Inhibition of Ligand-independent ERK1/2 Activity in Kidney Proximal Tubular Cells Deprived of Soluble Survival Factors Up-regulates Akt and Prevents Apoptosis

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Mouse kidney proximal tubular epithelial (MK-PT) cells die by apoptosis over 7–10 days when deprived of all survival factors. We show here that withdrawal of all survival factors from MK-PT cells is associated with a progressive increase in the activity of extracellular signal-regulated kinase-1 and -2 (ERK1/2) and a progressive decrease in phosphorylated Akt, a kinase critical to cell survival. Pharmacological inhibition of MEK1/2, the immediate upstream kinase for ERK1/2, not only prevented the decrease in phosphorylated Akt, but also prolonged MK-PT cell survival. Inhibition of ERK1/2, by itself, in the absence of any other known survival factors, was as potent as epidermal growth factor in maintaining MK-PT cell viability. ERK1/2 co-immunoprecipitated with Akt in a multimolecular assembly of signaling molecules, containing at a minimum ERK1/2, Akt, Rsk, and 3-phosphoinositide-dependent kinase 1 (PDK1). We hypothesize that the kinase Rsk, whose activation requires phosphorylation by both ERK1/2 and PDK1, acts as a bridge bringing ERK1/2 into proximity with PDK1-associated Akt. Although a number of interactions between the Raf-MEK-ERK and PI3K-Akt signaling pathways have been described, our results are the first to show modulation of Akt activity by signaling events originating with ERK1/2. Spontaneous activation of ERK1/2 occurs via MEK1/2 and appears to depend on oxidant stress, accompanying induction of the default pathway of apoptosis. Together, these data suggest that the spontaneous activation of ERK1/2, in the absence of known extracellular stimuli, represents a previously unrecognized major regulatory pathway determining the fate of cells destined to die by the default pathway of apoptosis.

An intimate relationship exists between the cell cycle and apoptosis (1–3). In general, the same soluble factors that induce cell proliferation are also potent inhibitors of apoptosis (2–4). When used to stimulate proliferation, these factors are also potent inhibitors of apoptosis (1–3). Inhibition of apoptosis by survival factors is therefore an example of negative regulation, meaning that, in the absence of survival factors, cells will automatically undergo apoptosis.

Recent studies have shown that activation by growth and survival factors of several key intracellular signaling molecules, including Ras (5), phosphatidylinositol 3-kinase (PI3K) (6), and protein kinase C (PKC) (7), occurs in two distinct waves of activity. The first wave begins almost immediately after stimulation and is typically transient, lasting no more than 30–60 min. After this time, activity subsides to baseline levels, despite the continued presence of growth factors (5–7). Cessation of signaling results from the combined effect of multiple negative feedback loops, including the internalization and degradation of growth factor receptors and the delayed activation of various antagonistic enzymes and phosphatases (8–10). Surprisingly, after several hours of signaling “silence,” a second wave of signaling activity spontaneously emerges, despite the absence of any additional extracellular stimuli (5–7). This second wave of activity persists for several hours and leads to cellular responses and downstream signaling events that are distinct from those associated with the initial signaling response to growth factors (6–8, 11, 12). Importantly, both waves of signaling are essential for progression through the cell cycle (6–8, 11, 12).

Given the close relationship between proliferation and the default pathway of apoptosis, we wondered whether withdrawal of survival factors might also be associated with delayed intracellular signaling events, analogous to those seen in the continuous presence of growth factors. We speculated that delayed waves of signaling activity, occurring in the absence of exogenous ligands, might play a role in the executionary phase of apoptosis, or, alternatively, they might promote survival by modulation of Akt activity.
mediating events that allow the cell to adjust to a now “hostile” environment.

We tested our hypothesis using primary cultures of mouse kidney proximal tubular epithelial (MK-PT) cells. We have previously shown that MK-PT cells undergo apoptosis over 7–10 days when deprived of all soluble survival factors (13–16). We focused our studies of the effects of growth factor deprivation on two distinct signaling pathways, the ERK1/2 and PI3K-Akt pathways, both of which play key roles in the regulation of proliferation and survival (1, 2, 17–19).

We show here that withdrawal of all soluble growth and survival factors is associated over the course of several days with a progressive increase in the activity of extracellular regulated kinase-1 and -2 (ERK1/2), kinases predominantly involved in cell proliferation (1, 2), and a progressive decrease in the activity of Akt a kinase critical to cell survival (2, 17–19). Pharmacological inhibition of ERK1/2 prevented the decline in Akt activity and prolonged the survival of MK-PT cells. Remarkably, pharmacological inhibition of ERK1/2 was as potent as epidermal growth factor (EGF) in inhibiting the default pathway of apoptosis and maintaining the viability of MK-PT cells.

These data demonstrate that activation of intracellular signal transduction pathways can occur in cells deprived of all extracellular growth or survival factors. The signaling events that we demonstrate are induced by survival factor withdrawal and include activation of ERK1/2 and inhibition of Akt. The fact that pharmacological modulation of these signaling events alters cell viability suggests that these events play an important role in regulating the fate of cells deprived of survival factors.

**EXPERIMENTAL PROCEDURES**

**Primary Culture of Mouse Kidney Proximal Tubular Epithelial (MK-PT) Cells**—MK-PT cells were cultured from collagenase-digested fragments of proximal tubules obtained from the cortices of C57BL/6 mice using a modification of previously described methods (13–17). Cortical tubules were plated in serum-free, defined culture medium (1:1 mixture of DMEM and Ham’s F-12 containing 2 mM glutamine, 15 mM HEPES, 5 μg/ml transferrin, 5 μg/ml insulin, 50 μg/ml streptomycin, and 50 μg/ml penicillin). MK-PT cells grew from the tubules and formed a confluent epithelial monolayer over 4 days. We have demonstrated that MK-PT cells grown by this technique have the morphological, biochemical, and transport features characteristic of proximal tubular cells (14–17). On the 5th day following plating of tubules, designated as time “zero” (t = 0) of the experimental period, confluent monolayers of MK-PT cells were subjected to experimental conditions and studied for an additional 5 days (14–17).

**Experimental Treatment of MK-PT Cells**—On day “zero,” MK-PT cells were subjected to one of two experimental conditions. MK-PT cells designated as “EGF present” were incubated in DMEM:F10 (1:1) containing EGF (10^{-8} M) as the only growth factor for 5 days. MK-PT cells designated as “EGF absent” were subjected to withdrawal of all soluble survival factors by incubation in DMEM:F10 (1:1) without any growth factors for the same time period. The culture medium was changed daily and replaced with fresh medium, either EGF-containing or EGF-free, as appropriate. After varying times of incubation, MK-PT cells were washed once with ice-cold phosphate-buffered saline and assessed immediately for viability by MTT (3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide) assay or snap-frozen at −70 °C for kinase assays or immunoblotting studies.

**MTT Assay for Viability**—The number of remaining viable MK-PT
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Fig. 2. Rsk, a downstream target of ERK1/2, is spontaneously phosphorylated in MK-PT cells subjected to withdrawal of all soluble survival factors. MK-PT cells were cultured in the presence or absence of EGF. Total and phosphorylated Rsk were determined by immunoblotting at the indicated times. Shown is a representative blot from \( n \) = 4 experiments.

Fig. 3. Pharmacological inhibition of MEK1/2 prevents the spontaneous increase in ERK1/2 activity following withdrawal of survival factors. MK-PT cells were cultured for 96 h in the presence or absence of EGF plus the indicated concentrations of the two MEK1/2 inhibitors, PD98059 or U0126. Total and phosphorylated ERK1/2 were determined by immunoblotting. Shown is a representative blot from \( n \) = 4 experiments.
gradual loss of the monolayer. We examined the kinetics of ERK1/2 activity in two parallel sets of MK-PT cells. At confluency \( t = H11005 \), the cell medium was changed to medium (DMEM: F-12) that either contained EGF ("EGF present") or did not contain EGF ("EGF absent"), as described under "Experimental Procedures." Because the culture medium we used was fully defined, EGF was the only soluble growth or survival factor present in the "EGF present" medium, whereas the "EGF absent" medium contained no growth factors.

The level of active phosphorylated ERK1/2 was elevated at the start of the experiment \( t = H11005 \). Subsequently, despite the continuous presence of EGF, the level of active ERK1/2 decreased over the 5 days of observation (Fig. 1A). In contrast, for MK-PT cells grown in the continuous presence of EGF, levels of phosphorylated ERK1/2 increased again and remained elevated for the remainder of the 5 days of observation. Remarkably, levels of phosphorylated ERK1/2 in MK-PT cells that have been deprived of all survival factors are comparable to or greater than those found in MK-PT cells incubated in the presence of EGF (Fig. 1A, compare "EGF present" to "EGF absent").

We confirmed these results through two independent approaches. First, we measured the in vitro kinase activity of immunoprecipitated total ERK1/2. In MK-PT cells subjected to EGF withdrawal, there was an abrupt increase in ERK1/2 kinase activity by 24 h that remained elevated through 96 h (Fig. 1B). In contrast, for MK-PT cells grown in the continuous presence of EGF, ERK1/2 kinase activity progressively declined (Fig. 1B). Thus, regardless of whether ERK1/2 activity was indirectly assessed by immunoblotting or directly by an in vitro kinase assay, MK-PT cells show a delayed but sustained increase in ERK1/2 activity in response to EGF withdrawal.

We next examined the state of phosphorylation of the ribosomal kinase Rsk, a downstream target of ERK1/2 (Fig. 2). After EGF withdrawal, levels of phosphorylated Rsk increased progressively over time in parallel with those of ERK1/2. In the continuous presence of EGF, levels of phosphorylated Rsk remained more or less constant, in a pattern comparable to that of ERK1/2. Taken together, these results show that, following withdrawal of all soluble survival factors, ERK1/2 activity spontaneously increases over time and is capable of activating downstream targets.

**Inhibition of Spontaneous ERK1/2 Activity Inhibits Apoptosis of MK-PT Cells Undergoing EGF Withdrawal**—To explore the consequences of the spontaneous activation of ERK1/2 in response to EGF withdrawal, we tested the effects of PD98059 (22, 23) and UO126 (22, 24), two highly specific inhibitors of MEK1/2, the immediate upstream kinase and activator of ERK1/2. PD98059 prevents the activation MEK1/2 by upstream kinases such as c-Raf (22, 23), whereas UO126 has a
dual effect, not only preventing activation of MEK1/2 by up-
stream kinases but also directly inhibiting MEK1/2 activity
(22, 24). Because PD98059 and UO126 are structurally unre-
related, any observed effects in our system are likely attributable
to inhibition of MEK1/2 rather than to potential nonspecific
effects of these drugs.

Inhibition of MEK1/2 with either PD98059 or UO126, as
expected, prevented the activation of ERK1/2 following 96 h of
EGF withdrawal (Fig. 3). Both drugs demonstrated a clear
dose-response effect in inhibiting the activation of ERK1/2. The
potency with which PD98059 and UO126 inhibited ERK1/2 is
in accord with their in vivo effectiveness as inhibitors of
MEK1/2 (22–24). These data establish not only that PD98059
and UO126 may be used in our system to probe the role of

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**Fig. 5.** Inhibition of spontaneous ERK1/2 activity prevents the de-
crease in phosphorylated Akt seen in MK-PT cells subjected to withdrawal
of all soluble survival factors. MK-PT cells were cultured in the presence or ab-
ence of EGF (A) minus or (B) plus the MEK1/2 inhibitors, PD98059 and UO126,
at the indicated concentrations. Total and phosphorylated Akt were determined by
immunoblotting (A) at the indicated times or (B) after 96 h of culture. Shown are
representative blots from \( n = 4 \) experiments.

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**Fig. 6.** Increased survival of MK-PT cells following inhibition of ERK1/2 is dependent on PI3K. MK-PT cells were cultured in the
absence of EGF and the presence of the MEK1/2 inhibitor UO126 (1 \( \mu \text{M} \)) plus the PI3K inhibitors, LY294002 (A) or wortmannin (B), at the
indicated concentrations. After 7 days, MK-PT cell viability (mean ± S.D., \( n = 6 \) experiments) was determined by MTT assay and expressed as a
percentage of the viability of MK-PT cells determined on day 0.
ERK1/2 but also that the spontaneous activation of ERK1/2 that occurs in response to the absence of EGF is dependent on activation of MEK1/2.

We next examined whether there is any association between the spontaneous ERK1/2 activity induced by EGF withdrawal and induction of the default pathway of apoptosis. As we have previously shown (13–16), most MK-PT cells (75%) remain viable up to 7 days in the continuous presence of EGF, whereas very few MK-PT cells (20%) are still alive 7 days after EGF withdrawal (Fig. 4A). Remarkably, when PD98059 (10 μM) or UO126 (1 μM) were present in the culture medium at concentrations sufficient to inhibit nearly all the increased ERK1/2 activity, apoptosis was inhibited and MK-PT cell survival increased to 100%, despite the absence of EGF (Figs. 3 and 4A). When MK-PT cell survival after EGF withdrawal was plotted as a function of the concentration of PD98059 or UO126 in the medium, classic dose-response curves were obtained (Fig. 4, B and C). The concentrations at which MK-PT cell survival was increased by 50% (5 μM for PD98059; 100 nM for UO126) are consistent with published values of the IC50 for inhibition of MEK1/2 activity (2–7 μM for PD98059; 70 nM for UO126) (22–24). Notably, addition of PD98059 (10 μM) or UO126 (1 μM) also modestly increased survival of MK-PT cells cultured in the continuous presence of EGF (Fig. 4A), suggesting that increased ERK1/2 activity may tend to inhibit survival even in the presence of survival factors. We conclude that inhibition of ERK1/2 activity in MK-PT cells subjected to EGF withdrawal is associated with inhibition of the default pathway of apoptosis and a consequent marked increase in MK-PT cell viability.

Inhibition of ERK1/2 Activity Prevents the Decline in Akt Phosphorylation in MK-PT Cells Undergoing Survival Factor Withdrawal—Activation of the kinase Akt mediates the survival activity of virtually all extracellular survival factors (2, 19). Conversely, loss or inhibition of Akt leads to activation of the default pathway of apoptosis (2, 19). Given the striking effects of ERK1/2 inhibition on MK-PT cell survival following EGF withdrawal, we examined the effects of ERK1/2 inhibition on the state of activation of Akt.

As expected, phosphorylated Akt was maintained at a high level in MK-PT cells cultured in the continuous presence of EGF, whereas levels of phosphorylated Akt declined in MK-PT cells subjected to EGF withdrawal (Fig. 5A). Strikingly, inhibition of ERK1/2 with PD98059 or UO126 prevented the decrease in the level of Akt phosphorylation observed in MK-PT cells 96 h after undergoing EGF withdrawal (Fig. 5B). In fact, levels of phosphorylated Akt in MK-PT cells undergoing EGF withdrawal and treated with PD98059 or UO126 (using concentrations at or above their respective IC50) were similar to or higher than those in MK-PT cells continuously stimulated with EGF. These results suggest that enhanced survival of MK-PT cells seen with inhibition of ERK1/2 activity is mediated, at least in part, by an increase in levels of phosphorylated Akt.

Increased Akt Phosphorylation following Inhibition of ERK1/2 Is Dependent on PI3K—We next addressed the mechanism by which inhibition of ERK1/2 might enhance levels of phosphorylated Akt in MK-PT cells subjected to EGF withdrawal. Activation of Akt by survival factors is dependent upon phosphorylation of PI3K (17–19). Interaction of Akt with these phosphorylated PI products leads to its recruitment to the cell membrane, where Akt is phosphorylated and activated by 3-phosphoinositide-dependent kinase-1 (PDK1) (25, 26). Membrane localization and activation of PDK1 is also dependent on phosphorylated PI products generated by PI3K (25, 26).

To determine whether the increased Akt phosphorylation observed in MK-PT cells treated with the MEK1/2 inhibitor UO126 is dependent on PI3K, we used two specific inhibitors of PI3K, wortmannin and LY294002 (22, 27, 28). As in the case of the two MEK1/2 inhibitors, wortmannin and LY294002 are structurally unrelated and exert their effects through distinct mechanisms (27, 28), so that any observed effects in our system are likely the result of PI3K inhibition as opposed to nonspecific effects of these drugs. Inhibition of PI3K with wortmannin or LY294002 prevented the increase in survival (Fig. 6, A and B) as well as the increase in levels of phosphorylated Akt at 96 h (Fig. 7) that occurred with inhibition of MEK1/2 by UO126 (1 μM). We conclude that increased levels of phosphorylated Akt seen in association with inhibition of ERK1/2 following EGF withdrawal are dependent on PI3K.

Activated ERK1/2 Co-immunoprecipitates with Akt in MK-PT Cells Subjected to Survival Factor Withdrawal—We next examined potential mechanisms by which spontaneous activation of ERK1/2 by EGF withdrawal may lead to decreased Akt activity. Because of the extended time course of our studies, involving up to 7 days of EGF withdrawal, spontaneous ERK1/2 activity can affect intracellular signal transduction through a wide variety of mechanisms, ranging from direct physical interactions with other signaling intermediates to ERK1/2-dependent transcription of new genes.

We used immunoprecipitation to look for evidence of a physical association between ERK1/2 and Akt. Immunoprecipitates of total Akt (active and inactive) obtained from lysates of MK-PT cells cultured in the presence of EGF for 96 h contained active phosphorylated ERK1/2 (Fig. 8A). Similarly, immunoprecipitates of total Akt obtained from lysates of MK-PT cells subjected to 96 h of EGF withdrawal contained total and phosphorylated ERK1/2. Inhibition of ERK1/2 activity by PD98059 (10 μM) or UO126 (1 μM) decreased the extent to which both total and active phosphorylated ERK1/2 co-immunoprecipitated with total Akt. Similar results were obtained when immunoprecipitates of total Akt were examined by in vitro kinase assay for ERK1/2 kinase activity (Fig. 8B). We confirmed these studies by transposing the roles of Akt and ERK1/2, that is, by immunoprecipitating total ERK 1/2 and then measuring the amount of co-immunoprecipitated Akt. As seen in Fig. 8C, similar results were obtained. Inhibition of ERK1/2 activity by
PD98059 (10 μM) or UO126 (1 μM) increased the co-immunoprecipitation of active phosphorylated Akt with total ERK1/2. These studies are consistent with the hypothesis that the spontaneous activation of ERK1/2 that occurs in MK-PT cells following withdrawal of survival factors may modulate Akt activity in MK-PT cells through a direct signaling effect. A potential mechanism for the co-localization of ERK1/2 and Akt is through bridging by the kinases Rsk and PDK1. Activation of Rsk requires distinct phosphorylation events by both ERK1/2 and PDK1 (29, 30), whereas activation of Akt requires phos-
phorylation by PDK1 (25, 26). Thus, association of Rsk with ERK1/2 and PDK1 may bring ERK1/2 into proximity with PDK1-associated Akt. Consistent with this possibility, immunoprecipitates of Akt contained both Rsk and PDK1 (Fig. 9A), in addition to ERK1/2 (Fig. 8A). c-Src, an abundant protein in MK-PT cells (31), was not detected in immunoprecipitates of Akt, demonstrating the specificity of the interaction between ERK1/2 and Akt (Fig. 9A). Correspondingly, immunoprecipitates of total ERK1/2 contained both PDK1 (Fig. 9B) and Akt (Fig. 8B). Thus, ERK1/2 and Akt may be part of a multimolecular complex containing PDK1 and Rsk.

**Activation of ERK1/2 in MK-PT Cells following Survival Factor Withdrawal Is Independent of Raf and Involves Oxidant Stress**—We have shown that spontaneous activation of ERK1/2 in MK-PT cells following EGF withdrawal is inhibited by PD98059 and UO126 and is therefore dependent on MEK1/2 (Fig. 3). The best characterized route for activation of MEK1/2 is via the canonical growth factor-dependent pathway, in which stimulation by a growth factor leads to the activation of the cytoplasmic G protein Ras, followed by activation of the kinase Raf, which lies immediately upstream to and activates MEK1/2 (32). However, EGF withdrawal was not associated with Raf activation, as evidenced by the lack of a change in either the level of phosphorylated Raf (Fig. 10A) or in the kinase activity of immunoprecipitated Raf (Fig. 10B) over a 96-h period. These data suggest that spontaneous activation of MEK1/2 and ERK1/2 does not occur through activation of Raf. Thus, it appears that EGF withdrawal activates MEK1/2 through a signaling pathway that is independent of the Ras-Raf pathway. It has previously been shown that MEK1/2 can be activated through a variety of signaling pathways other than the canonical Ras-Raf pathway. Alternate signaling pathways capable of activating ERK1/2 include PKC (33) or cyclic AMP (34).

We have previously shown that induction of the default pathway of apoptosis in MK-PT cells following withdrawal of growth factors is associated with oxidative stress and that antioxidants and scavengers of reactive oxygen species (ROS) can inhibit apoptosis of MK-PT cells following withdrawal of growth factors (14, 15). We therefore examined the role of oxidant stress in ERK1/2 activation following EGF withdrawal. For these studies, we used a number of different antioxidants and ROS scavengers that work through a variety of mechanisms. Desferroxamine inhibits the iron-dependent conversion of hydrogen peroxide to the highly reactive hydroxyl radical
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We report here several novel findings. First, withdrawal of all soluble growth and survival factors from primary cultures of MK-PT cells led to the emergence of ERK1/2 kinase activity, despite the fact that cells were cultured in a basal medium containing no extracellular ligands or stimuli. Second, pharmacological inhibition of ERK1/2, by itself, in the absence of any other known survival factors, was as potent as EGF in maintaining MK-PT cell viability. This finding suggests that increased ERK1/2 kinase activity in response to survival factor deprivation contributed to MK-PT cell death via the default pathway of apoptosis. Third, pharmacological inhibition of ERK1/2 in MK-PT cells deprived of all growth and survival factors not only prevented the decline in Akt activity, but resulted in levels of phosphorylated Akt comparable to those seen in MK-PT cells cultured in the continuous presence of EGF. Thus, the negative effect of spontaneous ERK1/2 activity on MK-PT cell survival appears to be mediated, at least in part, through ERK1/2-dependent down-regulation of the activity of the kinase Akt. Fourth, co-immunoprecipitation studies suggest that ERK1/2 co-localized with Akt in MK-PT cells deprived of all survival factors.

On the basis of these findings, we hypothesize that ERK1/2-dependent modulation of Akt activity takes place within a multimolecular assembly of signaling molecules, containing at a minimum ERK1/2, Akt, Rsk, and PDK1. Finally, the spontaneous emergence of ERK1/2 activity in MK-PT cells deprived of all survival factors occurred in a non-canonical manner, independently of Raf kinase, perhaps mediated by oxidant stress or the accumulation of ROS. A summary of these putative signaling events is depicted in Fig. 12.

The Raf-MEK-ERK and PI3K-Akt pathways play critical roles in the regulation of cell survival and proliferation (1, 2, 17–19). Most growth and survival factors simultaneously activate these two pathways. In some systems, coordinate activation of these pathways occurs via the cytoplasmic G protein Ras, which acts as a positive regulator of both Raf and PI3K (32, 35). Although a number of interactions between the Raf-MEK-ERK and PI3K-Akt signaling pathways have been described, our results are the first to show modulation of Akt activity by signaling events originating with ERK1/2. In contrast, several clear instances of cross-regulation originating with Akt exist in the literature (36, 37). For example, in MC-7 breast cancer cells, phosphorylation of Raf by Akt leads to inhibition of Raf kinase activity, presumably through binding of 14-3-3 protein (36). Negative regulation of Raf by Akt also occurred in differentiated C2C12 myotubular cells but not in undifferentiated C2C12 myoblast cells (37). Inhibition of Raf by Akt correlated with the co-immunoprecipitation of Raf and Akt in differentiated, but not undifferentiated, cells (37).

Our studies do not elucidate the mechanism by which ERK1/2 down-regulates Akt kinase activity. We have shown that ERK1/2 and Akt exist in a multimolecular complex containing at least ERK1/2, Akt, Rsk, and PDK1, suggesting that inhibition of Akt by ERK1/2 involves direct signaling events. Given the extended time course of our studies, we cannot eliminate the possibility that ERK1/2-dependent transcription of new genes also contributes to inhibition of Akt. Moreover, it is likely that other kinases, such as pp70S6k, whose activation, like that of Rsk, requires distinct phosphorylation events by both PDK1 and ERK1/2, will also be part of this signaling complex (29, 30, 38). Assembly of this complex likely entails phosphorylation of Akt by ERK1/2 involves direct signaling events by both PDK1 and ERK1/2, will also be part of this signaling complex (29, 30, 38). Assembly of this complex probably takes place at the cell membrane, where PDK1 and Akt are both recruited by interaction of their pleckstrin homology domains with phosphorylated PI products generated by PI3K (17–19, 25, 26). The fact that PI3K is required for the beneficial effect of ERK1/2 inhibition on cell survival and Akt activity may reflect this need for membrane recruitment.

The inhibitory effect of ERK1/2 on Akt need not necessarily entail phosphorylation of Akt by ERK1/2, nor even direct physical association between these two kinases, but may instead occur through the intermediary effects of a scaffolding, anchoring, or adaptor protein (39). Several negative regulators of Akt
activity have been described, including the phosphatase and tensin homolog on chromosome 10 (PTEN) (40), the Akt-interacting protein CTMP (41), and the caspase-generated cleavage product of the kinase PRK2 (42). Conceivably, sustained activation of ERK1/2 may lead to increased activity of one or more of these negative regulators of Akt. Alternatively, ERK1/2-mediated changes on scaffolding or adaptor proteins may affect the localization of Akt or its interaction with other essential molecules.

The spontaneous emergence of ERK1/2 kinase activity in MK-PT cells destined to die via activation of the default pathway of apoptosis raises several intriguing questions. The first is the mechanism by which ERK1/2 is activated. Our results show that activation of ERK1/2 is independent of Ras kinase and therefore does not occur through the canonical Ras-Raf-MEK-ERK pathway utilized by most growth and survival factors (32). One potential mechanism for ERK1/2 activation is through oxidant stress or accumulation of ROS, because the addition of several different antioxidants and ROS scavengers prevented activation of ERK1/2. In support of a role for ROS, we have previously shown that induction of the default pathway of apoptosis in MK-PT cells is accompanied by oxidant stress (14, 15). In addition, multiple studies indicate that ROS, such as hydrogen peroxide, are not only capable of activating ERK1/2 when added exogenously to cell cultures but also may function as normal signaling intermediates in the activation of ERK1/2 by growth factors, such as lysophosphatidic acid, that signal through G-protein-coupled receptors (43, 44).

A second important issue is the physiological role that spontaneous activation of ERK1/2 plays in cells deprived of all growth and survival factors. One possibility, consistent with inhibition of Akt, is that activation of ERK1/2 plays a role in the executionary phase of apoptosis. Alternatively, activation of ERK1/2 may represent an attempt by the cell to adjust to a "hostile" environment by turning on a new genetic program. For example, spontaneous activation of ERK1/2 may induce the transcription of genes encoding one or more autocrine survival factors or cell membrane survival factor receptors not normally expressed by MK-PT cells. Such initiation of new genetic programs to survive severe environmental stresses is an evolutionarily conserved process and can be found, for example, in bacteria that have been starved of an essential nutrient (45). Bacteria enter a so-called "stringent response," in which proliferative pathways are down-regulated and protective pathways leading to the synthesis of the missing nutrient are initiated (45). It should be noted that these two potential roles for spontaneous ERK1/2 activity, while opposite in nature, are not necessarily mutually exclusive, because their kinetics would be very different. Inhibition of Akt represents an early direct effect of ERK1/2 activity, whereas the induction of new genetic programs requires new gene transcription and would therefore occur after a delay of several hours or longer.

In summary, we have shown that withdrawal of all growth and survival factors from primary cultures of MK-PT cells is associated with the spontaneous emergence of ERK1/2 kinase activity. Activation of ERK1/2 occurs via MEK1/2 and appears to be dependent on oxidant stress, accompanying induction of the default pathway of apoptosis. We further show that activation of ERK1/2 induces down-regulation of Akt and that pharmacological inhibition of ERK1/2 not only prevents down-regulation of Akt but promotes MK-PT cell survival with a potency equal to that of EGF. ERK1/2-mediated inhibition of Akt apparently occurs within a multimolecular complex also including Rsk and PDK1. Taken together, our data suggest that activation of ERK1/2, in the absence of known extracellular ligands or stimuli, represents a previously unrecognized and major regulatory pathway determining the fate of cells destined to die by the default pathway of apoptosis.

**Fig. 12. Model of signaling events in MK-PT cells deprived of survival factors.** In MK-PT cells deprived of survival factors, oxidant stress, and/or the accumulation of reactive oxygen species (ROS) induces the activation of ERK1/2 via MEK1/2. Activation of MEK1/2 is independent of Ras1 kinase. Activated ERK1/2 then inhibits the activity of Akt, a kinase that plays a critical role in maintaining the viability of MK-PT cells. Co-localization of ERK1/2 and Akt occurs within a multimolecular complex at the cell membrane, initiated by recruitment of Akt and PDK1 to the cell membrane via specific interaction with the PI3K-generated phospholipids, phosphatidylinositol (3,4)-bisphosphate and (3,4,5)-trisphosphate (PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃). Recruitment of Rsk to this complex is dependent upon the kinase Rsk. Activation of Rsk requires distinct phosphorylation events by both ERK1/2 and PDK1, whereas activation of Akt requires phosphorylation by PDK1. Thus, association of Rsk with ERK1/2 and PDK1 brings ERK1/2 into proximity to PDK1-associated Akt. The precise mechanism by which ERK1/2 inhibits Akt is unclear. Inhibition may not necessarily require direct physical interaction between ERK1/2 and Akt but may instead occur through the intermediary effect of a scaffolding, anchoring, or adaptor protein. Pharmacological inhibition of MEK1/2 prolongs MP-KT cell survival by preventing activation of ERK1/2 and subsequent inhibition of Akt by ERK1/2.
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