Molecular Targeting of ERKs/RSK2 Signaling Axis in Cancer Prevention

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RSK2 is a downstream signaling protein of ERK1 and ERK2 and plays a key role in physiological homeostasis. For this reason, RSK2 is a highly conserved protein among the p90RSK family members. In its location in the signaling pathway, RSK2 is a kinase just upstream of transcription and epigenetic factors, and a few kinases involved in cell cycle regulation and protein synthesis. Moreover, activation of RSK2 by growth factors is directly involved in cell proliferation, anchorage-independent cell transformation and cancer development. Direct evidences regarding the etiological roles of RSK2 in cancer development in humans have been published by our research group illustrating that elevated total- and phospho-RSK2 protein levels mediated by ERK1 and ERK2 are higher in skin cancer tissues compared to normal skin tissues. Notably, it has been shown that RSK2 ectopic expression in JB6 Cl41 cells induces cell proliferation and anchorage-independent cell transformation. Importantly, knockdown of RSK2 suppresses Ras-mediated foci formation and anchorage-independent colony growth of cancer cells. Kaempferol is a one of the natural compounds showing selectivity in inhibiting RSK2 activity in epidermal growth factor-induced G1/S cell cycle transition and cell transformation. Thus, ERKs/RSK2 signaling axis is an important target signaling molecule in chemoprevention.

(Key Words: Carcinogenesis, Neoplastic cell transformation, Molecular targeting, ERKs/RSK2 signaling, Natural compounds

INTRODUCTION

Protein phosphorylation, a major regulatory mechanism of enzyme activity and stability, is a process of post-translational modification by addition of phosphate group into amino acid residues such as serine, threonine and tyrosine.¹ The phosphorylation of proteins is generally induced by cellular perturbations such as stress stimuli and cancerous transformation. Growth factors such as epidermal growth factor (EGF) and fibroblast growth factor, environmental stresses such as ultraviolet irradiation, and cytokines induce the phosphorylation of diverse proteins depending on the cellular context and the specific signaling pathway.² One of the well-known signaling pathways is the mitogen-activated protein kinases (MAPK) signaling pathway, which includes extracellular signal-regulated kinases (ERKs), Jun-N-terminal kinases (JNks) and p38 kinases (p38 MAPks) (Fig. 1).³ The 90 kDa ribosomal S6 kinases (p90RSKs: RSKs) superfamily, RSK1, RSK2, RSK3, RSK4, MSK1, and MSK2,⁴ is composed of RSKs and mitogen- and stress-activated protein kinases (MSKs) subfamilies, which are categorized together by amino acid similarity and structure.⁵ The amino acid sequence and structure of p90RSKs are also distinguishable from 70 kDa RSK (p70S6K).⁶ One of the striking features is that the RSK family of proteins contains two functional kinase domains. N-terminal kinase that phosphorylates the substrates and C-terminal kinase that stimulates the activation of RSK in one polypeptide.² The RSK family members shares very high amino acid similarity, about 80% among RSK isotypes including RSK1, RSK2, RSK3, and RSK4.
and cancer therapy.

**MAIN SUBJECT**

1. Tumor promoter signaling

MAPKs are Ser/Thr kinases that convert extracellular stimuli into a wide range of cellular responses including gene expression, mitosis, metabolism, motility, survival, apoptosis and differentiation. Binding of growth factors to the specific receptors induces dimerization and activation of receptor tyrosine kinases in the cytoplasmic membrane, resulting in induction of autophosphorylation at tyrosine residues (ERKs), p38 MAPKs and Jun-N-terminal kinases (JNKs). Currently, p90RSK superfamily is located in the downstream kinase of ERKs and p38 MAPKs and upstream of transcription factors, epigenetic factors and some of kinases regulating cell cycle distribution and protein synthesis. MAPKs, mitogen-activated protein kinases; Tpl-2, tumor promotion locus 2 (known as MAP3K8); MLK, mixed-lineage kinase; TAK, TGF-β-activated kinase; ASK, apoptosis signal-regulating kinase; DLK, dual leucine zipper kinase (known as MAP3K12); ZAK, sterile alpha motif and leucine zipper containing kinase; ATF1, activating transcription factor 1; Myt1, myelin transcription factor 1.
ERK2. Moreover, when cells are stimulated with growth factors RSK2 deficiency increases the total protein levels of ERK1 and RSK2 activity may contain a negative feedback loop because we have reported a critical clue that the interface between ERK1/2 is emphasized by many of human solid tumors containing constitutive active mutations in Ras and/or Raf with a high percentage in pancreatic, colon, breast, ovarian, prostate, lung, melanoma and other cancers. The gain-of-function mutation of Ras or Raf transduces their activation signal to downstream kinases through a phospho-conveyor system even without stimulation of upstream activation signals initiated by interaction of growth factors and specific receptors. Based on these reasons, many scientists have tried to identify or synthesize specific small molecules targeting Ras and/or Raf proteins. When cells were stimulated with tumor promoters such as EGF or 12-O-tetradecanoylphorbol-13-acetate (TPA), phosphorylation and activation of MAPK kinase signaling molecules were frequently observed. Because the phosphorylation signaling is activated within a few minutes after tumor promoter stimulation, MAPKs belong to immediate early response genes. However, increasing evidences have demonstrated that there are functional differences between isotypes of MAPKs. For example, strong or sustained activation of Raf signal induces a G1-specific cell cycle arrest through induction of p21, resulting in inhibition of cyclin-D- and -E-dependent kinases. Because of its high affinity with RSK1, which enhanced anchorage-independent cell transformation without EGF stimulation in JB6 Cl41 cells. Interestingly, colony formation in the RSK2 expressing JB6 Cl41 cells in soft agar without EGF stimulation was increased by stimulation of EGF indicating that overexpressed RSK2 activity was also enhanced by EGF stimulation. Moreover, proapoptotic BAD inactivation was achieved through RSK2 by activation of ERKs. Thus, ERKs/RSK2 signaling axis plays an important role in signaling node for the tumor promoter-induced cell proliferation and transformation.

2. ERK1/2-mediated RSK2 activation

The RSK2 activation mechanisms are very complicated due to the diverse nature of RSKs, the consecutive activity regulation by phosphorylation, the agonist-specific temporal activity regulation, the changing spatial distribution in cellular organelles, the existence of diverse interacting proteins, and the possession of two unique kinase domains in a polypeptide. The N-terminal kinase domain (NTKD) is classified as a AGC group kinase family (which includes PKA, PKG, and PKC), and the CTKD belongs to the calcium/calmodulin-dependent (CaMK) kinase family. The CTD of RSK2 contains a non-canonical ERK docking signal, Leu-X-X-Lys/Arg-Lys/Arg-X-X-X-X-X-Leu, which is different from classical D-type ERK docking domains and appears to fit the kinase interaction motif consensus sequences. An in vitro kinase assay demonstrated that RSK2 was activated by ERK1 and ERK2, not by p38 kinases. Generally, interaction of ERK1 or ERK2 with RSK2 has high affinity through D domain. The investigation of the interaction between ERK1/2 and RSK1 indicated that inactive ERK1/2 has a higher affinity with RSK1 than active forms of ERK1/2 following mitogen stimulation. Additionally, autophosphorylation of a serine residue near ERK1/2 docking domain of RSK1 promotes its dissociation from ERK1/2. Based on these evidences, a possible RSK2 activation mechanism is the activation of inactive ERK1 or ERK2 with RSK2 pre-complex by growth factor stimulation, which results in dissociation of ERK1/2 from CTD of RSK2. Activated ERK1 or ERK2 induces phosphorylation of RSK2 at Thr365/Ser369

directly with its downstream target effector proteins such as Raf proteins. Activated Rafs bind and phosphorylate the dual specificity kinases, MEK1/2, which in turn, phosphorylate ERK1/2 at a conserved Thr-Glu-Tyr motif. Eventually, activated ERK1/2 bind at the ERK docking motif in C-terminal (CTD) of RSKs and phosphorylate the linker domain (LD) and C-terminal kinase domain (CTKD) of RSKs, resulting in the activation of N-terminal kinase activity.

The importance of Ras/Raf/MEKs/ERKs/RSKs signaling pathway is emphasized by many of human solid tumors containing constitutive active mutations in Ras and/or Raf with a high percentage in pancreatic, colon, breast, ovarian, prostate, lung, melanoma and other cancers. The gain-of-function mutation of Ras or Raf transduces their activation signal to downstream kinases through a phospho-conveyor system even without stimulation of upstream activation signals initiated by interaction of growth factors and specific receptors. Based on these reasons, many scientists have tried to identify or synthesize specific small molecules targeting Ras and/or Raf proteins. When cells were stimulated with tumor promoters such as EGF or 12-O-tetradecanoylphorbol-13-acetate (TPA), phosphorylation and activation of MAPK kinase signaling molecules were frequently observed. Because the phosphorylation signaling is activated within a few minutes after tumor promoter stimulation, MAPKs belong to immediate early response genes. However, increasing evidences have demonstrated that there are functional differences between isotypes of MAPKs. For example, strong or sustained activation of Raf signal induces a G1-specific cell cycle arrest through induction of p21, resulting in inhibition of cyclin-D- and -E-dependent kinases. 

ERK1 or ERK2 induces phosphorylation of RSK2 at Thr365/Ser369 in cell proliferation and transformation. RSK2 deficiency attenuates cell proliferation in mouse embryonic fibroblasts (MEFs) compared with RSK2 wildtype MEFs. The potential of RSK2 as an oncokinase was suggested by the fact that ectopic expression of RSK2, which enhanced anchorage-independent cell transformation without EGF stimulation in JB6 Cl41 cells. Interestingly, colony formation in the RSK2 expressing JB6 Cl41 cells in soft agar without EGF stimulation was increased by stimulation of EGF indicating that overexpressed RSK2 activity was also enhanced by EGF stimulation. Moreover, proapoptotic BAD inactivation was achieved through RSK2 by activation of ERKs. Thus, ERKs/RSK2 signaling axis plays an important role in signaling node for the tumor promoter-induced cell proliferation and transformation.
located in the LD and at Thr577 located in the CTKD, resulting in the induction of RSK2 CTKD kinase activity. The activated CTKD phosphorylates another serine residue (Ser386) in the LD, which serves as a PDK1 docking motif. The PDK1 docking at the LD domain of RSK2 facilitates phosphorylation of Ser227 in the NTKD. Thus, the CTKD of RSK2 is essential for the initiation of RSK2 activation process, resulting in activation of RSK2 NTKD. The cascade kinase reaction indicated that the kinase domain, NTKD of RSK2, played a key role in substrate phosphorylation.9 The RSK2 protein purified from Escherichia coli did not have the ability to phosphorylate nuclear factor of activated T-cells (NFAT3)-261-365 protein, which is known to be the best substrate of RSK2 having about $K_m=0.3559 \mu M$.31 In contrast, when the RSK2 proteins were activated by active ERK2, RSK2 recovers the ability to phosphorylate NFAT3-261-365.9 Interestingly, RSK2 proteins not containing either NTKD or CTKD totally lost the ability to phosphorylate NFAT3-261-365 proteins, indicating that CTKD activation of RSK2 is indispensable to activate NTKD of RSK2.

3. ERK1/2-mediated RSK2 signaling in cell transformation

Up-regulation of the MAPK signaling pathway promotes cell proliferation and enhances cell survival in various cancer cells.21 When cells are stimulated with a survival growth factor, such as brain-derived neurotropic factor, RSK2 induces phosphorylation of proapoptotic BAD protein,32 resulting in enhancement of cell survival. Our research group found that the stimulation of tumor promoters, such as EGF or TPA, induces the phosphorylation of ERK1/2 and RSKs, resulting in induction of G1/S cell cycle transition and cell proliferation.22 These results were supported by experiments, which showed that RSK2 deficiency attenuates cell proliferation compared to RSK2 deficient MEFs.22 Recently, when cells were irradiated with ultraviolet light, RSK2 induced glycogen synthase kinase 3β (GSK3β) phosphorylation at Ser9.28 Since activation of GSK3β (non-phosphorylated GSK3β) at Ser9 induces cell cycle arrest and apoptosis, RSK2 antagonists MEFs showed resistance to apoptosis by ultraviolet irradiation.28 These results indicate that ERKs-mediated RSK2 signaling pathway induces not only cell proliferation but also cell survival. In this signaling pathway, RSK2 activity was correlated with cell transformation. When RSK2 was introduced to cells using an ectopic expression vector, the cells showed increased anchorage-independent colony formation without EGF stimulation.22 Furthermore, critical evidences highlighted the importance of Ras/MEKs/ERKs/RSK2 signaling pathway in cell transformation. For example, The Ras/MEKs/ERKs signaling axis-mediated RSK2 activation is proven by the knockdown of RSK2 with si-RNA RSK2 in cells stably expressing constitutively active Ras (CA-Ras) alone or CA-Ras and RSK2.23 The results showed that RSK2 knockdown suppressed foci formation in NIH3T3 cells.22 The RSK2 total protein profile indicates that RSK2 protein levels are higher in cancer cells than that of nonmalignant cells.9 Importantly, kaempferol, a natural compound harboring RSK2 selective inhibitory effect, inhibits cell proliferation in a dose dependent manner.9 The etiological evidence that RSK2 is involved in cancer development in humans was provided by skin cancer tissue array. Immunohistofluorescence array containing 70 core human skin cancer tissues and 10 normal skin tissues demonstrated that total RSK2 protein levels were higher in skin cancer tissues than that of normal tissues.9 Moreover, activated RSK2 protein, phospho-RSK2 at Thr577, was elevated in skin cancer tissues compared to normal skin tissues.10 Elevated total- and phospho-RSK2 protein levels were increased in sub-categorized human skin cancer tissues, such as squamous cell carcinoma, basal cell carcinoma and malignant melanoma, compared to normal skin tissues.10 Importantly, HaCaT cells, a premalignant human skin keratinocyte cell line, N/TERT-1 cells, a human skin keratinocyte cell line immortalized by telomerases,33 SCC-13 cells, a human skin epidermal squamous cell carcinoma cell line34 and SK-MEL-28 malignant melanoma cells contain different levels of endogenous RSK2 protein, and RSK2 knockdown effects on the cell proliferation were correlated with endogenous RSK2 protein levels.10 Based on these results, it can be concluded that ERK1/2-mediated RSK2 protein activation plays a key role in cell survival and cell proliferation, resulting in cancer development in humans.

4. Molecular Targeting of ERKs/RSK2 in chemoprevention

ERKs are one of the more well-known MAPK signaling molecules that are located of downstream of cell surface receptors and other cytoplasmic signaling proteins whose functions are deregulated in cancer and other human pathogenic disorders.3 Due to their importance and involvement in cell proliferation and survival, ERK1 and ERK2 have attracted intense research interest to identify small molecules that inhibit ERK1 and ERK2 activities. The rationale is supported by the mutational activation and/or overexpression of upstream signaling molecules that activate the ERKs. Our results also demonstrated that growth factors and environmental stresses induce the phosphorylation of ERK1 and ERK2 in a short time after treatment.10,22 To date, research for the identification and development of small molecules that target the Ras/Raf/MEKs/ERKs signaling axis have been focused on upstream proteins of ERKs and RSK2. Although, the Raf/MEKs/
Inhibitors targeting extracellular signal-regulated kinases (ERKs) and RSK2. Chemical compounds, AEZs-131, SCH772984, and magnolin, targeting ERKs activity are synthesized or identified. Magnolin, a natural compound abundantly found in Shin-Yi, inhibits cell proliferation and anchorage-independent transformation induced by tumor promoters such as EGF. BI-D1870 is a derivative from pyrido[2,3-d]pyrimidine group of Src inhibitors targeting N-terminal kinase domain of RSK2. Fmk is an irreversible C-terminal kinase inhibitor of RSK2 by covalent bond formation at Cys436. SL0101, kaempferol-3-O-(3, 4'-di-O-acetyl-α-L-rhamnopyranoside), is a natural compound abundantly found in Forsteronia refracta, which found in the South America Amazon rainforest, and have inhibitory effects of RSKs by targeting to N-terminal kinase domain. Kaempferol, a natural compound found in dietary foods such as leafs of green onion, targeting N-terminal kinase domain of RSK2, but not RSK1 and RSK3. Kaempferol also inhibits cell proliferation and anchorage-independent cell transformation. ATF1, activating transcription factor 1; Myt1, myelin transcription factor 1.

ERKs cascade is a critical signaling axis of Ras-mediated carcinogenesis, recent studies have clearly demonstrated that Ras is involved in the activation of effector signaling molecules. For example, the p110 catalytic subunits (p110α, β, γ, and δ) of class I phosphatidylinositol 3-kinases, the Ral small GTPase specific GEFs (RalGDS, Rgl, Rgl2, and Rgl3), the Tiam1 Rac small GTPase-specific GEF and the phospholipase C epsilon plays important roles in Ras-induced oncogenesis. This suggests that aberrant inhibition of Ras and Ras/Raf/MEKs by small molecules may express unwanted effects. Therefore, we have considered that targeting of signaling molecules closely located to transcription factors in signaling cascades may promise more specific effects against target diseases with fewer side effects (Fig. 2). BI-D1870, a derivative from pyrido[2,3-d]pyrimidine group of Src inhibitors, is a cell permeable inhibitor with relative specificity for the RSK family with about 10 to 30 nM of IC50 value. The results demonstrated that BI-D1870 inhibited the EGF- or TPA-mediated phosphorylation of GSK3β and LKB1 by targeting of NTKD of RSKs. A different type of RSK2 inhibitor, pyrrolopyrimidine fmk, was developed. The fmk targeted CTKD of RSK2 and inhibited RSK2 CTKD activity with 15 nM of IC50 and 200 nM of the half maximal effective concentration (EC50). The compound inhibits irreversible C-terminal kinase activity of RSK2 by covalent addition of chloromethylketone to thiol group of Cys436. Recently, our research group found that kaempferol, a natural compound abundantly found in edible dietary plants, targeted the N-terminal kinase activity by binding to the active pocket of RSK2. The docking score of kaempferol on the active pocket of RSK2 NTKD is about −12.8 kcal/mol which is similar to the SL0101 (kaempferol-3-O-(3, 4'-di-O-acetyl-α-L-rhamnopyranoside) docking score which is about −13.3 kcal/mol. Our computational model suggested that kaempferol formed three hydrogen bonds with the backbone atoms of Asp148, Leu150, and Leu74 or Lys100. Moreover, heterocyclic ring system of kaempferol lies in the hydrophobic region of the RSK2 NTKD catalytic site where it is occupied by ATP. More recently, X-ray crystal structure of RSK2 NTKD and kaempferol reveals that amino acids including Asp148, Glu197, and Lys100 participate in specific interactions between the inhibitor and the protein moiety of RSK2 NTKD to form specific hydrogen bonds. Interestingly, Ile50, Ile52, Phe79, Leu102, Val131, Leu147, Leu150, Leu155, Leu200, and Phe212 were shown to participate in the interaction between SL0101 and RSK2 N-terminal domain and NTKD. Our research group found that kaempferol inhibited not only RSK2 N-terminal kinase activity with a IC50 value of to
about 7 μM in vitro but also cell proliferation in cell culture with a IC₅₀ value of to about 30 μM. Although, the IC₅₀ values of SL0101 in the RSK2 NTKD activities were different (approximately from 89 nM to 7 μM) from kaempferol in vitro. The IC₅₀ values on cell proliferation were similar in both SL0101 and kaempferol, approximately from 20 to 40 μM. In many different premalignant and cancer cells, kaempferol proliferation and transformation. Based on these efforts, some research groups have found RSK inhibitors including ones that inhibit RSK2. One of the representative natural compounds having inhibitory effects on the RSK2 activity is SL0101, extracted from Forsteronia refracta, which found in the South America Amazon rainforest, and kaempferol, abundantly found in edible dietary plants such as leaves of green onion and endives. Although, SL0101 showed lower IC₅₀ values than that of kaempferol in vitro assay using holoenzymes, the effective IC₅₀ concentration of kaempferol is similar to that of SL0101 on cell proliferation and transformation. Although kaempferol is not suitable to be used in cancer therapy directly, kaempferol might be potentiated as a chemopreventive agent because of its role in cell proliferation and transformation. Important, after oral administration, kaempferol was detected in human plasma, about a concentration of 800 nmol/L, and urine, indicating that kaempferol is absorbed in the intestine. Thus, we believe similar concentration of SL0101 and kaempferol show similar effects on the cell proliferation.

FURTHER DIRECTIONS

Current studies on the p90RSK family are mainly focused on RSK2 because RSK2 mutation and genomic deletion causes a human genetic disease known as CLS. For the last decade, the research on the etiological role of RSK2 in human cancers have been gradually accelerating, and now research scientists believe that RSK2 is a key signaling molecule and playing an important role in cell proliferation and transformation. Based on these efforts, some research groups have found RSK inhibitors including ones that inhibit RSK2. One of the representative natural compounds having inhibitory effects on the RSK2 activity is SL0101, extracted from Forsteronia refracta, which found in the South America Amazon rainforest, and kaempferol, abundantly found in edible dietary plants such as leaves of green onion and endives. Although, SL0101 showed lower IC₅₀ values than that of kaempferol in vitro assay using holoenzymes, the effective IC₅₀ concentration of kaempferol is similar to that of SL0101 on cell proliferation and transformation. Although kaempferol is not suitable to be used in cancer therapy directly, kaempferol might be potentiated as a chemopreventive agent because of its distribution in edible plants. Moreover, kaempferol may use as a mother molecule to develop improved synthetic chemical compounds having higher selectivity and efficacy against RSK2.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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