Rsk1 mediates a MEK–MAP kinase cell survival signal

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Background: Growth factors activate an array of cell survival signaling pathways. Mitogen-activated protein (MAP) kinases transduce signals emanating from their upstream activators MAP kinase kinases (MEKs). The MEK–MAP kinase signaling cassette is a key regulatory pathway promoting cell survival. The downstream effectors of the mammalian MEK–MAP kinase cell survival signal have not been previously described.

Results: We identify here a pro-survival role for the serine/threonine kinase Rsk1, a downstream target of the MEK–MAP kinase signaling pathway. In cells that are dependent on interleukin-3 (IL-3) for survival, pharmacological inhibition of MEKs antagonized the IL-3 survival signal. In the absence of IL-3, a kinase-dead Rsk1 mutant eliminated the survival effect afforded by activated MEK. Conversely, a novel constitutively active Rsk1 allele restored the MEK–MAP kinase survival signal. Experiments *in vitro* and *in vivo* demonstrated that Rsk1 directly phosphorylated the pro-apoptotic protein Bad at the serine residues that, when phosphorylated, abrogate Bad's pro-apoptotic function. Constitutively active Rsk1 caused constitutive Bad phosphorylation and protection from Bad-modulated cell death. Kinase-inactive Rsk1 mutants antagonize Bad phosphorylation. Bad mutations that prevented phosphorylation by Rsk1 also inhibited Rsk1-mediated cell survival.

Conclusions: These data support a model in which Rsk1 transduces the mammalian MEK–MAP kinase signal in part by phosphorylating Bad.

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Background

Cell survival and proliferation require timely signals from extracellular growth factors for normal tissue development and maintenance. The molecular mechanisms underlying the remarkable efficiency with which growth factors inhibit the cellular apoptotic machinery have come under intensive investigation. The strength of any one growth factor's pro-survival effect is derived from the additive effects of the multiple downstream survival signaling pathways it activates [1,2]. Multiple survival agonists acting in concert target an even broader spectrum of survival pathways, providing synergistic survival signalling [3]. The multiplicity of survival signals and the gradation of their strength offer an organism exquisite regulatory control over the development and maintenance of cells for any given tissue. Molecular dissection of individual survival pathways emanating from a given growth factor is essential to elucidate the processes leading to both normal tissue activity as well as diseases characterized by inappropriate cell survival or excessive cell death.

The signal transduced by mitogen-activated protein (MAP) kinases and their upstream activators MAP kinase kinases (MEKs) is a key pathway promoting cell survival in response to growth factors [2,4–6]. Whereas the *Drosophila* pro-apoptotic protein Hid, which so far lacks known mammalian homologues, is a molecular target of the MAP kinase survival pathway during fly development [7,8], the mammalian downstream effectors of the MEK–MAP kinase cell survival signal have not been identified to date.

Mek1 and Mek2 activate the MAP kinases Erk1 and Erk2, which in turn play a critical role in the activation of the 90 kDa ribosomal S6 kinases (RSKs), serine/threonine kinases that are activated in response to mitogenic stimuli [9–12]. RSK activation is complex and requires phosphorylation at several sites, the regulation of which is only partially understood [10,13]. Members of the RSK protein kinase family contain two kinase domains. The carboxy-terminal kinase domain has not been shown to have phosphotransferase activity toward any exogenous substrates to date, and appears to be important for autophosphorylation and activation of the amino-terminal kinase domain. The latter is responsible for the phosphorylation of all known RSK substrates, which include the transcription factor c-Fos [14], the cAMP response element binding protein (CREB) [15], the transcriptional inhibitor IκB [16], the estrogen receptor [17] and the kinase Myt 1 [18]. As several RSK substrates have been implicated in the regulation of cell survival, we hypothesized that the MEK–MAP kinase survival signal could be transduced by RSKs.

Results

Interleukin-3 (IL-3) is a potent agonist of a number of signaling cascades in hematopoietic cells, including signals emanating from the phosphoinositide (PI) 3-kinase–protein kinase B/Akt pathway, the MAP kinase pathway and the protein kinase A (PKA) pathway [19–21]. The murine hematopoietic cell line 32D is dependent on IL-3 for survival [22]. The contribution of the MEK–MAP kinase pathway to IL-3-dependent survival was evaluated using chemical inhibitors of Mek1/2. In the continued presence of IL-3, either of the two MEK1/2-specific inhibitors PD098059 and U0126 induced apoptosis up to threefold compared to control vehicle-treated cells (Figure 1a). The diminished ability of IL-3 to suppress apoptosis in the absence of MEKs was consistent with other studies demonstrating a MEK-dependent survival signal in these cells [19]. As predicted from the multiplicity of survival pathways activated by IL-3, the effect of removing all IL-3-mediated survival signals was greater than the effect of inhibiting the MEK pathway alone and resulted in 80–90% apoptosis in this assay (data not shown). Introduction of constitutively active Mek1 (Mek-DD) into these cells promoted cell survival in the absence of IL-3 (Figure 1c). As expected from the pharmacological data in Figure 1a, Mek-DD alone was insufficient to restore the entire survival effect of IL-3 that is observed when all downstream survival pathways are activated in concert. Nonetheless, Figure 1a indicates that the remaining survival signals were not sufficient to compensate completely for the absence of the MEK signal.

To test the hypothesis that the MEK survival signal was transduced by Rsk1, the ability of a kinase-inactive Rsk1 mutant to block MEK-mediated cell survival was assayed. As shown in Figure 1c, cotransfection of Mek-DD together with a kinase inactive Rsk1 allele (Rsk1ΔΔ), which carries inactivating point mutations in both the amino-terminal and carboxy-terminal kinase domains (K112R,K464R), eliminated the protective effect of Mek-DD. As a control, co-expression of wild-type Rsk1 did not affect Mek-DD-mediated protection.

To further test this hypothesis, a novel constitutively active Rsk1 mutant was constructed. To date, RSKs have proven refractory to a point-mutant approach towards generating a constitutively active allele ([11] and data not shown). As the catalytic loop in Rsk1's amino-terminal kinase domain is phosphorylated and activated by PDK1 [23,24], a myristoylation sequence was added to the amino terminus of avian Rsk1. Whereas wild-type Rsk1 was inactive in the absence of serum, myristoylated Rsk1 maintained kinase activity following serum withdrawal (Figure 1b). Western blots confirmed that under these serum-starvation conditions, Erk proteins were inactive, as shown by the absence of the slower migrating phosphorylated polypeptides (Figure 1b). Consistent with its high degree of homology with avian Rsk1, human Rsk1 also exhibited constitutive activity following myristoylation (data not shown). When introduced into 32D cells, myristoylated avian Rsk1 protected cells from IL-3 withdrawal to an extent comparable to that observed with activated Mek-DD (Figure 1c). The inhibition of the MEK survival

Figure 1

Rsk1 mediates Mek1/2-dependent cell survival in 32D cells. **(a)** Inhibition of endogenous Mek1/2 promotes apoptosis in 32D cells in the presence of IL-3. Cells were treated with dimethyl sulfoxide (DMSO; vehicle), PD098059 or U0126 in the presence of IL-3 as described in the Materials and methods. The number of viable cells was scored by trypan blue staining after 24–30 h of treatment and converted to the percentage of cells that were nonviable. Error bars represent the SEM of three separate experiments. **(b)** Myristoylated avian Rsk1 (Myr–Rsk1) remains active in the absence of serum. Serum-starved HEK 293E cells transfected with either wild-type or myristoylated avian Rsk1 were stimulated for 10 min with serum as indicated. Whole cell lysates were analyzed by immunoblotting with an avian-specific anti-Rsk1 antibody (anti-Rsk1(Av)) and anti-Erk1/2 antibodies as indicated. Whole cell lysates were also immunoprecipitated with the anti-Rsk1(Av) antibody and immune-complex kinase assays were performed using GST–S6 as a substrate. Kinase activity was measured by the intensity of radioactive labeling of the
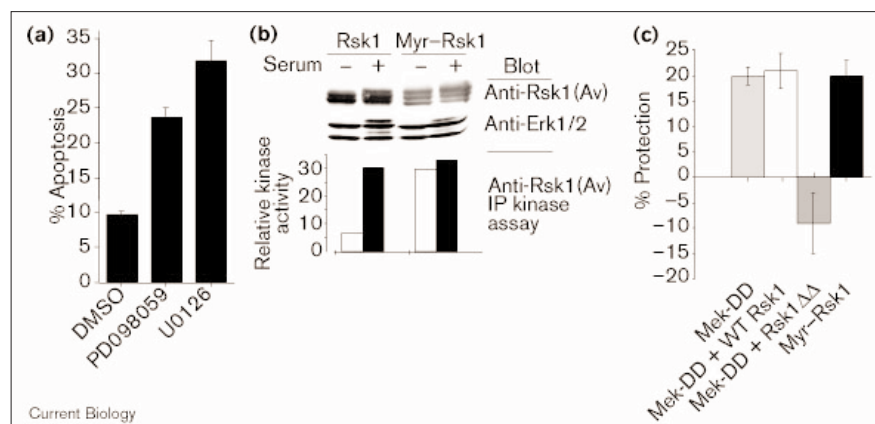
substrate band on a polyacrylamide gel (data not shown) and quantitated using phosphorimaging (graph). **(c)** Myristoylated Rsk1 and activated Mek1 (Mek1-DD) promote IL-3-independent survival, whereas kinase-dead Rsk1 eliminates Mek1-DD protection. Cells were transfected with the indicated constructs, as described in the Materials and methods; WT, wild type.

At 5–7 h post-transfection, the cells were starved of IL-3 for 14–18 h. GFP-positive cells were scored for apoptosis, as measured by PI uptake. The maximum level of apoptosis measured was around 60%. Results are expressed as percentage protection relative to that given by the vector for each independent experiment. Error bars represent the SEM for three independent experiments.

signal by kinase-inactive Rsk1, together with the restoration of the survival signal with activated Rsk1 supports a role for Rsk1 in transducing the MEK survival signal.

How might Rsk1 promote cell survival? As growth-factor-mediated survival has been shown to correlate with phosphorylation of the pro-apoptotic Bcl-2-related protein Bad in 32D cells in a manner dependent on MEKs [19], and as a growing number of reports implicate the MEK–MAP kinase pathway in phosphorylation of Bad [19,25,26], we explored the potential relationship between Rsk1 and Bad. To test whether Bad is phosphorylated by Rsk1 *in vitro*, HEK 293E cell lysates were immunoprecipitated using antibodies against endogenous Rsk and immune-complex kinase assays were performed using the bacterially expressed glutathione-S-transferase (GST)-conjugated substrate GST–Bad or, as a positive control, GST–S6. As an additional positive control, immune-complex kinase assays were performed from these same lysates using antibodies against Akt with GST–Bad and GST–glycogen synthase kinase (GSK)-3β as substrates. Following serum stimulation, immunoprecipitated RSKs directly phosphorylated both GST–S6 and GST–Bad (Figure 2a). Under these conditions in HEK 293E cells, Akt was poorly activated by serum but responded well to insulin. Conversely, Rsk was poorly activated by insulin. Transfected kinase-inactive Rsk1 demonstrated no detectable kinase activity toward recombinant GST–Bad (Figure 2b). Given these data, it is likely that Rsk1 phosphorylates Bad directly rather than via an additional coprecipitating kinase.

To determine which residues were phosphorylated by Rsk *in vitro*, recombinant GST–Bad point mutants carrying serine-to-alanine mutations at positions Ser112 (S112A) or Ser136 (S136A) or both (S112A,S136A) were used as kinase assay substrates for endogenous RSKs (Figure 2c,d). Consistent with studies demonstrating Akt's specificity for the Ser136 residue [27], Akt showed little kinase activity towards Bad mutants carrying the S136A mutation. Mutation of the Ser112 residue partially decreased Bad phosphorylation by Rsk. Although the phosphorylation of the S136A mutant was comparable to that of wild-type Bad under these conditions of substrate excess, mutation of both Ser112 and Ser136 further reduced Rsk phosphorylation of Bad below that seen with the S112A mutation alone. These *in vitro* data indicate that endogenous Rsk is capable of phosphorylating Bad at both Ser112 and Ser136, with a preference for the Ser112 site. Consistent with Rsk's phosphorylation of Bad *in vitro*, 32D cells treated with the Mek1/2 chemical inhibitors PD098059 or U0126 showed corresponding reductions in endogenous MAP kinase activation, endogenous phosphorylation of Bad at Ser112 and endogenous Rsk kinase activity (Figure 2e).

To determine whether myristoylated Rsk1 conferred constitutive phosphorylation of Bad in the absence of serum

in vivo, HEK 293E cells were transfected with GST–Bad together with either wild-type or myristoylated Rsk1. Following 24 hours of serum deprivation, cells were lysed before or after 10 minutes of serum stimulation. Expression levels of the transfected Rsk1 and GST–Bad constructs are shown in Figure 3a. Cotransfection of myristoylated Rsk1 reduced the mobility of the GST–Bad protein in both the presence and the absence of serum. To aid normalization of Bad expression levels, the lysates were run on a higher percentage gel, causing all forms to run at the same position. The blot was then stripped and reprobed with antibodies specific to Bad phosphorylated on Ser112 or Ser136 (anti-Bad pS112 and anti-Bad pS136). Transfection with myristoylated Rsk1 resulted in constitutive phosphorylation of Bad at both Ser112 and Ser136 (Figure 3a).

Kinase-inactive Rsk1 mutants were tested for their ability to block Bad phosphorylation *in vivo*. As shown in Figure 3b, a plasmid encoding GST–Bad was cotransfected with the kinase-inactive Rsk1 mutant (Rsk1ΔΔ). Cells were serum-starved and then stimulated and lysates were analyzed by western blotting. Following serum stimulation, the anti-Bad pS112 antibody signal was reduced in cells cotransfected with Rsk1ΔΔ. A similar result was obtained using a Rsk1 construct with a single point mutation in the amino-terminal kinase domain (Rsk1 K112R; data not shown). Probing with anti-Bad pS136 showed little effect of the kinase-inactive Rsk1 at that site (Figure 3b, lanes 5 and 6), consistent with the observation that alternative kinases such as Akt can also phosphorylate this site. An anti-GST western blot confirmed that these differences could not be attributed to differences in Bad expression levels.

To test whether crosstalk between the Akt and Rsk1 pathways could lead to Bad phosphorylation, myristoylated Rsk1 was tested for its ability to activate wild-type Akt and myristoylated Akt was tested for its ability to activate wild-type Rsk1. Figure 4 shows that, in the absence of serum, no wild-type Akt kinase activity was observed with or without cotransfected myristoylated Rsk1 (compare lanes 7 and 13). Wild-type Akt is poorly activated by serum in HEK 293E cells; in control experiments, however, this wild-type Akt construct showed strong Akt kinase activity in response to stimuli such as insulin (data not shown). Conversely, no wild-type Rsk1 kinase activity was observed in the presence of myristoylated Akt (compare lanes 9 and 11). Thus, Rsk1 phosphorylates Bad independently of Akt. Rsk1 phosphorylation of Bad is also independent of PKA, as the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), which stimulates the MAP kinase pathway and Rsk1 but not PKA in HEK 293E cells, also leads to Bad phosphorylation at Ser112, a result prevented by the Mek1/2 inhibitor PD098059 (data not shown).

To investigate the connection between the effect of Rsk1 on survival and its phosphorylation of Bad, we turned to

Figure 2
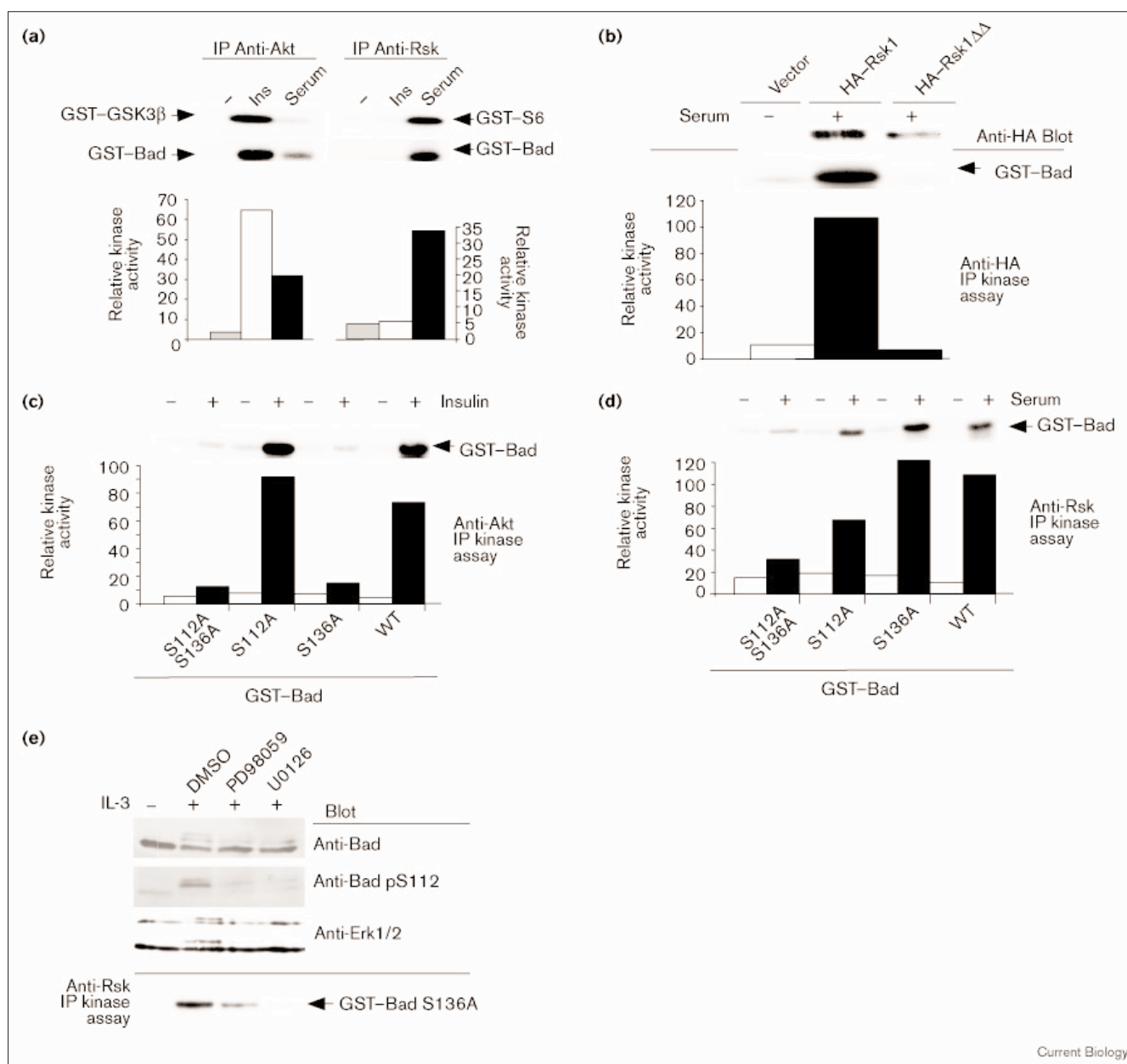
Rsk phosphorylates Bad *in vitro*. **(a)** Endogenous Akt or Rsk was immunoprecipitated (IP) from lysates of HEK 293E cells starved in serum-free media for 24 h prior to stimulation with 0.1 μM insulin (ins; 20 min) or 10% serum (10 min). Kinase assays using bacterially expressed GST–Bad, GST–S6 or GST–GSK3β as substrates were performed as described in the Materials and methods. Kinase activity was measured by the intensity of radioactive labeling of the substrate band on a polyacrylamide gel (top) and quantitated using phosphorimaging (bottom). Only the results with GST–Bad are quantitated. **(b)** Hemagglutinin-labeled kinase-inactive Rsk1 (HA–Rsk1ΔΔ) shows no associated kinase activity toward Bad. Lysates from serum-stimulated HEK 293E cells transfected with the indicated Rsk1 constructs were analyzed by immunoblotting with an anti-HA antibody. Anti-HA immune-complex kinase assays using GST–Bad as a substrate were performed. **(c,d)** Endogenous Rsk

phosphorylates Bad at Ser112 and Ser136 *in vitro*. Serum-starved HEK 293E cells were stimulated as in (a) and immune-complex kinase assays were performed using (c) anti-Akt or (d) anti-Rsk antibodies and GST–Bad mutant substrates as indicated. **(e)** Inhibition of endogenous Mek1/2 and Rsk blocks endogenous phosphorylation of Bad at Ser112 in 32D cells. Cells were starved of IL-3 for 3.5 h. DMSO, PD098059 (50 μM) or U0126 (5 μM) were added as indicated for 30 min prior to the re-addition of recombinant IL-3 for 10 min. Bad was immunoprecipitated and detected by western blot using the indicated antibodies. Densitometry analysis (data not shown) revealed a 3.6-fold decrease in the anti-BAD pS112 antibody signal with PD98059 and a 17-fold decrease with U0126 relative to the DMSO control. Anti-Erk1/2 immunoblots and anti-Rsk immune-complex kinase assays show that Mek1/2 inhibitors reduce the IL-3-induced kinase activity of Erk1/2 and Rsk, respectively.
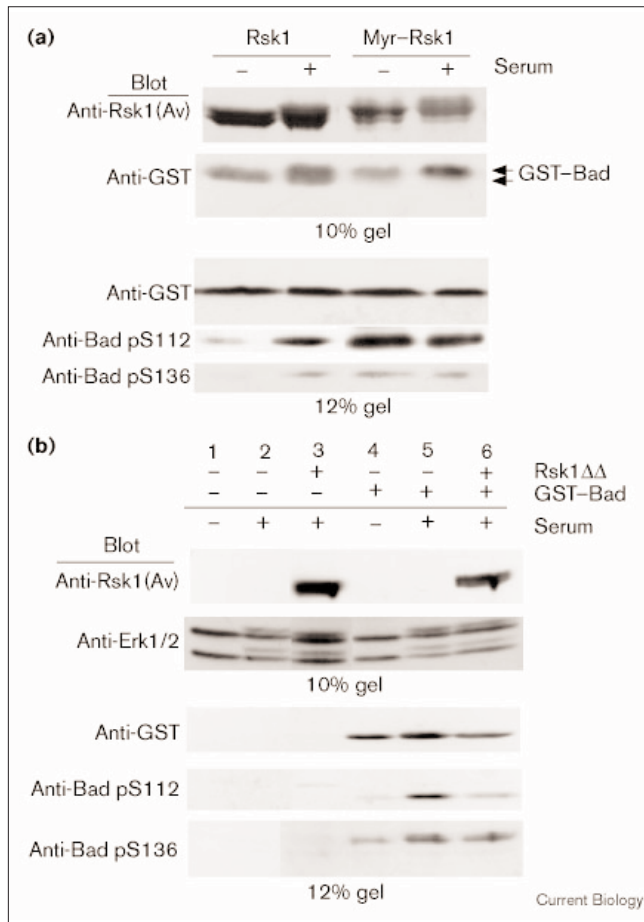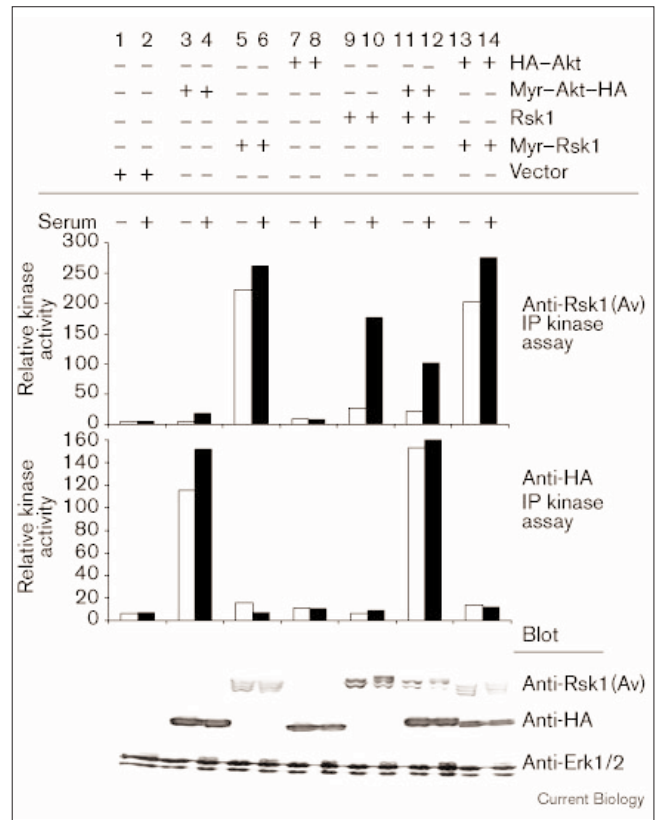
Rsk1 phosphorylates Bad *in vivo*. **(a)** Myr–Rsk1 induces growth-factor-independent Bad phosphorylation. HEK 293E cells transfected with GST–Bad together with either wild-type Rsk1 or Myr–Rsk1 were serum-starved then stimulated with serum for 10 min as indicated. Lysates were analyzed by immunoblotting with the indicated antibodies; anti-Rsk1(Av), avian-specific anti-Rsk1 antibody. Electrophoresis was performed on 10% polyacrylamide gels in the top two panels to visualize polypeptide mobility shifts and on 12% gels in the bottom three panels to facilitate quantitation. Densitometric analysis (data not shown), normalized for GST–Bad expression, showed that Myr–Rsk1 induced similar increases in phosphorylation of Bad at Ser112 or Ser136 in the presence or the absence of serum. **(b)** Kinase-inactive Rsk1 antagonizes Bad phosphorylation. HEK 293E cells transfected with GST–Bad and K112R/K464R Rsk1 (Rsk1ΔΔ) as indicated were serum-starved and stimulated as in (a). Lysates were analyzed by immunoblotting with the indicated antibodies. 12% polyacrylamide gels, which abrogated polypeptide mobility shifting, were run in the bottom three panels to facilitate quantitation. Densitometric analysis (data not shown) showed that kinase-inactive Rsk1 induced a 40% decrease in signal with the anti-Bad pS112 antibody (lane 6), but no decrease in signal was detected with the anti-Bad pS136 antibody (lane 6) relative to controls (lane 5).

Rsk1 and Akt phosphorylate Bad independently. HEK 293E cells transfected with the indicated constructs were serum-starved and then stimulated with serum as indicated. Immune-complex kinase assays using either avian-specific anti-Rsk1 (anti-Rsk1(Av)) or anti-HA antibodies and the substrate GST–Bad were performed. Protein expression was determined by immunoblotting with the indicated antibodies.

the FL5.12 cell system developed by Stan Korsmeyer and colleagues [28]. We used FL5.12 cells that stably express both Bad and the related anti-apoptotic protein Bcl-x$_L$ (FL5.12–Bcl-x$_L$–Bad cells), which have been shown to undergo apoptosis following IL-3 withdrawal in a manner dependent on the phosphorylation state of Bad. The phosphorylation of the Bad Ser112 and Ser136 sites in these cells antagonizes Bad's binding to and disabling of Bcl-x$_L$ [28]. IL-3 activated both endogenous MAP kinase and endogenous Rsk in FL5.12–Bcl-x$_L$–Bad cells (Figure 5a). Constitutively active Rsk1 conferred constitutive phosphorylation of Bad at Ser112 (Figure 5b) and, as in 32D cells, partially restored protection from apoptosis following IL-3 withdrawal (Figure 5c). To test whether part of Rsk1's survival effect occurred through Bad phosphorylation, the survival effect of constitutively active Rsk1 was assayed in FL5.12 cells stably expressing a Bad construct containing serine-to-alanine substitutions at both Ser112 and Ser136. Western blot analysis confirmed that equivalent levels of Bad were expressed in each cell line ([28] and data not shown). Two-thirds of Rsk1's protective effect was abrogated by co-expression of this mutant form of Bad (Figure 5c). To control for potential cell-line-dependent variations, the survival in

Figure 5
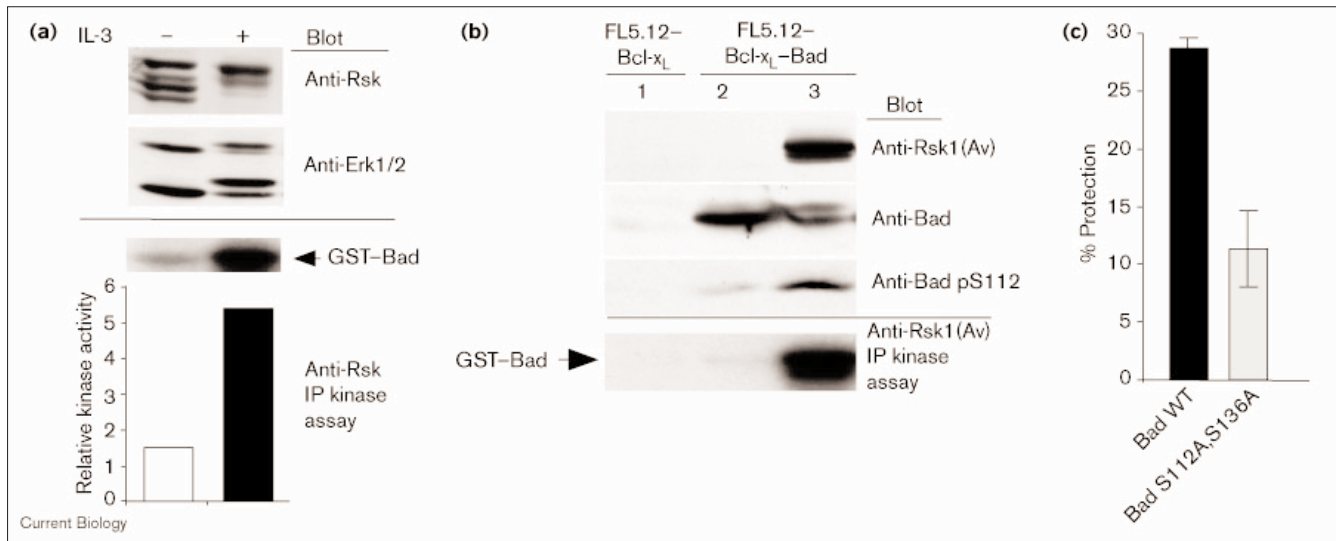
Rsk1 phosphorylates Bad and protects FL5.12–Bcl-x$_L$–Bad cells from the apoptosis induced by IL-3 withdrawal. **(a)** IL-3 activates Rsk in FL5.12–Bcl-x$_L$–Bad cells. Cells were deprived of IL-3 for 4 h prior to 10 min of IL-3 stimulation (0.5 ng/ml). Cell lysates were analyzed by immunoblotting with the antibodies indicated. Immune-complex kinase assays of endogenous Rsk were performed using a GST–Bad substrate. **(b)** Myr–Rsk1 promotes Bad phosphorylation in FL5.12–Bcl-x$_L$–Bad cells. Cells transfected with either empty vector (lane 2) or Myr–Rsk1 (lane 3) were lysed 4 h post-transfection and whole cell lysates were analyzed by immunoblotting with the indicated antibodies; anti-Rsk1(Av), avian-specific anti-Rsk1 antibody. Lane 1 shows an untransfected FL5.12-Bcl-x$_L$ parental cell line as a control. By densitometric analysis, Myr–Rsk1 induced a sixfold increase in the anti-Bad pS112 antibody signal (lane 3) relative to vector (lane 2). Anti-Rsk1(Av) immune-complex kinase assays using GST–Bad as substrate are shown in the bottom panel. **(c)** Bad S112A,S136A double mutants antagonize the Rsk1 survival signal. FL5.12–Bcl-x$_L$–Bad cells containing either the wild-type (WT) or the S112A,S136A version of Bad were electroporated with GFP and Myr–Rsk1. Apoptosis was assayed as described in the Materials and methods; the maximal level of apoptosis was between 46% and 56%. Percentage protection against apoptosis is measured relative to vector control for each cell line for each independent experiment. Error bars represent the SEM for three independent experiments.

the presence of myristoylated Rsk1 was reported relative to that with vector controls for each cell line. The residual Rsk1 survival effect observed with the double Bad mutant raises the exciting possibility that Rsk1, like Akt, may target multiple survival pathways.

Discussion

The molecular mechanisms underlying the mammalian MEK–MAP kinase survival pathway have not been previously described. This study supports a novel role for Rsk1 as a downstream effector of the MEK–MAP kinase survival signal. Kinase-inactive Rsk1 blocked the MEK survival signal and constitutively active Rsk1 restored the survival effect observed with activated Mek1. Furthermore, these studies identified Bad as one target through which Rsk1 could promote cell survival.

The emerging complexity and interplay between multiple survival signaling pathways has been the focus of two recent reviews [1,2]. It is intriguing to note that upstream regulators of Rsk1, such as the small GTPase Ras, can activate multiple survival pathways. The MEK pathway is one component of the Ras survival pathway, and Ras point mutations that prevent Ras-dependent activation of

MEKs partially inhibit the Ras survival effect [29]. Our data support the observation that these additional survival signals are insufficient to fully compensate for the loss of the MEK signal in certain cell types. The magnitude of Ras-mediated cell survival represents the additive effects of multiple downstream survival mechanisms of which the MEK–MAP-kinase–Rsk1 pathway is one. It is likely that the interplay between multiple signals influences the final outcome with regard to cell survival and that the MEK–Rsk1 survival signal to Bad and other substrates acts within this context.

An important link between kinase signaling pathways and the cellular apoptotic machinery was made following the discovery that Bad undergoes phosphorylation at the Ser112 and Ser136 positions in response to IL-3 or insulin-like growth factor 1 (IGF-1) [21,27,28,30]. Bad promotes apoptosis at least in part by dimerizing with and inactivating Bcl-x$_L$ [28]. Phosphorylation of Bad at either Ser112 or Ser136 abrogates its pro-apoptotic function by promoting its dissociation from Bcl-x$_L$ [28]. Once phosphorylated at either or both sites, Bad binds to 14-3-3 proteins in an interaction that has been proposed to sequester Bad from Bcl-x$_L$ [28]. A strikingly extensive

array of separate signaling pathways regulates Bad's apoptotic activity. Akt phosphorylates Bad at Ser136 upon IGF-1 or IL-3 stimulation [21,27]. PKA can phosphorylate Bad at Ser112 [20] and calcineurin can promote apoptosis by dephosphorylating and hence activating Bad [31]. Mek1/2-dependent Bad phosphorylation has also been described in some cell systems [19,25,26], and our data now identify Rsk1 as a MEK-dependent Bad kinase.

Although Bad's antagonism of Bcl-x$_L$ activity plays an important role in apoptosis in some cell systems, Bad is not universally required for apoptosis to occur [25,32]. One reason for this could be the composite variability of expression and regulation of pro- and anti-apoptotic members of the Bcl-2 family. Bad expression levels are variable between different tissues [33] and the ratios between the pro- and anti-apoptotic members of the Bcl-2 family have been proposed to function as a rheostat, with the combined expression levels and functional state of all family members finally tipping the scales of cell fate toward either life or death [34].

Conclusions
This study has identified Rsk1 to be a critical mediator of the MEK–MAP kinase cell survival signal and identifies Bad as one target of the Rsk1 survival pathway. Like the kinases Akt and PKA, Rsk1 probably promotes cell survival via targets in addition to Bad (Figure 6). The Akt cell survival signal is mediated by several mechanisms in addition to Bad phosphorylation, such as phosphorylation of Caspase-9 [35], CREB [36], GSK-3β [37], the Forkhead transcription factor FKHRL1 [38], and IκB kinase [39,40]. Likewise, PKA has also been shown to phosphorylate both Bad [20] and CREB [41]. As we observed a residual Rsk1 survival effect in FL5.12 cells expressing a form of Bad that could no longer be phosphorylated at Ser112 and Ser136, it is likely that Rsk1 substrates in addition to Bad play a role in apoptosis. It is intriguing that Rsk1 also phosphorylates a number of substrates associated with apoptosis, such as IκB [16] and the transcription factors CREB [15] and c-Fos [14]. Thus, in addition to regulating cell survival via post-translational mechanisms, Rsk1, like Akt, probably contributes to transcriptionally governed survival pathways. Further investigation into the novel pro-survival function of Rsk1 is needed to elucidate the roles of additional Rsk1 substrates in MEK–MAP kinase-mediated cell survival.

Materials and methods
Cell culture, transfection and electroporation
E1A-transformed human embryonic kidney 293 (HEK 293E) cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS). Transfection was by calcium phosphate precipitation; the total amount of DNA per transfected 60 mm dish was 3–5 μg. FL5.12 cells stably expressing Bcl-x$_L$ together with either wild-type or mutant Bad (FL5.12–Bcl-x$_L$–Bad cells; a gift of S. Korsmeyer) were cultured as described [28] except that recombinant IL-3 (0.5 ng ml^{-1}) was used instead of WEHI-cell-conditioned media.

Figure 6
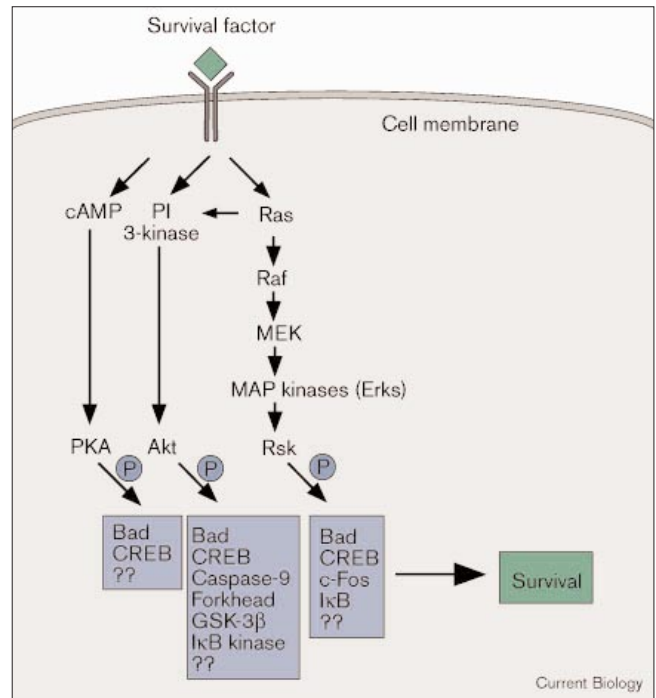
Model in which the Bad kinases Akt, PKA and Rsk1 mediate cell survival by phosphorylating an array of effector molecules. Survival factors trigger diverse signaling pathways, which activate kinases that can neutralize the pro-apoptotic effects of Bad. These three known kinases, the cAMP-dependent kinase PKA, the PI 3-kinase-dependent kinase Akt and now the MEK–MAP kinase-dependent kinase Rsk1, not only regulate the phosphorylation state of Bad but also of a variety of effector molecules implicated in apoptotic regulation. This offers the cell a sensitive combinatorial means by which it can regulate the triggering of the apoptotic program. P, phosphorylation.

The 32D cells (a gift of M. Meyers) were maintained in RPMI medium supplemented with 10% FBS and 5% WEHI-conditioned medium as a source of IL-3. For electroporation, the cells were pelleted and resuspended in 10% FBS in RPMI1640 without phenol red (Gibco BRL) at a concentration of 1–1.5 × 10^7 cells per 500 μl. A 500 μl aliquot of this cell suspension was added to a total of 20 μg DNA for each electroporation. The Rsk1 mutant plasmids were transfected at a 9:1 ratio (μg:μg) relative to the GFP plasmid to ensure that GFP-positive cells would also carry the gene of interest. Electroporation settings were 300 V and 800 μF for FL5.12 cells and 250 V and 800 μF for 32D cells. Electroporation was performed using a BioRad electroporation unit and 0.4 cm cuvettes. Cells were permitted to recover for 5–10 min and then gently transferred to 15–25 ml of antibiotic-free culture media for 5–10 h prior to apoptosis assays and biochemical analysis. Transfection efficiencies ranged between 25–40% for FL5.12 cells and 10–20% for 32D cells.

Immunoprecipitation, in vitro *kinase assays, immunoblotting and antibodies*
For HEK 293E cells, cells were washed once with serum-free DMEM and then returned to the same medium for 24 h prior to stimulation with either 10% FBS for 10 min or insulin (0.1 μM) for 20 min as indicated in the figure legends. For FL5.12 and 32D cells, the cells were washed three times in 10–15 ml volumes of serum-free, IL-3-free Iscove's modified Dulbecco's medium (IMEM) or RPMI and then incubated in the same for 2–4 h prior to stimulation with recombinant IL-3 (0.5 ng ml^{-1})

for 10 min. Prior to lysis, cells were washed once in ice-cold PBS and then lysed in lysis buffer (pH 7.2) composed of 0.5% NP40 (or IPEGAL), 0.1% Brij-35, 0.1% sodium deoxycholate (NaDOC), 1 mM EDTA, 7 mM K_2HPO_4, 3 mM KH_2PO_4, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerolphosphate, 1 mM Na vanadate, 2 mM DTT, 5 μg ml^{-1} pepstatin A, 10 μg ml^{-1} leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Lysates were then cleared by spinning for 10 min at 4°C and 14,000 rpm in an Eppendorf microcentrifuge. Immunoprecipitation kinase assays were performed on lysates containing 250–500 μg protein. Lysates were incubated with the indicated antibodies for 1–3 h, then incubated for an additional hour with 20–30 μl of a 50% slurry of Protein-A–Sepharose beads (Sigma) in PBS. The beads were washed once with Buffer A composed of 1% NP40, 0.5% NaDOC, 100 mM NaCl, 10 mM Tris pH 7.2, 1 mM EDTA, 1 mM Na vanadate, 2 mM DTT and 1 mM PMSF; once with Buffer B composed of 1M NaCl, 0.1% NP40 (or IPEGAL), 10 mM Tris pH 7.2, 1 mM Na vanadate, 2 mM DTT and 1 mM PMSF; once with ST buffer composed of 150 mM NaCl, 10 mM Tris pH 7.2 and 1 mM PMSF; and once with 20 mM HEPES, 10 mM $MgCl_2$. The kinase assays were performed by adding 30 μl of kinase buffer composed of 20 mM HEPES pH 7.2, 10 mM $MgCl_2$, 100 μg ml^{-1} BSA, 3 mM β-mercaptoethanol, 50 μM ATP, 10 μCi [γ^{32}P]ATP and 2–3 μg of recombinant substrate. Reactions were incubated for 30 min at 30°C and were stopped by adding 10 μl of 4× sample buffer and boiling for 3 min. Reactions were subjected to SDS–PAGE on 12% gels and quantitation was accomplished by phosphorimaging. For immunoblots, 20–100 μg of protein lysate per lane were analyzed by SDS–PAGE on 10% gels, except as otherwise indicated in the figure legends, and as noted below for endogenous Bad. The proteins were transferred to nitrocellulose membranes, blocked in 5% milk in PBST (PBS + 0.2% Tween-20), probed with the indicated antibodies and visualized using enhanced chemiluminescence (NEN). For detection of endogenous Bad in 32D cells, 5×10^8 cells were lysed in 750 μl of lysis buffer, immunoprecipitated with 5 μl of anti-Bad antibody (Santa Cruz), washed three times in lysis buffer, boiled in sample buffer and subjected to SDS–PAGE on a 10% gel. Protein was then transferred to a nitrocellulose membrane and blocked in 5% milk in Tris-buffered saline containing 0.2% Tween-20. Anti-Bad antibody (R&D Systems) was used as primary probe and Protein-A–horseradish peroxidase (HRP) was used as secondary probe. Visualization was accomplished using enhanced chemiluminescence (NEN). Densitometry measurements were performed using a Biorad imaging system and Quantity One software. Commercial antibodies were obtained and used as directed from the following sources: anti-Akt, UBI; anti-Bad, R&D Systems, Santa Cruz; anti-Bad pS112, NEB; anti-Bad pS136, NEB; Protein-A–HRP secondary probe, Pierce. The rabbit polyclonal anti-GST antibody was a gift from T. Rapaport. Rabbit polyclonal anti-Rsk, avian-specific anti-Rsk1 and anti-Erk1/2 antibodies were made as described [10]. Results presented are representative of three independent experiments.

Apoptosis assays
To induce apoptosis, FL5.12 (or 32D) cells were washed three times in 10–15 ml volumes of IMEM (Gibco BRL; or RPMI for 32D cells). Electroporated cells were then subjected to centrifugation through 3 ml Ficoll cushion for 30 min at 2,000 rpm and viable cells at the interface were collected. Viable cells were then incubated in IMEM containing 10% FBS (or RPMI containing10% FBS for 32D cells) without IL-3 (or without WEHI medium for 32D cells) for 13–18 h. Cells were then incubated with PI (40 mg ml^{-1}) to identify nonviable cells and analyzed by fluorescence-activated cell sorting, gating on GFP-positive transfected cells. Markedly diminished GFP-positivity was noted by 48–72 h post-transfection. Percentage protection relative to vector controls was calculated as $100 \times$ [1–(% apoptosis of cells transfected with test plasmid(s)/% apoptosis of cells transfected with vector plasmid)]. For drug-induced apoptosis and inhibition of Bad phosphorylation, 32D cells were starved of IL-3 for 3.5 h. DMSO, PD098059 (50 mM in DMSO, Calbiochem) or U0126 (5 mM in DMSO, RBI) were added as indicated for 30 min prior to the re-addition of recombinant IL-3 (0.01 ng ml^{-1}). After 10 min, lysates were made for immunoanalysis or, for cell death

analysis, viable cells were scored by trypan blue after 24–30 h of treatment. Rsk1 activation following IL-3 starvation was greatly inhibited by prior drug treatment whereas activated Rsk1 was not turned off by the subsequent addition of the drug (data not shown). Correspondingly, the pro-apoptotic effect of these drugs required a short period of IL-3 starvation to quiesce Rsk1 prior to drug treatment and IL-3 addition. This argues against a non-specific toxicity of the drugs. Importantly, the vehicle control demonstrated little apoptosis, so the initial short starvation was insufficient to account for the increased apoptosis.

Plasmids and constructs
All transfected Rsk1 constructs used in these studies were derived from the avian Rsk1 cDNA, and were detected using avian-specific Rsk1 antibodies. Avian Rsk1 shares greater than 80% identity with murine, human and Xenopus Rsk1 and they are regulated in a similar fashion [12,13,42,43]. GST–GSK3β was generated by placing Met1–Ala22 of human GSK3β (a gift of B. Neel) into pGEX-3X (Pharmacia). GST–S6 contains Lys218–Lys249 of Rat S6 in pGEX-3X (Pharmacia). GST–Bad was generated by placing full-length murine Bad (from pEBG-Bad, NEB) into pGEX-4T-1 (Pharmacia). Point mutants of Bad (S112A, S136A and S112A,S136A) were generated using the quick-change approach (Stratagene). Generation of an activated allele of Rsk1 was done by cloning either the avian [10] or human Rsk1 cDNA downstream of a DNA linker encoding six amino acids (MGSSKS) of the Src myristoylation signal in pCMV6 (a gift of P. Tsichlis). Plasmids pCMV6-HA-Akt and pCMV6-Myr-Akt-HA were gifts of P. Tsichlis. HA–Rsk1 and HA–Rsk1ΔΔ (K112R/K464R) were generated by placing avian Rsk1 wild-type and mutant cDNAs into pKH$_3$. Plasmid pRK7-Mek-DD was a gift from R. Erikson. pCMV-GFP-PH encodes the pleckstrin homology domain of spectrin fused to GFP (gift of R.F. Kalejta, T. Shenk and A.J. Beavis). For cotransfection experiments, Rsk1 constructs were cloned into pMT2, which is driven by an SV40 promoter and allowed for efficient production of the cotransfected protein(s).

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