DNA isolated from primary cultures of mammary epithelial cells maintained under proliferating conditions (lane 2); and cultured in the presence (+) or absence (−) of ECM-overlay for 48 h (lanes 3 and 4, respectively). DNA molecular weight markers (M) (100 bp ladder, Promega) are shown in lanes 1 and 5. (**B**) As (**A**) (left), nucleosomal laddering of low molecular weight DNA from cells cultured in the presence (+) or absence (−) of ECM-overlay for 48 h (lanes 2 and 3, respectively). Also shown, cells cultured in the absence of EHS ECM but in the presence of soluble Laminin (L) or on Collagen (C) for 48 h (lanes 4 and 5, respectively). DNA molecular weight markers (M) are shown in lanes 1 and 6. (**C**) top panels: FACS analysis of nuclei from primary cultures of mammary epithelial cells maintained in the absence or presence of ECM, as in (**A**) (above). Bottom panel: Graphical representation of the FACS analysis data shown in top panels. Results shown are mean ± S.E.M. for seven independent experiments. (**D**) Apoptotic cell death measured *in situ* by nuclear morphology. Cells were cultured in the presence (lane 1) the absence (lane 2) of ECM-overlay or in the presence of an ECM-overlay and PD 98059 (10 μM)+SB 203580 (1.0 μM) (lane 3) or LY 294002 (10 μM) (lane 4). Shown are mean per cent values (± S.E.M.) of three determinations per treatment where >1300 cells were assessed in four fields for each determination. Statistical analysis, students' *t*-test: values 2, 3 and 4 were >than 1, $P<0.001$; value 4 was >3, $P<0.05$ and value 4 was >2, $P<0.01$

that inhibition of the Erk 1/Erk 2 pathway does not affect cell survival for the first 24 h (lanes 2–8) but induces a dramatic effect on cell survival between 24 and 48 h, comparable to that observed in cells not overlaid with ECM (compare lanes 9 and 11). However, a combination of the MAP kinase pathway inhibitors, PD 98059 + SB 203580, caused a rapid and reproducible impairment of survival of ECM-overlaid cells (Figure 2A, bottom panel): This effect was apparent within 3 h and well established by 9–12 h (lanes 4–7). This suggests a co-operative action between these two MAP kinase pathways in modulating ECM-determined mammary epithelial cell survival.

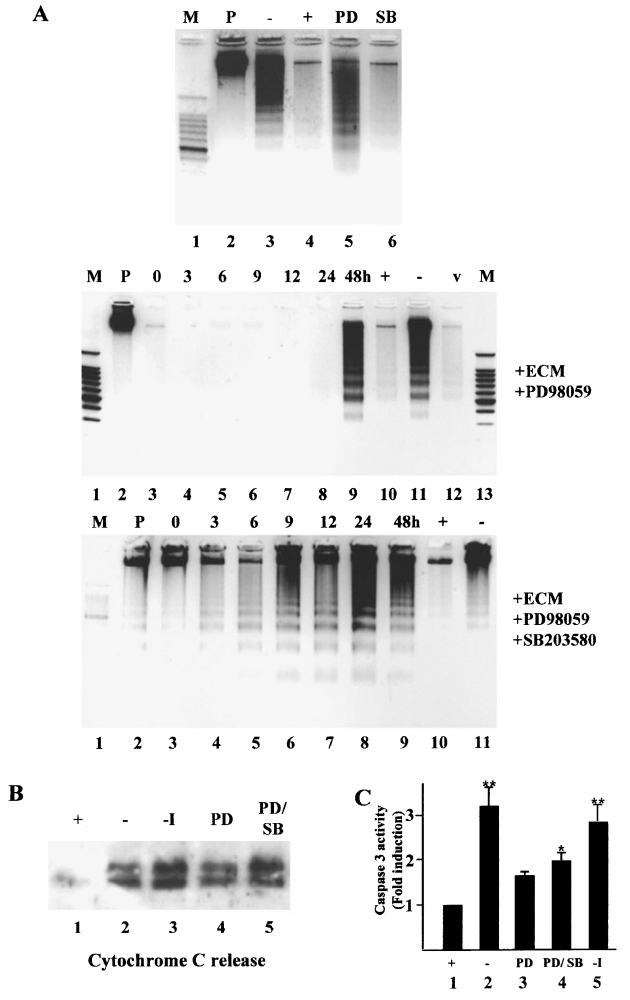

Figure 2 MAP kinases are essential modulators of ECM driven epithelial cell survival. (**A**) Images of ethidium bromide stained agarose gels of low molecular weight DNA isolated from primary cultures of mammary epithelial cells. Top panel: cells cultured under proliferating conditions (P) (lane 2) and for 48 h in the absence (−) or presence (+) of ECM-overlay (lanes 3 and 4), and in the presence of ECM-overlay and PD 98059 (10 μM) (PD), or SB 203580 (1.0 μM) (SB) (lanes 5 and 6, respectively). DNA molecular weight markers (M) are shown in lane 1. Middle panel: cells cultured, as in top panel, under proliferating conditions (P) and in the presence of ECM-overlay and PD 98059 over a 48 h time course (lanes 2–9), and, again, cells cultured in the presence (+) or absence (−) of ECM or cells cultured in the presence of ECM and vehicle (lanes 10–12). Bottom panel: cells cultured as in top panel, in the presence of ECM and PD 98059 + SB 203580 over a 48 h time course (lanes 3–9) and, again, cells cultured under proliferating conditions (P) and for 48 h in the presence (+) or absence (−) of ECM-overlay (lanes 2, 10 and 11). Markers (M), as in Figure 1A. (**B**) Western blot analysis of cytosolic cytochrome c. Cells cultured for 3 h in the presence of the lactogenic hormones (hydrocortisone, insulin and prolactin) and: in the presence (+) or absence (−) of ECM-overlay, ECM-overlay without insulin (−I), ECM-overlay and PD 98059 (PD) and ECM-overlay and PD 98059 + SB 203580 (PD/SB) (lanes 1–5, respectively). Cytochrome c ran at approximately 12.5 kDa on a 12% SDS gel. (**C**) Fluorimetric assay of caspase 3 activity. Cells cultured for 3 h in the presence of the lactogenic hormones and: in the presence (+) or absence (−) of ECM-overlay, with ECM-overlay and PD 98059 (PD), with ECM-overlay and PD 98059 + SB 203580 (PD/SB) and with ECM-overlay without insulin (−I), (lanes 1–5, respectively). *, $P<0.05$; **, $P<0.01$ (students *t*-test)

We measured the commitment of the cells to apoptosis at an early time point (3 h) after ECM-overlay and MAP kinase inhibitor treatment by analysing cytochrome c loss to the cytosolic compartment[42–46] and caspase 3 activation.[42–49] The expected hierarchical chronology[46] would predict the cytochrome c loss and caspase 3 activation at times earlier than we had observed apoptosis (Figures 1 and 2). In the absence of ECM-overlay and 3 h after ECM-overlay, in the presence of PD 98059 and SB 203580, significant levels of cytosolic cytochrome c were detectable (Figure 2B, lanes 2 and 5). In the presence of PD 98509 alone, less release was detectable (compare lanes 4 and 5). This difference is maintained at 16 h after overlay (result not shown). With ECM-overlay and no inhibitors little or no release was detectable (lane 1). Cytosolic cytochrome c is also detectable in ECM-overlaid cells cultured in the absence of insulin or in the presence of insulin and the specific PI-3 kinase inhibitor LY 294002 (lane 2 and result not shown). Caspase 3 activity was determined fluorimetrically 3 h after overlay of the mammary epithelial cells. From Figure 2C it can be seen that cells cultured in the absence of ECM-overlay show a 3.1-fold higher level of caspase 3 activity than cells cultured with ECM overlay (compare lanes 2 and 1). Cells cultured with an ECM overlay but in the presence of PD 98059 did not show a significant increase in caspase 3 activity, over basal, at this time (lane 3 and see Discussion); however, cells cultured with the ECM-overlay but in the presence PD 98059 and SB 203580 did show a significant induction (twofold) in caspase 3 activity (lane 4). Finally, cells cultured with an ECM-overlay but in the absence of insulin (lane 5) also showed a significant induction (2.9-fold) in caspase 3 activity at the 3 h time point. The apparent lesser release of cytochrome c at 3 h in the presence of PD 98059 (compared to PD 98059 + SB 203580 treatment) (Figure 2B) and the lower degree of activation of caspase 3 by PD 98059 treatment (again compared to the use of both MAP kinase inhibitors) (Figure 2C) suggests a slowness in the commitment to apoptosis when the Erk 1/Erk 2 pathway inhibitor is used and corroborates the observance of apoptosis (Figure 2A) only after 24 h with inhibition of the Erk pathway only. Use of both inhibitors led to a higher level of cytochrome c release (Figure 2B), significant increase in caspase 3 activity (Figure 2C) and the presence of clear nucleosomal laddering even at 3 h (Figure 2A).

Overlay of the mammary epithelial cells with ECM rapidly and transiently activated the Erk 1/Erk 2 MAP kinase pathway as monitored by Western analysis of Erk 1/Erk 2 phosphorylation using a bis-phospho (Thr 202/Tyr 204)-

specific antibody (Figure 3A, upper panel). ECM-induced Erk 1/Erk 2 phosphorylation was apparent within 5 min of overlay but had dissipated by 30 min (lanes 2–4). This activation was inhibited by PD 98059 (lanes 7–8). A similar response was detectable by immunocytochemistry using the same antibody and this was again inhibited by PD 98059 (results not shown). No sustained activation of the Erk 1/Erk 2 pathway was observed. However, by assaying every 10 min after overlay for 4 h, a pulsatile induction of Erk 1/Erk 2 phosphorylation was demonstrated (Figure 3B, upper panels, open arrows). The lower panel shows a graphical representation of this data, using Scion Image® software, by computing the levels of phospho-Erk 1/Erk 2 relative to total Erk 1/Erk 2 levels.

No such rapid transient induction of p38 MAP kinase phosphorylation was seen in response to ECM-overlay (results not shown). Nor was an induction of p38 MAP kinase phosphorylation, as analyzed by Western blot using a phospho-p38 MAPK specific antibody, detectable after ECM overlay in the longer term, over 24 h (Figure 4A, upper panels). However, after ECM overlay in the presence of the Erk 1/Erk 2 pathway inhibitor, PD 98059, a reproducible but slower onset of p38 MAP kinase phosphorylation was detectable. This was observed at 6 h after overlay (Figure 4A, lower panels, lane 4) or at 3 and 6 h in other representative experiments. This 'slow' phosphorylation of p38 MAP kinase in the presence of PD 98059 was paralleled by a transient induction of p38

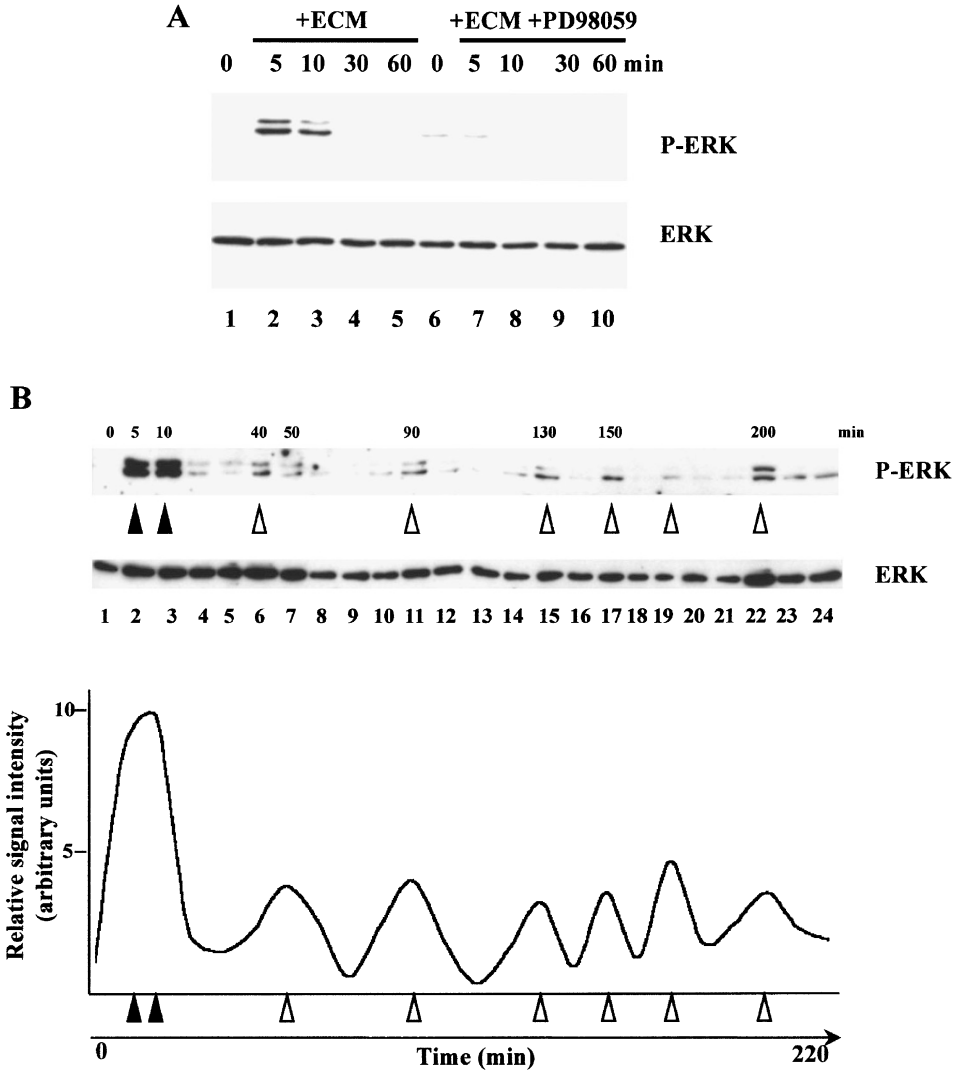

Figure 3 Extracellular matrix induces MAP kinase activity. (**A**) Western blot analysis of phosphorylated Erk 1/Erk 2 (P-ERK) and Erk 1/Erk 2 (ERK). Extracts of cells cultured as in Figure 2A for 0–60 min following ECM-overlay (lanes 2–5) or ECM-overlay in the presence of PD 98059 (lanes 7–10) were subjected to Western analysis using anti-phospho-p42/44 MAP kinase (Thr202/Tyr204) (NEB) (top panel) and anti-p42/44 MAP kinase (NEB) antibody (bottom panel). (**B**) Extracts of cells cultured for 0–220 min following ECM-overlay were subjected to Western blot analysis, as in (**A**) above, using anti-phospho-p42/44 MAP kinase (Thr202/Tyr204) (NEB) (top panel) and anti-p42/44 MAP kinase (NEB) antibody (bottom panel). Closed arrows mark the early transient induction of phosphorylation whilst open arrows indicate subsequent pulsatile phosphorylation. Lower panel shows graphical representation of the data from (**B**) upper panels. The data represents the ratio of the intensity of each phospho-Erk 1/Erk 2 band to the total Erk 1/Erk 2 band signal measured using Scion Image® software

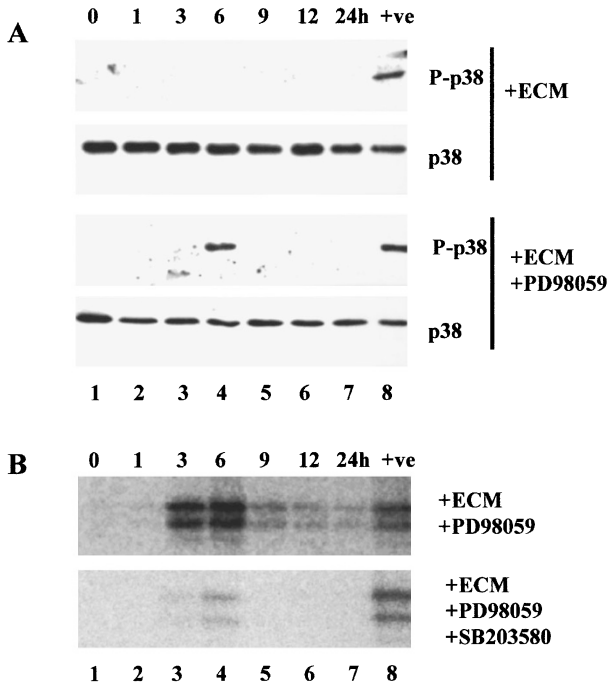

Figure 4 Inhibition of extracellular matrix induced-Erk 1/Erk 2 signalling induces p38 MAP kinase activity. (**A**) Western blot analysis of phosphorylated-(P-p38) and p38 MAP kinase (p38). Extracts of cells cultured, as in Figure 2A, over a 24 h time course (lanes 1–7) in the presence of ECM-overlay (top panels) or ECM-overlay and PD 98059 (bottom panels) were analyzed with anti-phospho-p38 MAP kinase (Thr180/Tyr182) (NEB) (top panel) or anti-p38 antibody (NEB) (bottom panel). (**B**) p38 MAP kinase *in vitro* kinase assay. A 24 h time course (lanes 1–7) of cells cultured, as in (**A**), above, with ECM-overlay and PD 98059 or PD 98059 + SB 203580 (upper and lower panels, respectively); cell extracts were immunoprecipitated with an anti-p38 MAP kinase antibody and incubated with GST-ATF 2 (NEB, 2 μg) in the presence of [γ-^{32}P]ATP (10 μCi), resolved on an SDS–PAGE gel and exposed to X-ray film. Lane 8 (+ve) shows an identical assay carried out with extract from anisomycin stimulated cells, provided as positive control by the manufacturer, NEB

MAP kinase activity, as measured by GST-ATF 2 phosphorylation *in vitro* by immunoprecipitated p38 MAP kinase, 3 and 6 h after ECM-overlay (lanes 3 and 4, top panel). In the presence of both PD 98059 and the p38 MAP kinase inhibitor, SB 203580, no induction of p38 MAP kinase activity was seen (lanes 3 and 4, bottom panel). These studies suggest that activation of the Erk 1/Erk 2 pathway is indeed detectable in response to ECM overlay in mammary epithelial cells but that further drive through the pathway occurs in a pulsatile manner. The triggering of drive through the p38 MAP kinase pathway upon inhibition of Erk 1/Erk 2 signalling may be compensatory and suggests cross talk between the pathways.

The ECM-overlaid mammary epithelial cells were maintained in the presence of insulin (5 μg/ml). Farrelly *et al*[16] have established the requirement for insulin (or IGF-1) for the survival of overlaid mammary epithelial cells in primary culture. It most likely functions by activating a PI-3 kinase/Akt-dependent signalling cascade. Omission of insulin from the ECM-overlaid cultures led to mammary epithelial cell apoptosis, as judged by nucleosomal DNA laddering (Figure 5A, top panel), in a similar time-course to that observed with PD 98059 + SB 203580 treatment. Apoptosis was also induced by exposure of the insulin-treated ECM-overlaid cells to the specific PI-3 kinase inhibitor, LY 294002 (10 μM),[50] again, in a similar time frame, (Figure 5A, bottom panel). This demonstrates the requirement of insulin as a survival factor for the ECM-overlaid cells and its dependence on PI-3 kinase signalling. Treatment of insulin deprived ECM-overlaid cells with insulin led to the rapid activation of the PI-3 kinase/Akt pathway as judged by induction of Akt phosphorylation, measured by Western blot analysis (Figure 5B). Akt phosphorylation was demonstrated within 5 min of insulin treatment (lane 2) and this insulin-induced activation of Akt phosphorylation could be inhibited by LY 294002 (lane 4). Insulin induction of Akt phosphorylation was not affected by treatment of the cells with PD 98059 + SB 203580 (lane 3) (for a period of time shown to cause substantial apoptosis (16 h) (see Figure 2A, lower panel). In addition, surviving, insulin-treated ECM-overlaid cells showed maintained levels of Akt phosphorylation over a period of 48 h after overlay (Figure 5C, upper panels). In contrast, removal of insulin at the time of overlay led to a rapid reduction in phosphorylated Akt levels (Figure 5C, second panels). Exposure of the insulin treated ECM-overlaid cells to LY 294002 also led to a loss of phosphorylated Akt levels in a similar time frame (Figure 5C, third panels). However, as seen with acute insulin induction of Akt phosphorylation, treatment of the cells with PD 98059 + SB 203580 did not affect insulin-associated long-term maintenance of Akt phosphorylation (Figure 5C, bottom panels) although inducing significant levels of cell death (see Figure 2A, bottom panel). Similarly, the Erk 1/Erk 2-pathway inhibitor, PD 98059, alone, had no effect on the insulin-maintained phospho-Akt levels (results not shown).

Lastly, we observed that inhibition of the PI-3 kinase dependent pathway (with LY 294002) was significantly more efficient ($P < 0.05$) than inhibition of the MAP kinase pathways (with PD 98059 + SB 203580) in causing apoptosis of the ECM-overlaid cells cultured in the presence of insulin (Figure 1D, compare lanes 3 and 4). It is noteworthy also that the presence of insulin seemed to partially protect the non-ECM-overlaid cells from apoptosis (compare lanes 2 and 4). Thus, the insulin/PI-3 kinase pathway may be the more potent survival pathway; however, inhibition of the ECM/MAP kinase pathway precipitated high levels of apoptosis (lane 3).

The data suggest that ECM overlay determines cell survival through a MAP kinase dependent mechanism that is seemingly independent of the insulin-triggered PI-3 kinase/Akt-dependent intracellular signalling pathway. It also provides evidence for a hitherto undescribed Erk 1/Erk 2-p38 MAP kinase pathway 'cross-talk' that may modulate the Erk 1/Erk 2-regulated ECM-driven survival stimulus.

Discussion

Mid-pregnant mouse mammary epithelial cells in primary culture and a number of extracellular matrix dependent

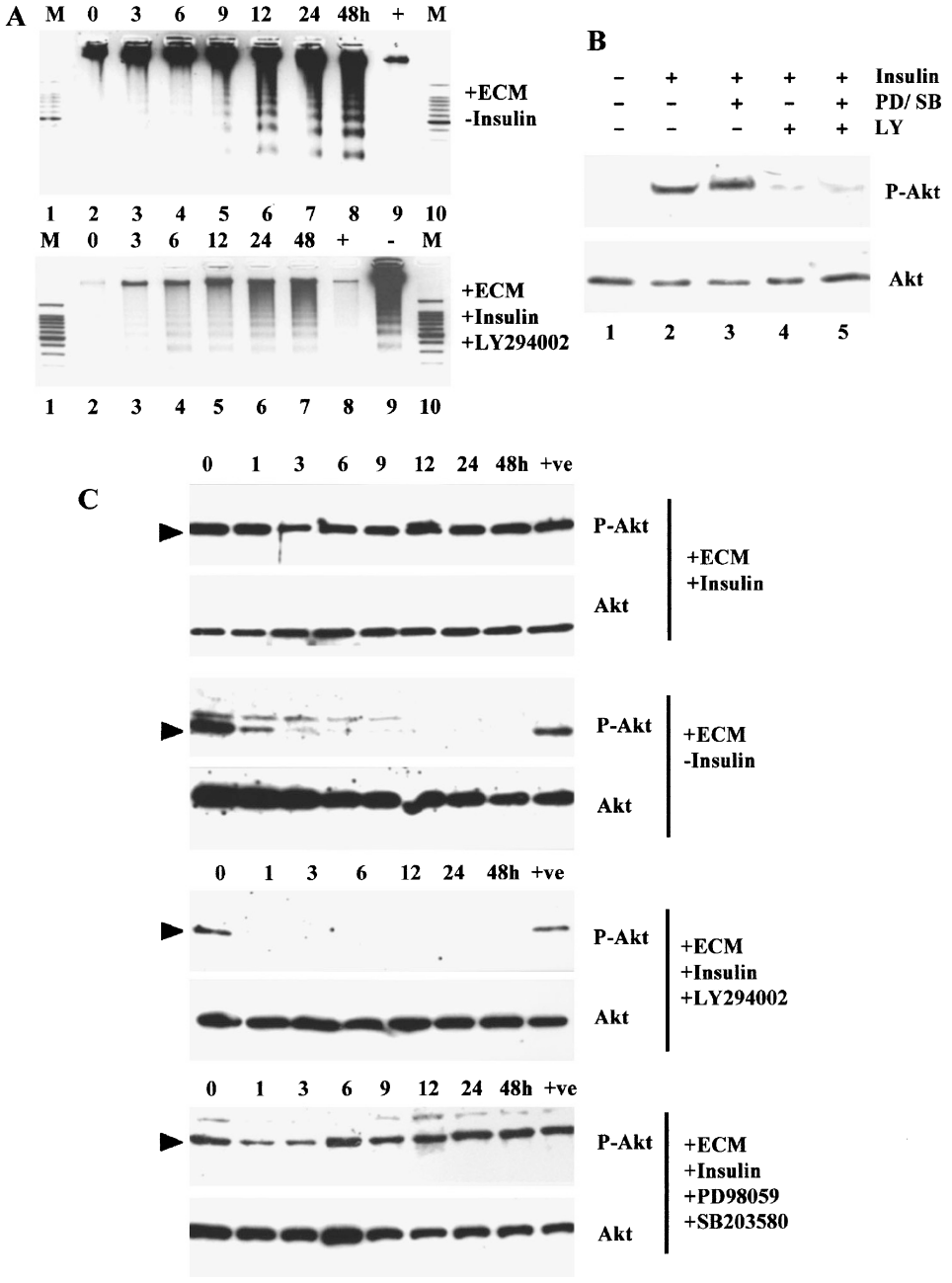

Figure 5 The PI 3-kinase dependent insulin determined mammary epithelial cell survival pathway induces Akt/PKB phosphorylation; apoptosis induced by MAP-kinase inhibitors does not affect Akt/PKB phosphorylation. (**A**) Images of ethidium bromide stained agarose gels of low molecular weight DNA isolated from primary cultures of mammary epithelial cells. Top panel: cells cultured as in Figure 2A for 0–48 h in the presence of ECM-overlay and in the absence of insulin (lanes 2–8, respectively) and in the presence of ECM-overlay for 48 h (lane 9). Bottom panel: cells cultured over a 48 h time course (lanes 2–7) in the presence of ECM-overlay, insulin and LY 294002 (10 μM) and, again, cells cultured in the presence (+) or absence (−) of ECM-overlay (lanes 8 and 9). Markers (M), as in Figure 1A. (**B**) Acute treatment with insulin (5 μg/ml) causes Akt phosphorylation. Western blot analysis of phosphorylated-Akt/PKB (P-Akt) and Akt/PKB (Akt) in cells cultured, as in (**A**) above, with ECM-overlay for 16 h, and then treated with insulin for 2 min, in the absence or presence of PD 98059 + SB 203580, or LY 294002 or both (lanes 1–5, as indicated), Phospho-Akt (Ser473) and Akt antibodies from NEB were used. (**C**) Insulin determined cell survival and Akt phosphorylation are correlated. Western blot analysis of phosphorylated-Akt/PKB (P-Akt) and total Akt/PKB (Akt) in extracts of cells cultured, as in (**A**) above, with ECM-overlay and in the presence of insulin (top panels), ECM-overlay, but without insulin (second set of panels), ECM-overlay, with insulin, but with LY 294002 (third set of panels) or ECM-overlay, with insulin, but with PD 98059 + SB 203580 (bottom panels), 48 h time courses

immortalised cell lines have been used successfully to model the mammary alveolar epithelial cell of late pregnancy.[12–14,17,18,35,36] They require survival factors which if removed cause cell loss by apoptosis. The primary cultures of mid-pregnant mammary epithelial cells that we describe here require overlay with ECM or with soluble

laminin and the presence of insulin to maintain cell survival (Figure 1). The recent study of Farrelly *et al*[16] and other earlier studies[15,51] show also that the survival influence of the EHS-ECM can be substituted with soluble laminin and that its influence can be negated with blocking antibodies for α6 or β1 integrins.

Treatment of the ECM-overlaid cells with the Erk 1/Erk 2 pathway inhibitor, PD 98059, in the presence of insulin, caused significant cell death after 24–48 h (Figure 2A) suggesting a dependence of mammary epithelial cell survival on an Erk 1/Erk 2-modulated pathway. ECM overlay acutely stimulated Erk 1/Erk 2 phosphorylation demonstrating a potential functional dependence of ECM-induced intracellular signalling on the Erk 1/Erk 2 pathway (Figure 3A) and Erk 1/Erk 2 pathway signalling may be maintained by ECM, in the longer term, by pulsatile activation reflected by pulsed Erk 1/Erk 2 phosphorylation (Figure 3B). The cells exist in a dynamic state, whereby they must constantly be capable of perceiving cell survival signals in order to protect against apoptosis. This pulsative drive through Erk 1/Erk 2 phosphorylation may act as a potential longer-term ECM driven-epithelial cell survival pathway. The pulsatile nature of activation of the Erk 1/Erk 2 pathway is possibly consistent with the hypothesis that Erk 1/Erk 2 signalling must be down regulated in order for differentiation responses to be transduced[52] (reviewed in[31]). However, maintained intracellular read-outs from this pulsatile Erk 1/Erk 2 signalling that are required for cell survival remain to be identified. In a fibroblast cell culture model using fibronectin stimulation, Miyamoto *et al*[32] have elegantly demonstrated that an ECM-β1-integrin receptor engagement, on inducing formation of focal contacts, led to a recruitment of Erk 1/Erk 2 into such contacts. These contacts also contained FAK, Ras, Raf-1, MEKK and MEK1, proven upstream modulators of Erk 1/Erk 2 phosphorylation/activation. This provides a conceptual model of how ECM-dependent survival signals would selectively activate MAP kinase pathways. FAK recruitment to focal adhesions and activation by phosphorylation on tyrosine has been functionally linked to MAP-kinase (Erk 1/Erk 2) activation in a range of model systems.[53–55] Several studies have also demonstrated that ECM-integrin association with FAK initiates a survival pathway.[56,57] In human mammary (BT474) cells Xu *et al*[58] demonstrated that a dominant negative form of FAK localises to focal adhesions, causes reduced FAK phosphorylation, loss of adhesion and subsequent cell death. However, FAK-dependent activation of MAP-kinases in mammary epithelial cells has not been reported to date. This, in fact, is the first report of an Erk 1/Erk 2 survival pathway in mammary epithelial cells.

The Erk 1/Erk 2 pathway inhibitor, PD 98059 caused apoptotic death only after 24 h (Figure 2A) and had a weak effect on cytochrome c release and activation of caspase 3, at 3 h after ECM-overlay, while in combination with the p38 MAP kinase inhibitor, SB 203580, it caused mammary epithelial death much more rapidly and triggered cytochrome c release and caspase 3 activation more effectively (Figure 2A). SB 203580, alone, had no effect on mammary epithelial cell survival (Figure 2A) and ECM overlay did not trigger p38 MAP kinase phosphorylation. However, in the presence of the Erk 1/Erk 2 pathway inhibitor, p38 MAP kinase phosphorylation and activity, are slowly but transiently induced (Figure 4A,B). This together with the more rapid cell death associated with blocking both pathways suggests that activation of the p38 MAP kinase pathway may compensate for Erk 1/Erk 2 pathway inhibition in protecting mammary epithelial cells from apoptosis. These effects could reflect either direct or indirect functional interactions between these two MAP kinase pathways. There is evidence demonstrating opposing effects of these two signalling pathways (e.g.[59–61]) however, there are few reports of co-operative interactions between the Erk 1/Erk 2 and p38 MAP kinase pathways.[62–64] Deak *et al*[64] have demonstrated that several growth factors stimulate both p38 MAP kinase and Erk 1/Erk 2, and that activity of both these pathways is essential for maximal activation of the novel kinase, MSK1 (mitogen and stress activated kinase 1).

Removal of insulin from our cultures or the addition of the PI-3 kinase inhibitor, LY 294002, precipitated apoptotic cell death (Figure 5A) and activation of death associated processes (Figure 2B,C). This would reflect earlier reports by others of a role for insulin/IGF-1 in mammary epithelial cell survival[5,65] and the existence of an insulin/IGF-1-determined PI-3 kinase-dependent survival pathway in mammary epithelial cells.[16] Activation of this pathway and the associated mammary epithelial cell survival has been linked with recruitment of PI-3 kinase to the insulin receptor-associated protein, IRS-1, and maintained phosphorylation of Akt/PKB.[16] We show (Figure 5B) that acute insulin stimulation of ECM-overlaid mammary epithelial cells causes PI-3 kinase-dependent Akt/PKB phosphorylation but that this is unaffected by MAP kinase pathway inhibition. In addition, cell death triggered by insulin removal or PI-3 kinase inhibition was paralleled by a rapid loss of cellular phospho-Akt while in contrast, cell death triggered by PD 98059 + SB 203580 did not affect phospho-Akt levels (Figure 5C). This would suggest that the MAP kinase-modulated mammary epithelial cell survival is not PI-3 kinase dependent. Although both pathways are essential for cell survival, and are seemingly independent, quantitative evaluation of the apoptotic effectiveness of inhibiting each pathway suggests a somewhat greater dependence on the PI-3 kinase Akt/PKB pathway for cell survival (Figure 1D).

What cellular elements do these signalling pathways target to facilitate cell survival? Members of the Bcl-2 family of pro- and anti-apoptotic proteins would seem the most likely. The pro-apoptotic protein, Bad, is maintained in an inactive form by both Akt- and PKA-catalysed phosphorylation.[23,24,66] There also exists evidence indicating a role for MAPK kinase (MEK1) pathway signalling in Bad phosphorylation.[67] These authors showed that GM-CSF can induce PI-3 kinase/Akt-independent phosphorylation of Bad, that inhibition of a MEK1 signalling pathway, by PD 98059, abrogated this phosphorylation and most recently that MEK-dependent phosphorylation of Bad is required for its release from its possible pro-apoptotic association with Bcl-$_{xL}$.[68] These, or other modulations of Bcl-2 superfamily

member activity, might provide the integrating target for the mammary epithelial cell survival pathways. This will be the focus of our future studies. Thus, inhibition of ECM-driven Erk 1/Erk 2 MAP kinase signalling slowly impairs ECM-determined mammary epithelial cell survival. A novel compensatory triggering of the p38 MAP kinase may contribute to the slow death response to Erk 1/Erk 2 pathway inhibition alone and whilst this response is likely to be PI-3 kinase independent, its exact nature remains to be elucidated.

Materials and Methods

Primary mammary epithelial cell culture

Mouse mammary epithelial cells were prepared from mid-pregnant CD-1 mice, essentially as previously described.[13,17–20] Cells were seeded on tissue culture plastic at a density of 2.4×10^6 cells/ml and grown for 48 h in a proliferation medium of Hams F12 (Gibco) containing 10% heat inactivated foetal bovine serum (Gibco), epidermal growth factor (EGF), 5 ng/ml (Promega), insulin, 5 μg/ml (Sigma), hydrocortisone, 1 μg/ml (Sigma), fetuin, 1 mg/ml (Sigma) and gentamicin, 50 μg/ml (Sigma) and cultured at 37°C in a humidified atmosphere of 5% CO_2. The cells were washed and then cultured for a further 48 h in F12 medium (with gentamicin) in the presence of 10% serum, EGF, insulin and hydrocortisone. The cultures were washed extensively (with PBS) and 'starved' for 6 h in F12 medium in the absence of EGF and serum, in the presence of hydrocortisone and in the presence or absence of insulin. The cultures were subsequently washed extensively and the medium changed to differentiation medium (F12, gentamicin, in the presence of hydrocortisone and in the presence or absence of insulin and prolactin, 3 μg/ml (Sigma)). The cells were then cultured in the absence or presence of an overlaid laminin-rich ECM matrix (Matrigel®, Becton Dickinson, 280 μg/ml, 2%) which had been diluted in the above medium[20] with or without the specific intracellular signalling pathway inhibitors, PD 98059, 10 μM, SB 203580, 1 μM or LY 294002, 10 μM, as required. After the desired time period the cells were harvested by scraping and were pelleted by centrifugation. The cell pellets were snap frozen in liquid N_2 and stored at −80°C prior to protein extract preparation.

Flow cytometric analysis (FACS analysis)

Cell nuclei were prepared for FACS analysis as described by Gurley *et al*.[69] Following isolation of the cell nuclei, the unfixed nuclei were either stored at 4°C or used immediately for DNA analysis; 50 μl of propidium iodide solution (400 μg/ml) was added directly to the nuclear suspension without centrifugation and the nuclei stained for 30 min at room temperature in the dark. The stained nuclei were then filtered through a needle (0.50 × 16 mm). DNA content (10 000 events) was analyzed using a FACStar Plus flow cytometer (Becton Dickinson) with laser excitation at 488 nm using a 639 nm band pass filter to collect the red propidium iodide fluorescence. The DNA content profile of the cell nuclei was analyzed using LYSIS 11 software.

Isolation of low molecular weight DNA and analysis of nucleosomal DNA laddering

Low molecular weight DNA was isolated from cultured primary mammary epithelial cells using a protocol modified from that originally described by Hirt:[38] 9-cm plates of cells were grown to confluency ($\sim 5 \times 10^7$ cells) for each experiment. The medium was aspirated from the plates and the cells were lysed by scraping and subsequent resuspension in 10 mM Tris-HCl (pH 7.9), 5 mM EDTA (pH 8.0) and 0.5% SDS. The suspension was then adjusted to a final concentration of 1 M NaCl by the addition of ¼ volume of 5 M NaCl and incubated on ice at 4°C overnight. The solution was cleared by centrifugation at 14 000 × *g* for 30 min at 4°C, 400 μl was removed and the DNA precipitated at −20°C overnight by the addition of 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol. The precipitated low molecular weight DNA was recovered by centrifugation at 14 000 × *g* for 20 min at 4°C and the pellet dried, resuspended and incubated in 10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, pH 8.0 (TE) containing 100 μg/ml RNaseA at 37°C for 2 h. The resultant dissolved low molecular weight DNA was subjected to electrophoresis at 4 V/cm in a 2% agarose gel stained with ethidium bromide and the DNA subsequently visualised by UV transillumination.

Nuclear morphology assay

Primary mammary epithelial cells were grown as described above in the presence or absence of the desired inhibitors on LabTek® 2-well Chamber Slides. After 48 h the medium was aspirated and the cells fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. They were then permeabilised in 0.1% Triton X-100 in 1 × TBST and washed twice in 1 × TBST. The cells were then incubated with 5 μM 4,6-diamidino-2-phenylindole (DAPI) (Sigma Research Reagents) in PBS for 3 min and washed by immersion in 50 ml of PBS. They were then mounted in 20 μl Citifluor (Agar Scientific) before analysis on either an Olympus BX50, or a Leica Leitz DMRB, fluorescent microscope. Apoptotic cells were scored based on nuclear morphology.[16] Each treatment was analyzed three times by counting four fields (cumulatively > 1300 cells per four fields). Statistical analysis was carried out using the Students' *t*-test.

Preparation of total cellular extracts and subcellular fractions

Primary mammary epithelial cells were lysed with high salt lysis buffer (10 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM DTT, 400 mM KCl, 1 mM Na_3VO_4, 1 mM β-glycerophosphate, 5 mM benzamidine, 0.2% NP-40, 10% glycerol and 1 μg/ml each of aprotinin, leupeptin and pepstatin A) by resuspension and incubated on ice for 30 min. The suspension was then cleared by centrifugation at 14 000 × *g* for 15 min at 4°C to yield supernatants comprising the total cell extract. The protocol was adapted from Eul *et al*[70] and Robidoux *et al*.[71]

Cytosolic extracts for cytochrome c analysis were prepared by resuspending cells in 200 μl of isotonic extraction buffer (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPES, pH 7.5, 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml each of leupeptin, pepstatin A and aprotinin). After incubation on ice for 1 h, cells were subjected to 30 strokes in a dounce homogeniser and subcellular particulate material removed by centrifugation at 14 000 × *g* for 15 min at 4°C to yield supernatants comprising the cytosolic extract.[72]

Nuclear extracts were prepared using a protocol modified from that originally described by Osborn *et al*.[73] Harvested cells were resuspended in 100 μl of lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate, 5 mM benzamidine, 0.2% NP-40, and 1 μg/ml each of aprotinin, leupeptin and pepstatin A) and incubated on ice for 15 min. The suspension was then centrifuged at 14 000 × *g* for 10 min and the nuclear extract was obtained by

resuspending the resultant pellet in 50–100 μl of nuclear extraction buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 mM Na_3VO_4, 1 mM β-glycerophosphate, 5 mM benzamidine, 0.2% NP-40, and 1 μg/ml each of aprotinin, leupeptin and pepstatin A) followed by incubation on ice for 30 min. The suspension was subsequently cleared by centrifugation at 14 000 $\times g$ for 10 min at 4°C and the supernatant retained.

Immunoblotting analysis

Cellular and subcellular extracts were resolved on SDS-polyacrylamide gels and were then electrophoretically transferred onto nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked for 1 h in TBST (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween) containing 5% non-fat dried milk and were incubated overnight with the appropriate primary antibody: anti-ERK (#9102, 1 : 2000), anti-phospho-ERK (#9101, 1 : 2000), anti-p38 (#9212, 1 : 2000), anti-phospho-p38 (#9211, 1 : 2000), anti-Akt (#9272, 1 : 2000) and anti-phospho Akt (#9271, 1 : 2000) (all from New England Biolabs); anti-cytochrome c (PC333, 1 μg/ml, Oncogene Research Products). After incubation for 1 h with secondary antibody raised against rabbit IgG (P0260) or mouse IgG (P0448) conjugated to horseradish peroxidase (Dako, Cambridge, UK) bands were visualised using enhanced chemiluminescence (Supersignal® West Dura, 34075ZZ, Pierce).

p38 MAP kinase assay

Total cellular extracts were prepared as described above and p38 MAP kinase was immunoprecipitated with anti-p38 antibody (5 μl/ml) overnight at 4°C in immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 1% Triton X-100 and 0.5% NP-40). Immunocomplexes were captured on protein A Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C. Beads were washed three times by centrifugation with immunoprecipitation buffer and once with p38 kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4 and 10 mM $MgCl_2$). The activity of the immune complex was assayed at 30°C for 30 min in 50 μl of kinase buffer containing 10 μCi [γ-^{32}P]ATP, 30 μM ATP with 2 μg of GST-ATF-2 (New England Biolabs) as substrate. The reactions were terminated with Laemmli sample buffer[74] and proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualised in a GS-250 PhosphorImager (Bio-Rad).

Caspase 3 assay

Fifty μg of total cell extract, from cells treated as required, were added to 3 ml of incubation buffer (100 mM HEPES, pH 7.5, 10% (w/v) sucrose, 0.1% (w/v) CHAPS, 10 mM DTT) containing 12 μl of caspase 3 specific substrate linked to a fluorescent moiety (Ac-DEVD-amc, 5 mM) and incubated for 30 min at 25°C. Fluorescence was measured on an LS-5 Luminescence Spectrophotometer (Perkin Elmer) at 380 nm excitation and 460 nm emission peaks.

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