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Rapamycin Induces Transactivation of the EGFR and Increases Cell Survival

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Abstract

The mammalian target of rapamycin (mTOR) signaling network regulates cell growth, proliferation and cell survival. Deregulated activation of this pathway is a common event in diverse human diseases such as cancers, cardiac hypertrophy, vascular restenosis, and nephrotic hypertrophy. Although mTOR inhibitor, rapamycin has been widely used to inhibit the aberrant signaling due to mTOR activation that plays a major role in hyperproliferative diseases, in some cases rapamycin does not attenuate the cell proliferation and survival. Thus, we studied the mechanism(s) by which cells may confer resistance to rapamycin. Our data shows that in a variety of cell types the mTOR inhibitor rapamycin activates Erk1/2 signaling. Rapamycin-mediated activation of the Erk1/2 signaling requires (a) the epidermal growth factor receptor (EGFR), (b) its tyrosine kinase activity, and (c) intact auto-phosphorylation sites on the receptor. Rapamycin treatment increases tyrosine phosphorylation of EGFR without the addition of growth factor and this transactivation of receptor involves activation of c-Src. We also show that rapamycin treatment triggers activation of cell survival signaling pathway by activating the pro-survival kinases Erk1/2 and p90RSK. These studies provide a novel paradigm by which cells escape the apoptotic actions of rapamycin and its derivatives that inhibit the mTOR pathway.

Keywords

mTOR; Rapamycin; Transactivation; EGFR; c-Src; p90RSK; apoptosis

Introduction

Growth-regulating signals originating from within and outside the cells are integrated through serine/threonine protein kinase mammalian target of rapamycin (mTOR) that has a

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key role in many aspects of cellular physiology. The mTOR signaling network has been implicated in the regulation of many cell functions including protein synthesis, cell growth and cell survival (Abraham, 1998; Guertin & Sabatini, 2005; Huang & Houghton, 2003; Sabatini, 2006). There are several links between mTOR signaling and human diseases such as cancer (Wullschleger et al., 2006). A number of pathways converge to regulate the activity of mTOR. These include AMP kinase (AMPK) that is activated by low ATP levels and, therefore, nutritional status of the organism, receptor tyrosine kinases such as EGFR and Insulin receptor as well as Wnt signaling pathway (Hardie et al., 2006; Inoki et al., 2006; Kudo et al., 1996; Tzatsos & Kandror, 2006).

Rapamycin is a macrolide antibiotic whose main mammalian target is mTOR. mTOR in complex with raptor is known as mTOR complex 1 (mTORC1) while association of mTOR with rictor and mSin1/5 forms mTOR complex 2 (mTORC2) (Jacinto et al., 2006). mTORC1, but not mTORC2, is the form of mTOR that is inhibited by rapamycin (Jacinto et al., 2004). Essentially, rapamycin in complex with its intracellular receptor FKBP12 binds directly to mTOR and disrupts the binding of raptor to mTOR (Lorenz & Heitman, 1995). Since raptor is required to access the mTOR substrates, rapamycin suppresses the mTORC1 mediated phosphorylation of the downstream substrates S6 kinase 1 and 4EBP1 resulting in decreased protein synthesis. In contrast to mTORC1, FKBP12-rapamycin cannot bind to mTORC2 (Jacinto et al., 2004; Sarbassov et al., 2004). However, the binding of FKBP12-rapamycin to mTOR seems to block subsequent binding of the mTORC2-specific components, rictor (Sarbassov et al., 2006) and mSin1 (Frias et al., 2006). This latter mechanism may explain why rapamycin inhibits mTORC2 in some cells. Since mTORC2 phosphorylates Akt on S473 (Sarbassov et al., 2005), the phosphorylation of this site is often used as a reporter of mTORC2 activity.

Because mTORC1 increases protein synthesis, cell growth, and cell survival, it has a profound role in regulating hypertrophy of the heart, vascular smooth muscle cell growth in stenosis and restenosis following balloon angioplasty, as well as survival of cancer cells (Boluyt et al., 2004; McMullen et al., 2004; Sharma et al., 2006). Therefore, rapamycin has important uses in cardiology and in the treatment of certain forms of cancer such as rhabdomyosarcoma, neuroblastoma, glioblastoma, small-cell lung carcinoma, osteosarcoma, pancreatic carcinoma, RCC, Ewing sarcoma, prostate cancer and breast cancer (Bjornsti & Houghton, 2004).

One of the substrates of mTOR is the p70 S6 kinase (p70S6K) which upon phosphorylation by mTOR is activated. Studies have shown that p70S6K- elicited phosphorylation of the insulin receptor substrate 1 (IRS-1) results in inhibition of IRS-1 binding to the insulin or insulin like growth factor (IGF-I) receptors or degradation of IRS-1 (Harrington et al., 2004; Manning, 2004; Um et al., 2004). In this manner, mTOR via p70S6K can inhibit insulin or IGF-I receptor- mediated prosurvival and proliferative signaling. Thus, rapamycin- induced inhibition of mTOR and, therefore, suppression of p70S6K activity alleviates the “negative feed-back” inhibition of IRS-1 and results in increased IRS-1 protein levels to further activate Akt by augmenting IGF-I receptor signaling. Indeed, the antitumorigenic action of rapamycin in breast and colon cancers has been shown to be decreased due to increased Akt activation (Wan et al., 2007). These studies suggest the presence of compensatory

mechanism(s) in rapamycin-induced apoptotic process. Other than the possible role of a compensatory increase in Akt pathway in response to rapamycin, there is a paucity of information on how other mechanisms activated by rapamycin may decrease sensitivity to the drug.

Here, we show that rapamycin activates the upstream kinases, Erk1/2 and RSK, of mTOR in rat aortic vascular smooth muscle cells, mouse lung fibroblasts (B82L cells), human adrenal cortex adenocarcinoma cells (SW13) and COS7 cells. Studies using epidermal growth factor receptor (EGFR) null and mutant EGFR expressing cells suggest that mTOR negatively regulates transactivation of the EGFR. Relieving this inhibition by rapamycin permits the transactivation of EGFR via c-Src and stimulation of downstream kinases that lead to anti-apoptotic actions of rapamycin. Thus, our findings unravel a novel paradigm by which rapamycin increases cell survival via the c-Src - EGFR transactivation pathway.

Results and Discussion

Although rapamycin has been shown to inhibit cellular proliferation and increase apoptosis, we observed that treatment of mouse lung fibroblast (B82L) cells expressing wt-EGFR with low (1 nM) concentrations of rapamycin activated signaling pathways that promote cell proliferation and survival. Essentially rapamycin increased the active, phosphorylated forms of Erk1/2 and its immediate downstream kinase, p90RSK (RSK) in the absence of any agonist (Fig. 1A). As expected, stimulation of cells with EGF (positive control) increased the activation and phosphorylation of these kinases. Similar data were also observed in primary rat aortic vascular smooth muscle cells (VSMC), COS-7 and human adrenal cortex adenocarcinoma (SW13) cells (Fig. 1B). The inhibition of phosphorylation of its downstream substrate p70S6 kinase (Fig. 1A and 1B) shows that mTOR was effectively inhibited by rapamycin. Rapamycin mediated activation of Erk1/2 and RSK is cell type specific since in HeLa and HEK293T cells rapamycin did not activate these kinases (Fig. 1B). Although rapamycin has been reported to inhibit only the mTORC1, in some cells mTORC2 is also inactivated by rapamycin (Dormond et al., 2007; Vega et al., 2006). As monitored by phosphorylation of Akt on S473, the mTORC2 phosphorylation site, rapamycin also inhibited mTORC2 in B82L cells (not shown). Among the four isoforms of RSKs (RSK1-4), RSK4 is structurally different and apparently also functionally distinct from the other three isoforms (Dummler et al., 2005; Myers et al., 2004). To determine whether rapamycin increased the phosphorylation of RSK1, RSK2, and RSK3, B82L cells expressing the wt-EGFR were treated with or without rapamycin and the three isoforms of RSK were immunoprecipitated with isoform- specific antibody. As shown in the supplementary data (Fig. S1), rapamycin increased the phosphorylation of all three isoforms of RSK. To determine whether rapamycin increased the kinase activity of the RSKs, we studied RSK1 as an example and performed in-vitro kinase activity assays. As shown in figure 1C, in-vitro kinase activity assays of RSK1 immunoprecipitated from B82L cells showed that rapamycin increased its activity in the absence of any other agonist. As expected exposure of cells to EGF elevated RSK1 activity that was not further augmented by rapamycin (Fig. 1C). These findings suggest that in unstimulated cells, the basal mTOR activity attenuates Erk1/2 and RSK activation in a cell type specific manner and that Western analyses with the site-specific anti-phospho-RSK antibody faithfully reflects the

activation state of RSKs. Moreover, as shown in figure 2 the effects of rapamycin on Erk1/2 and RSK activation coincide with inhibition of mTOR activity, i.e, decrease in S6 kinase phosphorylation. While Erk1/2 and RSK activation in B82L cells were maximal 30 minutes after rapamycin treatment and lasted for at least 2 hr (Fig. 2A) the activation of these kinases by rapamycin in SW13 cells was more sustained (Fig. 2B).

Erk1/2 pathway represents an integration point where signals emanating from other pathways converge to regulate proliferation, migration, growth and survival of cells (Banko et al., 2006; Roux et al., 2007). One mechanism by which the Erk1/2 pathway regulates cell survival is via the activation of the immediate downstream kinases, RSKs (Roux et al., 2003). When activated by growth factors, RSKs translocate to the nucleus (Chen et al., 1992) and phosphorylate their nuclear substrates such as CREB and c-fos (Frodin & Gammeltoft, 1999). Therefore, next we determined the cellular distribution of the rapamycin- activated RSK. Immuno-flourescence studies using anti-phospho- T573 antibody which recognizes phospho-forms of RSK1, RSK2, and RSK3 showed that in B82L cells rapamycin not only increased the phosphorylation of RSKs but also translocated the active RSKs into the nucleus (Fig. 3A). This is similar to what we and others have observed with growth factor activated RSKs (Chaturvedi et al., 2006; Shimamura et al., 2000).

To investigate the functional significance of rapamycin-induced increase in the activation of the pro-survival proteins Erk1/2 and RSKs we performed apoptosis assays using B82L cells. As compared with controls, rapamycin attenuated the TNFα & CHX induced cellular apoptosis (Fig. 3B). To determine if RSK1/2 are involved in the rapamycin mediated decrease in apoptosis, the RSK1/2 specific inhibitor “fmk” was used (Cohen et al., 2005). As shown in figure 3B, fmk increased apoptosis in the absence of TNFα & CHX. Moreover, fmk obliterated the anti-apoptotic actions of rapamycin in the presence of TNFα & CHX (Fig. 3B). These findings indicate that in the absence of TNFα & CHX basal RSK1/2 activity contributes toward cell survival and rapamycin mediates its anti-apoptotic actions in the presence of TNFα & CHX via activation of RSK1/2 (Fig. 3B). Our findings that rapamycin increases cell survival are similar to a report in Jurkat cells in which despite a decrease in cell size upon rapamycin treatment and mTOR inhibition, the cells were protected from Fas/Apo-1 death-receptor activation- mediated apoptosis (Fumarola et al., 2005). However, that study did not delineate the mechanism(s) involved in the pro-survival actions of rapamycin. That fmk, inhibits the C-terminal kinase domains of RSK1/2 and, therefore, their autophosphorylation on S380 (Cohen et al., 2005) in response to rapamycin and EGF is shown in figure 3C. Unexpectedly, we also observed that in B82L cells, fmk inhibited rapamycin- mediated, but not EGF- elicited Erk1/2 phosphorylation (Fig. 3C). Moreover, fmk, to some extent, revived the rapamycin- inhibited phosphorylation of S6K (Fig. 3C). These observations suggest that other additional feedback mechanisms may exist. For instance, RSK1/2 may activate, or no longer inhibit, a phosphatase that dephosphorylates Erk1/2 in the presence of rapamycin when mTOR is inhibited. Thus, in the absence of rapamycin the regulation of this phosphatase by RSK1/2 may not be observed. Likewise, when mTOR is inhibited, fmk may inhibit a phosphatase that dephosphorylates S6K, thereby increasing its phosphorylation. The precise reasons for these intriguing observations are not presently clear and will be investigated in the future.

To identify the mechanisms by which rapamycin increased Erk1/2 and RSK activities, the ability of rapamycin to regulate Erk1/2 and RSK activities in naïve B82L (PRN-B82L) cells was tested; naïve B82L cells do not express EGFR (Li et al., 1999). As shown in Fig. 4A, rapamycin did not increase the activation of RSKs and Erk1/2 in these cells suggesting that the expression of wt-EGFR is necessary for the rapamycin- mediated activation of the Erk1/2 cascade. Next, using B82L cells that overexpress the kinase inactive mutant of EGFR (B82L K721M), we tested the importance of EGFR kinase activity in this process. As illustrated in figure 4B, although the K721M mutant of the EGFR was expressed, rapamycin failed to increase Erk1/2 activation in B82L K721M cells suggesting that EGFR kinase activity is necessary for the rapamycin mediated increase in Erk1/2 and RSK activity. The selective inhibitor of EGFR kinase activity, AG1478, also obliterated the ability of rapamycin to activate RSKs and Erk1/2 cascade in B82L cells expressing wt-EGFR and SW13 cells (supplementary data, Fig. S2), confirming that rapamycin-mediated activation of Erk1/2 and RSK requires the EGFR kinase activity. Similarly, experiments using B82L cells expressing the EGFR Y5F mutant, which lacks the five major autophosphorylation sites (Y992, Y1068, Y1086, Y1148, Y1173) but in which the tyrosine kinase activity is intact, also showed that the autophosphorylation sites on the EGFR are necessary for rapamycin-mediated increase in Erk1/2 and RSK phosphorylation (Fig. 4C). Notably, upon long exposure of Western blots (Fig. 4C) EGF modestly activated of RSK and Erk1/2 in B82L cells expressing the Y5F EGFR. This may be due to signal mediated by the minor phosphorylation sites on the EGFR or dimerization of the EGFR with other receptor tyrosine kinases such as the PDGF receptor (Saito et al., 2001) to activate Erk1/2 and RSK. However, even long exposures of immunoblots did not show any activation of Erk1/2 or RSK by rapamycin (Fig. 4C). Moreover, the time course of rapamycin-mediated transactivation of the EGFR was similar to that observed for activation of Erk1/2 and RSK (c.f. Fig. 4D and Fig 2A). Together, these data demonstrate that rapamycin- mediated activation of the Erk1/2 signaling requires (a) expression of the EGFR, (b) its tyrosine kinase activity, and (c) intact major autophosphorylation sites on the receptor. These data strongly suggest that rapamycin, perhaps by inhibiting mTOR, permits transactivation of the EGF receptor and that in the absence of rapamycin this transactivation is inhibited (negative feedback) by mTOR or its downstream signaling element(s).

The EGFR can be transactivated either by enhanced “inside-out signaling” that would increase cell surface shedding of EGFR ligands such as pro HB-EGF by stimulating metalloproteinases of the ADAM (a disintegrin and metalloproteinase) family (Asakura et al., 2002; Gschwind et al., 2003; Prenzel et al., 1999) or by intracellular activation of non-receptor tyrosine kinase c-Src (Daub et al., 1997), which phosphorylates the EGFR on tyrosine residue 845 and 1101 and results in receptor activation (Tice et al., 1999). To distinguish between these two mechanisms in rapamycin- induced EGFR transactivation, first we determined the role of ADAMs and EGFR ligand shedding. While the EGFR monoclonal antibody 528 (mAb 528) that binds to the extracellular ligand binding domain of the EGFR markedly reduced the ability of EGF to autophosphorylate the EGFR, rapamycin-mediated phosphorylation of the EGFR was not altered (supplementary data Fig. S3A). Additionally, the inhibitors of ADAMs GM6001 and TAPI-2 did not alter the ability of rapamycin to transactivate the EGFR (supplementary data Fig. S3B and S3C). These data

suggest that rapamycin does not transactivate the EGFR by augmenting the extracellular shedding of its ligand(s). Therefore, next we determined the role of c-Src in rapamycin-mediated transactivation of EGFR. Evidence that c-Src may be involved in EGFR transactivation was provided by our findings in COS7 (supplementary data Fig. S4A), SW13 and B82L cells (Figs. 5A–5C) where rapamycin increased phosphorylation of EGFR on Y845, the c-Src site (Tice et al., 1999). This notion was further validated by the findings that the amount of active c-Src (pSrc Y416) increased (Fig. 5A, 5B, and 5D) while the amount of inactive c-Src (pSrc Y527) decreased (Fig. 5A) upon rapamycin treatment. To further confirm the role of c-Src in the transactivation of EGFR we tested the ability of the c-Src kinase inhibitor PP2 to attenuate rapamycin- mediated transactivation of the EGFR. In control SW13 cells treated with the inactive analog of the cSrc inhibitor, PP3, rapamycin increased phosphorylation of the EGFR at Y845 (Fig. 5B). This increase in the receptor phosphorylation was significantly reduced by treatment of the cells with the c-Src specific inhibitor PP2. Further, PP2, but not the inactive analog, PP3, by inhibiting the EGFR phosphorylation obliterated the ability of rapamycin to increase Erk1/2 or RSK phosphorylation (Fig. 5B and 5C). That PP2 inhibited Src activity is evident by the inhibition of phosphorylation of the Src on Y416 (Fig. 5B). Similar results with PP2 were also observed in B82L cells (Fig. 5C), COS7 cell (supplementary data, Fig. S4B) and with another c-Src inhibitor, dasatinib (supplementary data Fig. S4C). The observation that the c-Src kinase inhibitor PP2 inhibits rapamycin- elicited EGFR phosphorylation suggests that c-Src is upstream of the EGFR. This notion was directly tested using B82L cells expressing a form of the EGFR that is mutated at the c-Src phosphorylation site (Y845F). As shown in figure 5D, although rapamycin activated c-Src as evident by the increase in amount of pSrc Y416, it did not increase phosphorylation of either the EGFR or its downstream kinases Erk1/2 and RSK (Fig. 5D). Consistent with the notion that cSrc is upstream of the EGFR, our findings also show that although the EGFR kinase inhibitor AG1478, inhibited EGFR autophosphorylation on Y1173 (not shown) and Erk1/2 as well as RSK activation (Fig. 5E), the activation of c-Src by rapamycin was not altered (Fig. 5E); these data also demonstrate that AG1478 did not inhibit c-Src activity. Interestingly, although c-Src activation was not altered by AG1478, the phosphorylation of Y845 on the activation segment of the EGFR was inhibited (Fig. 5E). A previous study has also shown that AG1478 inhibits the ability of c-Src to phosphorylate the EGFR on Y845 (Kiley & Chevalier, 2007). AG1478, by binding the ATP site on EGFR, has also been reported to cause dimerization of the inactive EGFR (Arteaga et al., 1997). Thus, it is possible that AG1478 induced dimerization of the inactive receptor and alterations in its structure preclude c-Src- mediated phosphorylation of Y845 in the activation segment of the EGFR.

Depending upon the cell type, rapamycin induced either a transient or sustained activation of the survival kinases (Fig. 2). Therefore, next we investigated whether rapamycin increased cell survival over a longer time period. SW13 and B82L cells were treated with or without TNF-α in the presence and absence of rapamycin. Without TNF-α, rapamycin did not significantly alter cell survival (Fig. 6). However, in SW13 cells, rapamycin significantly decreased cell death induced by different TNF-α concentrations over the entire 24 hour period studied (Fig. 6A). On the other hand, in B82L cells expressing the EGFR, rapamycin inhibited cell death up to 8 hours after TNF-α addition but did not significantly affect the

rate of cell death thereafter (Fig. 6B). That the pro-survival actions of rapamycin involve the transactivation of the EGFR is demonstrated by the lack of an effect of rapamycin on cell survival in naïve B82L cells that do not express the EGFR (Fig. 6B). The ability of rapamycin to protect against cell death over a longer time course in SW13 cells vs. B82L cells expressing EGFR can be explained by the sustained vs transient activation of the survival kinases, respectively, in these two cell types. Hence, it would appear that the time course of rapamycin-induced Erk1/2 and RSK activation would determine the duration of the pro-survival actions of rapamycin. Unlike the in-vitro cell studies with a single addition of rapamycin, replenishing doses in patients being treated with rapamycin may further prolong activation of the survival kinases to inhibit death of certain cells.

The data presented here show that rapamycin, perhaps by inhibiting mTOR and its downstream signaling element(s), activates c-Src which then transactivates the EGFR to stimulate the Erk1/2 pathway and increases cell survival. Transactivation of the EGFR by Src kinase has already been reported (Tice et al., 1999) and aberrant signaling due to overexpression of EGFR has also been shown to play an important role in hyperproliferative diseases (Fischer et al., 2003). Based on these findings preclinical and clinical data have strongly suggested the potent effect of tyrosine kinase inhibitors as anticancer agents (Metro et al., 2006) and EGFR family kinase inhibitors such as gefitinib, erlotinib, and lapatinib are already in clinical use or trials. Similarly, Src kinase inhibitor SU6656 (perhaps, in part by inhibiting EGFR transactivation) has also been shown to attenuate the activation of Ras-Erk1/2 and PI-3K/Akt cascades (Jin et al., 2007) and is currently used in preclinical studies.

Although rapamycin is used in treatment of certain forms of tumors, inhibition of mTORC1 signaling by rapamycin in some cancers such as carcinomas of lung, breast, liver, gastrointestinal tract and ovary (Vivanco & Sawyers, 2002) has been shown to restore resistance of cancer cells to chemotherapy (VanderWeele et al., 2004; Wendel et al., 2004) at least in part, via activation of Akt-dependent signaling through a feedback mechanism (O'Reilly et al., 2006; Sun et al., 2005). However, other mechanisms may also contribute toward the drug resistance. In this context, our findings that rapamycin, via the Src mediated transactivation of the EGFR and activation of its downstream kinases Erk1/2 and RSK increases cell survival, may represent an additional mechanism of drug resistance in certain forms of tumors. Because of the involvement of the EGFR in mediating the pro-survival actions of rapamycin, our studies also suggest that combinatorial therapy of tumors with EGFR inhibitors and rapamycin may be more efficacious and less prone to drug resistance. Indeed, the combination of rapamycin or its analogs with EGFR inhibitors has been found to be more effective at inhibiting the proliferation of cells derived from a number of different cancers including those of the pancreas (Azzariti et al., 2008), kidney (Costa et al., 2007), and glioblastoma (Goudar et al., 2005). Based on these studies, clinical phase II trials in patients with non-small cell lung carcinoma (Milton et al., 2007; Kris et al., 2007) and gliobastoma multiforme (Galanis et al., 2005) have been conducted and show an improved outcome when rapamycin derivatives are combined with EGFR inhibitors. Our findings that rapamycin activates the survival kinases Erk1/2 and RSKs in rat aortic vascular smooth muscle cells may also explain why rapamycin (sirolimus) coated stents do not increase apoptosis of vascular smooth muscle cells (Parry et al., 2005). Here again, the addition of receptor tyrosine kinase inhibitor(s) with rapamycin to coat stents could be beneficial.

Currently, how rapamycin activates c-Src is not known and this forms the subject of future studies. However, our findings reported here unravel a novel paradigm by which rapamycin regulates the EGFR via c-Src kinase and identify a hitherto undefined mechanism by which rapamycin promotes cell survival and drug resistance. Moreover, our study also provides the rationale for combinatorial drug therapy with rapamycin and EGFR inhibitors.

Material and Methods

Reagents

Mouse lung fibroblasts (B82L) overexpressing EGF receptor (EGFR) were obtained from Dr. Paul J. Bertics, University of Wisconsin, Madison, WI. Anti-phospho Src (Ser-416 and Ser-527), anti-phospho-Akt (Ser-473), anti-phospho-S6 kinase (Thr 389), anti-phospho-RSK (Thr-573), and anti-phospho-Erk1/2 (Thr-202/Tyr-204) antibodies were purchased from Cell Signaling (Beverly, MA). Anti-phospho-RSK1 (Ser-380) (Epitomics, Burlingame, CA), anti-Erk1/2 (Upstate Biotechnology, Lake Placid, NY) and anti-RSK1 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were also used. The anti-phospho-EGFR (Tyr-845 and Tyr-1173) antibodies were from Biosource International Inc. (Camarillo, CA). Rapamycin, AG1478, TAPI-2, GM6001 and PP2/PP3 were from Calbiochem Inc. (San Diego, CA). All other reagents were obtained from Sigma Chemicals Co. (St. Louis, MO) unless otherwise mentioned.

Immunoprecipitations (IP)

Overnight serum starved B82L cells were treated with 1 nM rapamycin for 30 min at 37 °C. RSK1 immunoprecipitations (IPs) were performed using 250 μg of total cell lysate proteins and 700 ng of anti-RSK1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 2h at 4 °C. For IPs, cells were lysed in a buffer containing 50 mM Hepes, pH 7.5, 1% Triton X-100, 0.5% CHAPS (ICN Biomedicals Inc., Aurora, Ohio), 150 mM NaCl, 1 mM DTT, 1 mM sodium orthovanadate, 50 mM NaF, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 100 μM PMSF, 1 μM microcystin and 1 μg/ml each pepstatin A, aprotinin, and leupeptin. The immune complexes were precipitated by incubation with 30 μL of protein G-agarose beads (Roche, Indianapolis, IN) for 2hr at 4 °C. Immune complexes were washed 3 times with lysis buffer followed by elution with reducing Laemmli sample buffer and proteins were separated on 10% polyacrylamide gels before immunoblotting.

RSK1 activity in IPs

Triplicate IPs of RSK1, performed as indicated above, were resuspended in 20 mM Hepes, pH 7.5, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM DTT and 25 mM β-glycerophosphate, 5 mM $MgSO_4$, 200 μM Kemptide, 125 μM ATP and 10 μCi [γ-^{32}P]ATP for 10 min at room temperature. Reactions were terminated by the addition of equal volume of 20% trichloroacetic acid and following centrifugation (16,000 × g, 5 min), aliquots of each supernatant were spotted onto P81 paper (Whatman), air-dried, and washed three times with 0.5% phosphoric acid. The filters were then dried and counted in a liquid scintillation counter. One parallel set of IP'd proteins were denatured in Laemmli sample buffer, separated on 10% polyacrylamide gels and analyzed by immunoblotting to ensure that equal amount of RSK1 was IP'd under different conditions.

Immunocytochemistry

Serum starved B82L cells were treated with 1 nM rapamycin for 30 min at 37 °C. Cells were fixed with 100% methanol for 10 min at −20 °C, followed by methanol/acetone (1:1) for 10 min at −20 °C. After permeabilizing the cells with 0.3% Triton-X100 in PBS for 5 min, anti-phospho RSK (T573) antibody (1:250 dilution, Cell Signaling, Danvers, MA) was added. Alexa fluor 488 conjugated goat anti-rabbit secondary antibody (1:500 dilution) was used. Images were captured using an Olympus fluorescence microscope. Confocal images were obtained using a multiphoton Zeiss LSM 5 Pa laser scanning microscope.

Cellular Apoptosis

B82L cells (50,000 cells/well) plated on a 24 well dish were treated with rapamycin as described above. When indicated, the RSK1/2 inhibitor (fmk, 3 μM) was added 15 min before rapamycin treatment 37 °C. Cells were then treated with or without 20 ng/ml TNF-α plus 25 μg/ml cycloheximide (CHX) to induce apoptosis. After one hour of TNF/CHX treatment DNA fragmentation was measured using the Cell Death detection kit (Roche, Indianapolis, IN) as described previously (Chaturvedi et al., 2006).

Cell Survival

Naïve and EGFR expressing B82L cells or SW13 cells were seeded in 35 mm dishes (200×10^3 cells/dish) and exposed to different concentrations of TNF-α in the presence or absence of rapamycin (1 nM); controls without any addition and rapamycin alone were also performed. At different times after TNF-α addition, the dead cells were washed off and the detached surviving cells that excluded trypan blue were counted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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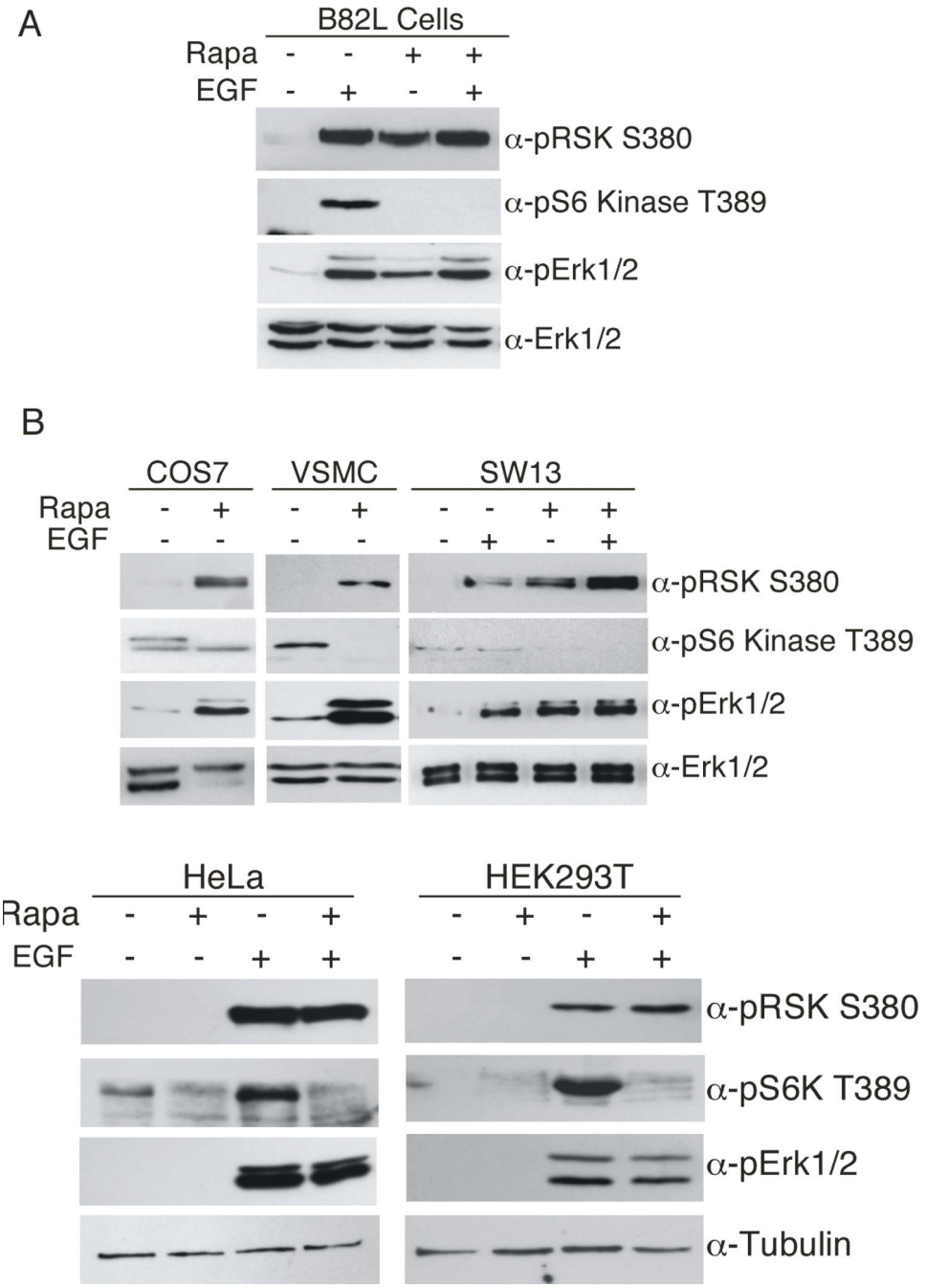


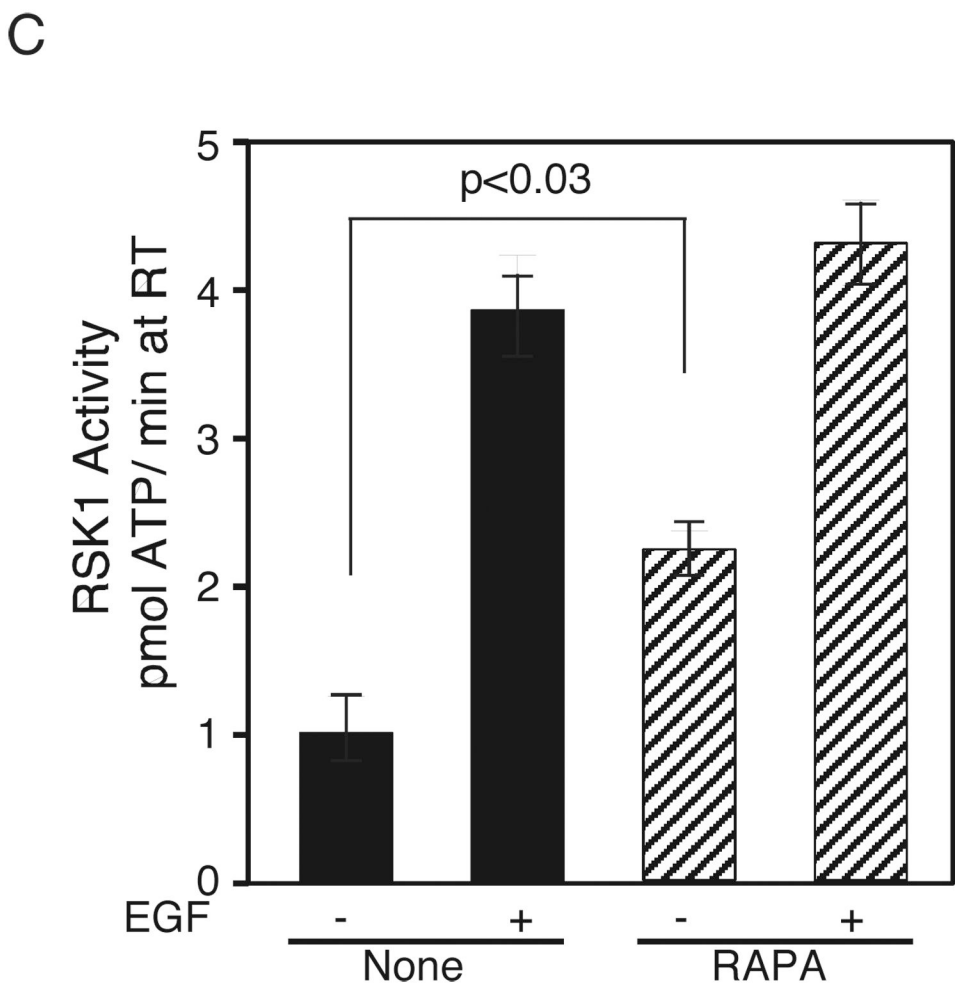


Figure 1. Rapamycin induces activation of RSK and Erk1/2 in a number of different cell types

Overnight serum starved B82L cells expressing the wt-EGFR (**Panel A**), COS-7, VSMC, SW13, HeLa and HEK293T cells (**Panel B**) were treated with or without 1nM rapamycin for 30 min followed by stimulation with or without 50 nM EGF for 10 min. All cells were plated at a density of 2×10^5 cells/35 mm dish. Cells were lysed in Laemmli sample buffer and proteins were immunoblotted with the indicated antibodies. Total Erk1/2 was used as loading controls. **Panel C**: Overnight serum starved B82L cells expressing the wt-EGFR were treated with or without 1 nM rapamycin for 30 minutes followed by immunoprecipitation using RSK1 specific antibody. Triplicates of immunoprecipitates were used in the kinase activity assay using Kemptide as substrate as described under “Materials and Methods”. Statistical significance was assessed by Student’s unpaired *t* test (n=3). For all panels a representative of 3 similar experiments is shown.

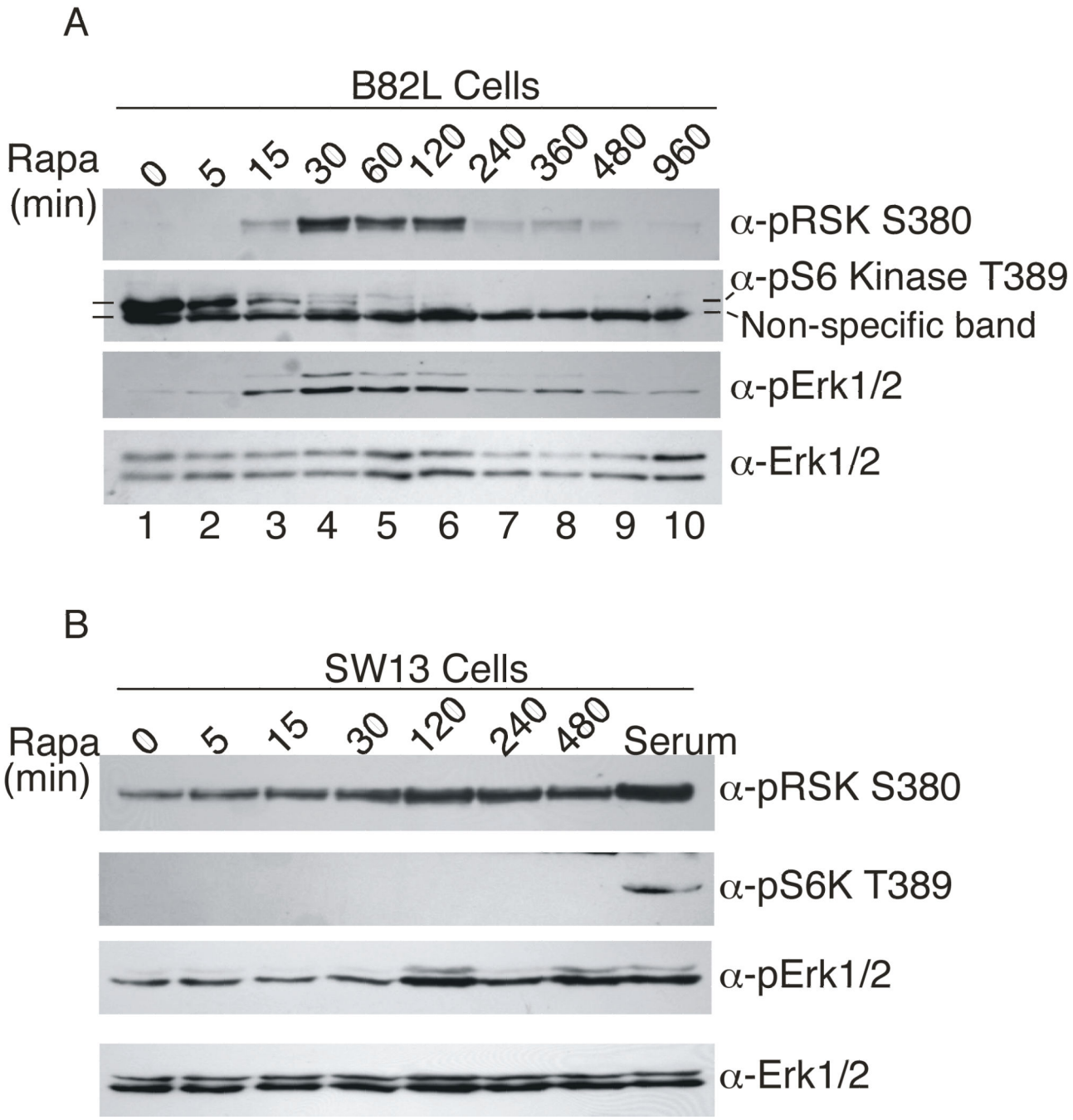
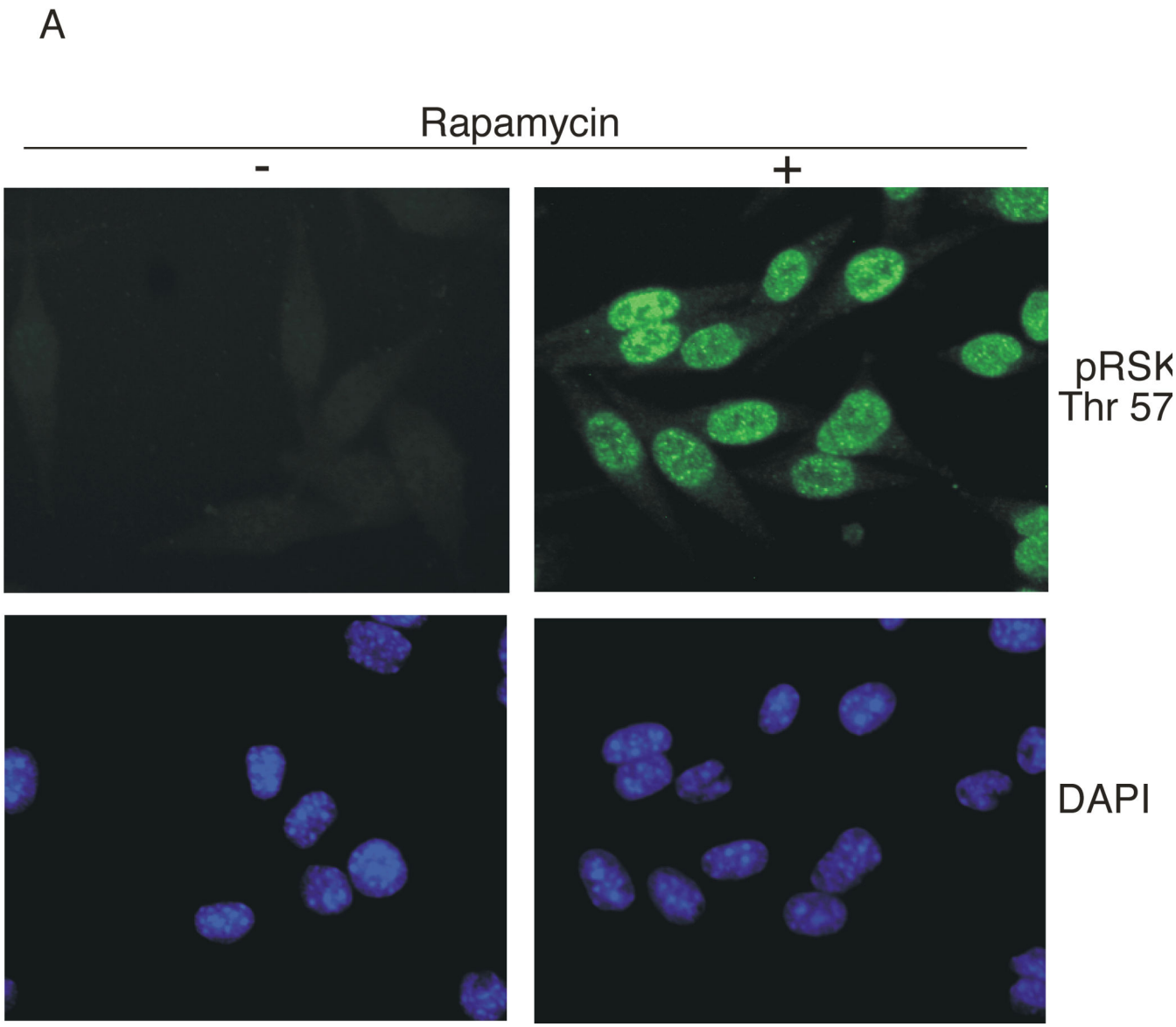


Figure 2. Time course of RSK and Erk1/2 activation by rapamycin

Overnight serum starved B82L cells expressing wt-EGFR (**Panel A**) and SW13 cells (**Panel B**) were treated with 1nM rapamycin for the indicated times. Cells were lysed in Laemmli sample buffer and proteins were immunoblotted with the indicated antibodies. Total Erk1/2 was used to assess loading. A representative of 4 similar experiments is shown.



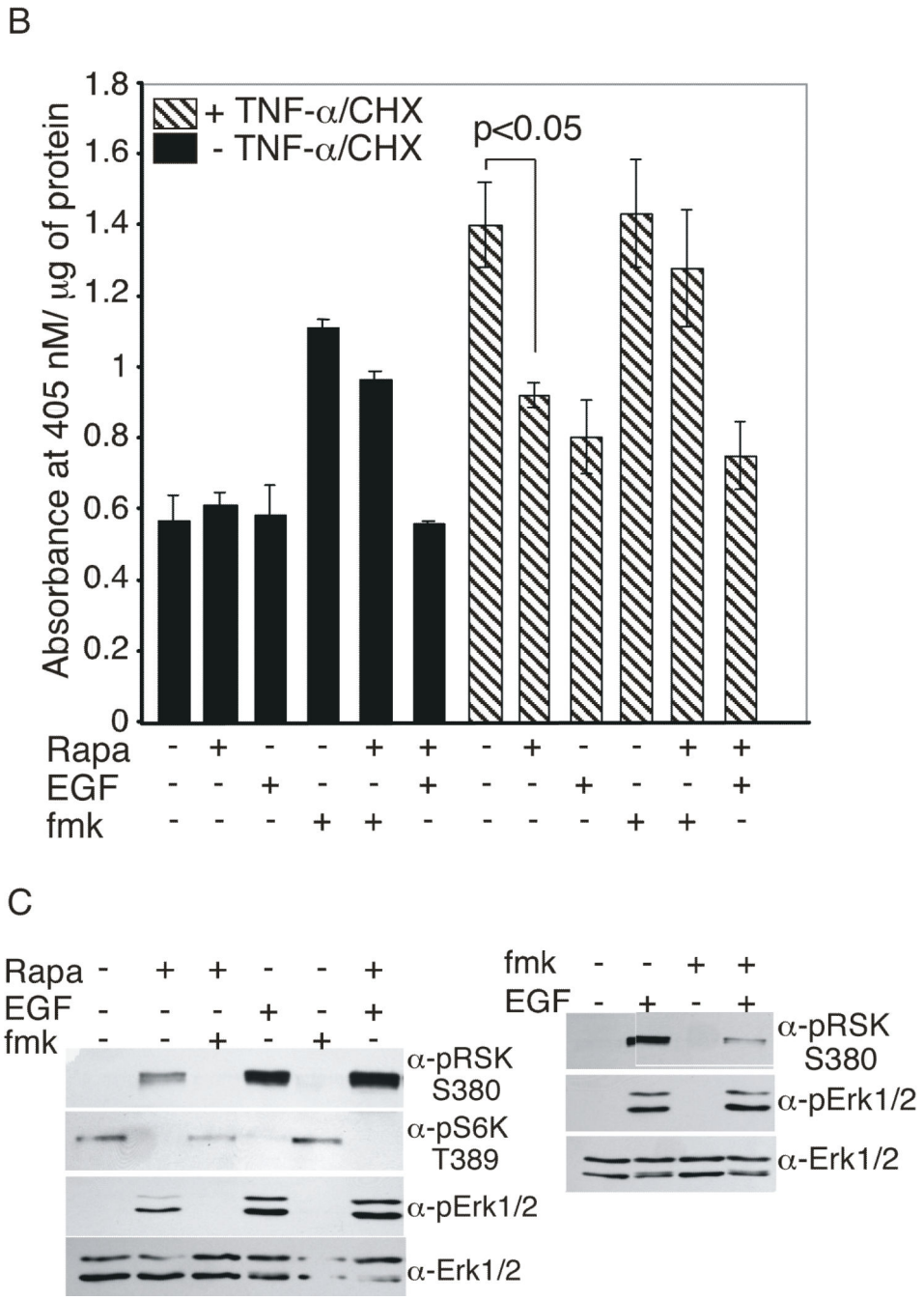


Figure 3. Rapamycin- activated RSK is localized in the nucleus and rapamycin inhibits cellular apoptosis

Panel A: B82L cells expressing wt-EGFR were treated with 1 nm rapamycin for 30 min before fixing the cells. The active, phospho-RSK was detected using anti-phospho-T573 RSK antibody and goat anti-rabbit conjugated to Alexa flour 488. DAPI was used to stain nucleus of the cells. **Panel B**: Overnight serum starved B82L cells expressing the wt-EGFR (50,000 cells/well in 24 well plate) were treated with or without 3 μM fmk for 15 min followed by 1nM rapamycin for 30 min. TNF-α (20 ng/ml) plus cycloheximide (25 μg/ml)

(TNF-α/CHX) or vehicle were then added for 1 hr and DNA fragmentation was monitored, using ELISA based Cell death detection kit (Roche), as described under “Materials and Methods”. Data are mean ± SEM of $OD_{405\ nm}$ per μg of the protein (n=3). **Panel C**: Cells treated as described in Panel B were lysed in Laemmli buffer. Proteins were separated on SDS-PAGE followed by immunoblotting for the detection of phospho-RSK S380 antibody. Total Erk1/2 was used as loading control. For all panels a representative of 4 similar experiments is shown.

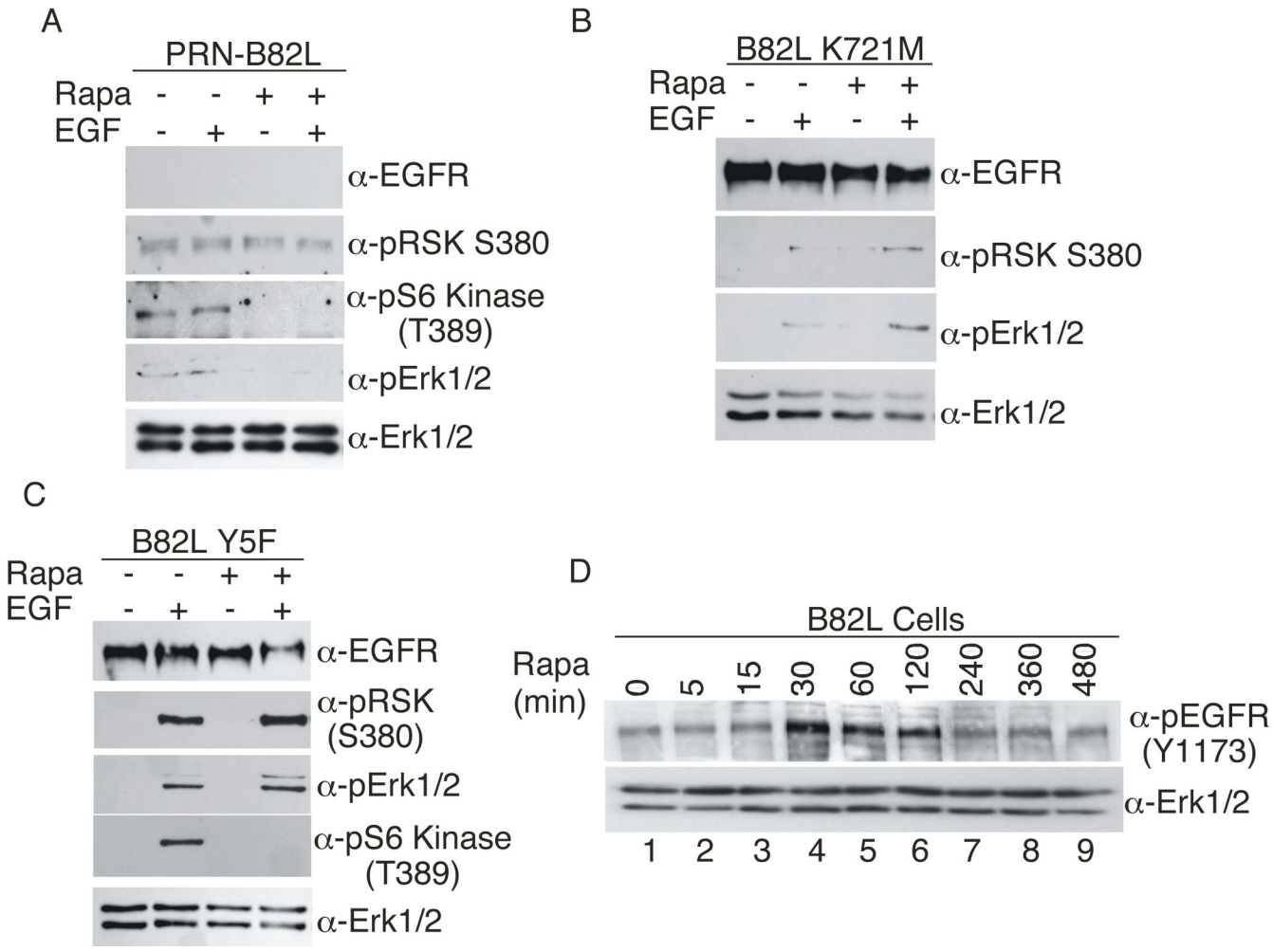
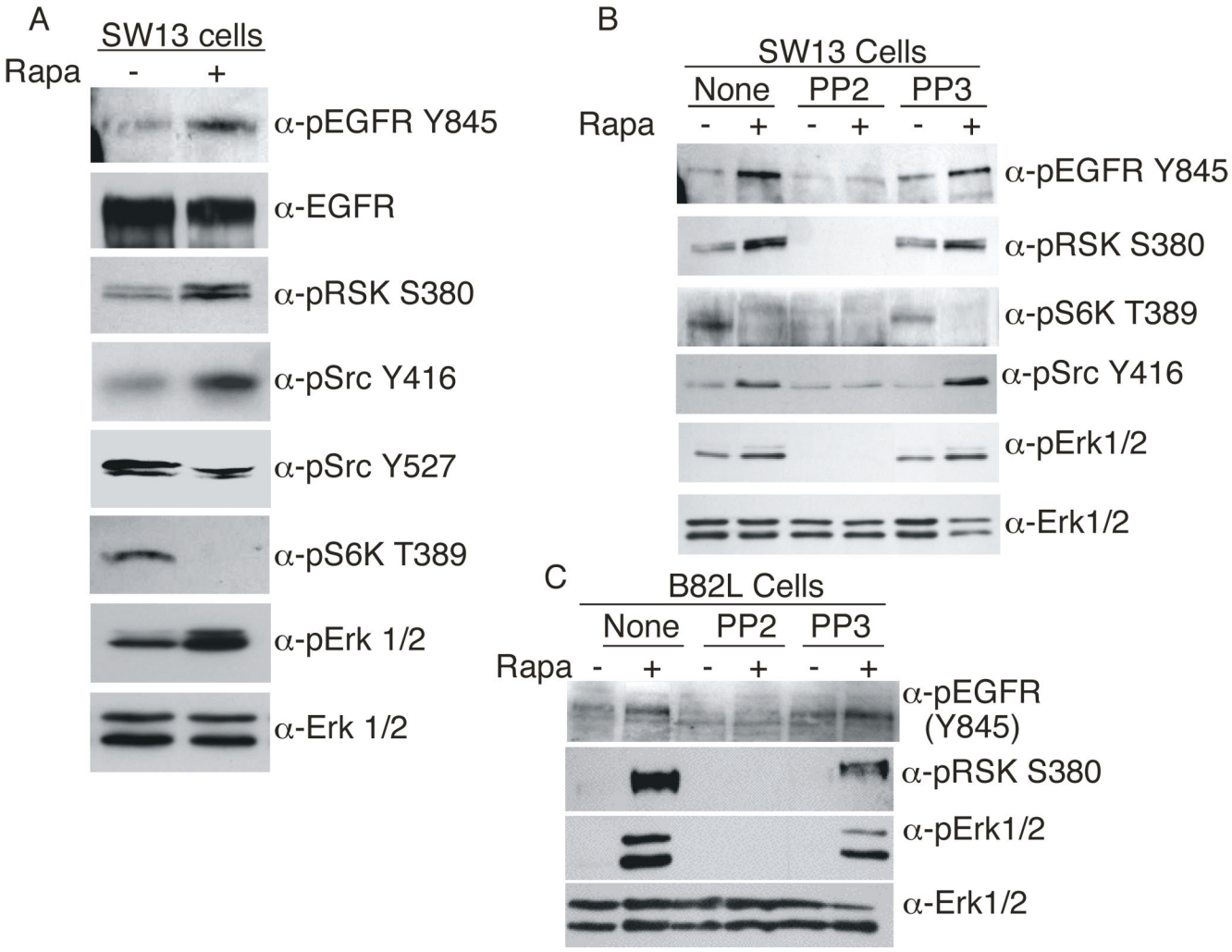


Figure 4. The EGFR, its kinase activity and intact auto-phosphorylation sites on the GFR are necessary for the rapamycin mediated activation of the receptor

Overnight serum starved Parental B82L cells (PRN-B82L) which lack endogenous EGF receptor (**Panel A**), B82L cells that overexpress the kinase dead mutant of EGFR K721M (B82L K721M, **Panel B**) and wt-EGFR expressing B82L cells, which lack the five autophosphorylation sites on the EGFR (B82L Y5F, **Panel C**) were treated with or without 1nM rapamycin for 30 min followed by 50 nM EGF for 10 min. Cells were lysed in Laemmli buffer and samples were immunoblotted for the detection of EGFR, phospho-RSK, phospho-S6 kinase and phospho-Erk1/2 using indicated antibodies. Total Erk was used as loading control. **Panel D**: Overnight serum starved B82L cells expressing wt-EGFR were treated with 1nM rapamycin for the indicated times. Cells were lysed in Laemmli sample buffer and proteins were immunoblotted with the pEGFR-Y1173 and Erk1/2 (loading control) antibodies. For all panels a representative of 3 similar experiments is shown.



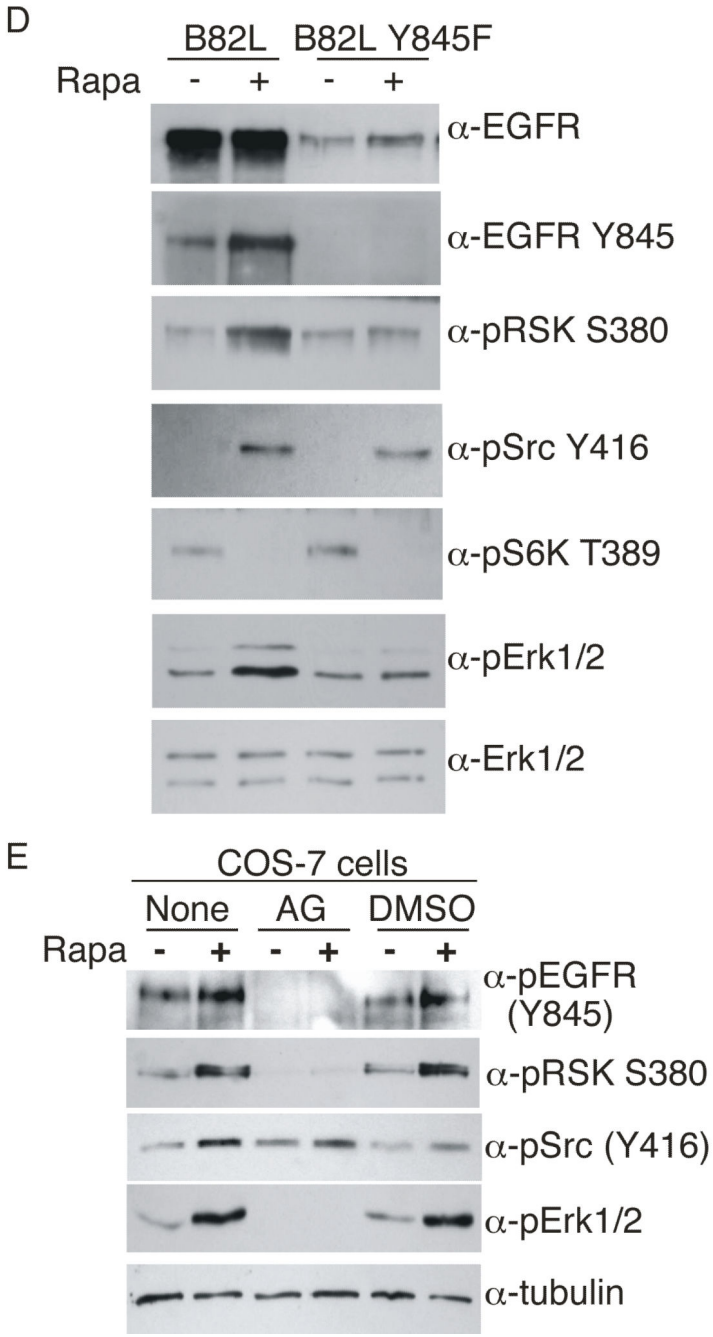


Figure 5. c-Src mediates Rapamycin- elicited transactivation of the EGFR

Panel A: SW13 cells were serum starved overnight followed by treatment with 1 nM rapamycin for 30 min and lysed in Laemmli buffer. Cell lysates were analyzed for active (p-Src Y416) and inactive (p-Src Y527) c-Src, phospho-RSK, phospho-Erk1/2, phospho-S6 kinase, EGFR and phospho-EGFR, as indicated. Erk1/2 was used as loading control. Overnight serum starved SW13 (**Panel B**) and B82L cells expressing wt-EGFR (**Panel C**) were treated with 1 μM each of the Src inhibitor PP2 or its inactive analog PP3 for 45 min followed by treatment with or without 1nM rapamycin for 30 min and subsequently lysed in

Laemmli buffer. Proteins in the lysates were immunoblotted for the presence of phospho-Y845 EGFR, phospho-RSK, phospho-S6 kinase, active phospho-c-Src (pSrc Y416), phospho-Erk1/2 and total Erk1/2 (loading control). **Panel D**: B82L cells expressing either the wt- EGFR or Y845F mutant of the EGFR were treated with and without rapamycin (1 nM) for 30 minutes. Proteins in the lysates were analyzed for the various proteins and their phosphorylated counterparts as described for panels A–C. **Panel E**: COS-7 cells were treated with 1 μM AG1478 for 45 min or control DMSO as illustrated. Cells were then treated with or without 1nM rapamycin for 30 min and lysed in Laemmli sample buffer. Cell lysates were immunoblotted for phospho-Y845 EGFR, phospho-RSK, active phospho-c-Src (pSrc Y416), phospho-Erk1/2 and tubulin (loading control). For all panels a representative of 3 similar experiments is shown.

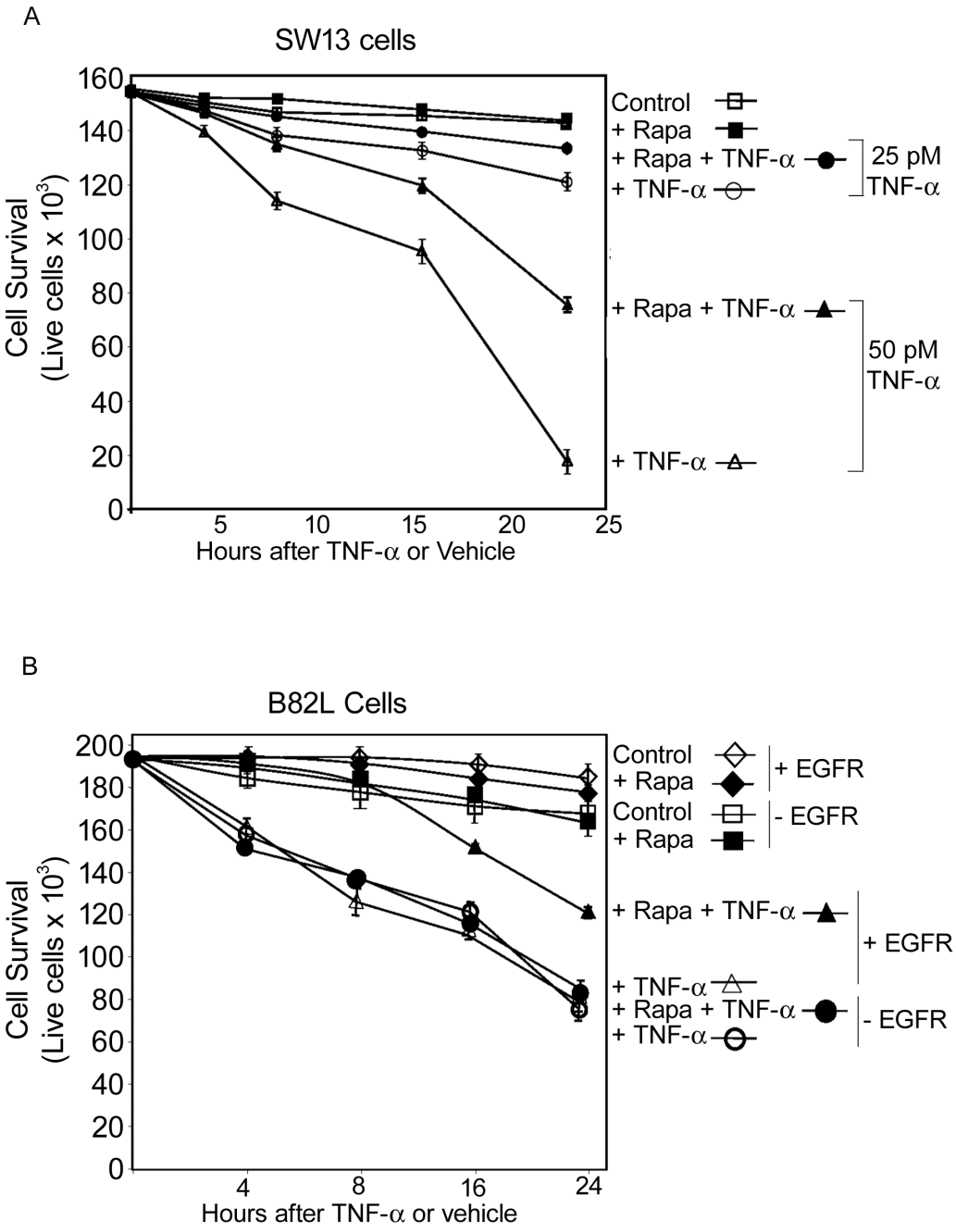


Figure 6. Time course of rapamycin induced cell survival

Overnight serum starved SW13 cells (**Panel A**) and B82L cells (**Panel B**) were treated with 1 nM rapamycin for 30 min followed by addition of TNF-α (0.05 nM & 0.025 nM in SW13 cells, and 0.05 nM in B82L cells). The cells were incubated at 37 °C and live cells were counted at the indicated times. A representative with mean ± SD from triplicate measurements in at least 3 similar experiments is shown.