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Sumin Kang, Emory University
Shaozhong Dong, Emory University
Ailan Guo, Cell Signaling Technology, Inc. 01923
Hong Ruan, Cell Signaling Technology
Sagar Lonial, Emory University
H Jean Khoury, Emory University
Ting-Lei Gu, Cell Signaling Technology, Inc.
Jing Chen, Emory University

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Epidermal Growth Factor Stimulates RSK2 Activation through
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Sumin Kang†, Shaozhong Dong‡, Ailan Guo§, Hong Ruan‡, Sagar Lonial‡, Hanna Jean Khoury†, Ting-Lei Gu†, and Jing Chen†1

From the †Winship Cancer Institute, Emory University School of Medicine, Atlanta, Georgia 30322 and §Cell Signaling Technology, Inc., Danvers, Massachusetts 01923

The Ser/Thr kinase ribosomal S6 kinase 2 (RSK2) has been demonstrated to phosphorylate transcription factor CREB (cyclic AMP-responsive-binding protein) and histone H3 in response to mitogenic stimulation by epidermal growth factor (EGF). EGF activates the MEK/ERK pathway to activate RSK2. We recently reported that receptor tyrosine kinase fibroblast growth factor receptor 3 (FGFR3) directly tyrosine phosphorylates RSK2 at Tyr-529, which consequently regulates RSK2 activation by facilitating inactive ERK binding to RSK2 that is required for ERK-dependent phosphorylation and activation of RSK2 (Kang, S., Dong, S., Gu, T. L., Guo, A., Cohen, M. S., Lonial, S., Khoury, H. J., Fabbro, D., Gilliland, D. G., Bergsagel, P. L., Taunton, J., Polakiewicz, R. D., and Chen, J. (2007) Cancer Cell 12, 201–214). Here we report that upon treatment of EGF, RSK2 was tyrosine-phosphorylated at Tyr-529 and activated in 293T and COS7 cells that do not express FGFR3. In contrast to FGFR3, the receptor tyrosine kinase EGF receptor did not directly phosphorylate RSK2 at Tyr-529 in an in vitro kinase assay using recombinant RSK2 and active EGF receptor or FGFR3. By mass spectrometry-based studies, we identified Src tyrosine kinase family members Src and Fyn as upstream kinases of RSK2 Tyr-529. Treatment of Src inhibitor PP2 effectively attenuated EGFinduced activation and Tyr-529 phosphorylation of RSK2, suggesting that Src family members are the kinases that phosphorylate RSK2 at Tyr-529 in response to EGF. Src and Fyn were able to directly phosphorylate RSK2 at Tyr-529 in the in vitro kinase assay. In vitro reconstitution of Tyr-529 phosphorylation by Src in glutathione S-transferase-tagged RSK2 enhanced inactive ERK binding to RSK2 wild type, but not the Y529F mutant. Together, our findings suggest that Src-dependent phosphorylation at Tyr-529 facilitates inactive ERK binding to RSK2, which might be a general requirement for RSK2 activation by EGF through the MEK/ERK pathway.

In mammalian cells, various mitogenic stimuli, including epidermal growth factor (EGF),2 induce a Ras-dependent mitogen-activated MEK/ERK kinase cascade that results in the transcriptional activation of immediate-early responsive genes (1, 2). The Ser/Thr kinase RSK2 is a substrate of ERK and belongs to a family containing four members, RSK1–4. RSK2 has been implicated to play an active role in EGF-dependent transcriptional activation by phosphorylation and regulation of the transcription factor CREB (cyclic AMP-responsive binding protein) (3). Phosphorylation at Ser-133 dictates the ability of CREB to interact with the coactivator CBP (CREB-binding protein) that mediates functional contacts with the basal transcriptional machinery (3). RSK2 is also required for EGF-activated phosphorylation of histone H3, which contributes to chromatin remodeling during mitosis and transcriptional activation (4, 5).

Among the four RSK family members, RSK2 is so far the best characterized. RSK2 contains two distinct kinase domains, both of which are catalytically functional (reviewed in Refs. 6, 7). The C-terminal kinase domain (CTD) is responsible for autophosphorylation at Ser-386 that is critical in RSK activation, whereas the N-terminal kinase domain is believed to phosphorylate exogenous substrates of RSK (8). However, the precise mechanism of RSK2 activation remains elusive. The current model suggests that ERK-dependent activation of RSK2 involves a series of sequential events. First, inactive ERK binds to the C terminus of RSK2 in quiescent cells, and this interaction is an absolute requirement for activation of RSK2 (9–11). Second, when stimulating signal such as mitogen comes, ERK is activated and phosphorylates RSK2 at Thr-577 in the activation loop of the CTD domain and Ser-369 and Thr-365 in the linker region between the two kinase domains, leading to activation of the RSK2 CTD domain. Third, activation of CTD domain results in autophosphorylation of Ser-386 in the linker region, which provides a docking site for 3-phosphoinositide-dependent protein kinase 1 (PDK1) (12). PDK1 in turn phos-

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† A Georgia Cancer Coalition Distinguished Cancer Scholar. To whom correspondence should be addressed. Tel.: 404-778-5274; Fax: 404-778-5520; E-mail: jchen@emory.edu.

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2 The abbreviations used are: EGF, epidermal growth factor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; CREB, cyclic AMP-responsive-binding protein; RSK, ribosomal S6 kinase; CTD, C-terminal kinase domain; GST, glutathione S-transferase; HA, hemagglutinin; WT, wild type; MAPK, mitogen-activated protein kinase; FGFR3, fibroblast growth factor receptor 3.
phorylates Ser-227 in the activation loop of the N-terminal kinase domain, allowing RSK2 to phosphorylate its downstream targets (13). Last, activated N-terminal kinase domain auto-phosphorylates Ser-749 at the C-terminal domain of RSK2, which results in dissociation of active ERK from RSK2 (11).

We have recently reported that FGFR3 signals through activation of RSK2 to provide proliferative and pro-survival signals in human t(4;14) multiple myeloma cells with dysregulated expression of FGFR3 (14). We proposed that FGFR3 activates RSK2 by a two-step model in which, in addition to FGFR3-mediated activation of the MEK/ERK pathway, FGFR3 directly phosphorylates RSK2 at Tyr-529, which regulates the ERK-dependent activation of the serine/threonine kinase RSK2 by allowing inactive ERK to bind RSK2 in the initial step (14). However, it remains unclear whether tyrosine phosphorylation at Tyr-529 of RSK2 is a specific requirement of FGFR3 signaling in hematopoietic cells or whether this might represent a more general mechanism for RSK2 activation.

Here we report that upon treatment of EGF, RSK2 is tyrosine-phosphorylated at Tyr-529 and activated in 293T and COS7 cells that do not express FGFR3. However, this phosphorylation was not mediated directly by activated receptor tyrosine kinase EGFR but by Src tyrosine kinase family members. Phosphorylation at Tyr-529 by Src facilitates ERK binding to RSK2, which represents a general requirement for RSK2 activation by EGF through the MEK/ERK pathway.

**EXPERIMENTAL PROCEDURES**

*Cells and Reagents—* 293T and COS7 cells were cultured in Dulbecco modified Eagle’s medium supplemented with 10% fetal bovine serum. Antibodies against EGF, phospho-RSK (Ser-380), Pyk2, JAK2, ERK1/2, and Src were from Cell Signaling Technology Inc. (Danvers, MA). Specific antibody against...
Src Phosphorylates and Regulates RSK2

Phospho-RSK2 (Tyr-529) was generated and provided by Cell Signaling Technology. Phospho-Tyr antibody (pY99) and antibodies against EGFR, RSK2 and Fyn were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against GST, HA, and β-actin were from Sigma. Src inhibitor PP2 was from Calbiochem. Glutathione-Sepharose 4B and protein G-conjugated agarose beads were from Amersham Biosciences. Recombinant active Src and Fyn were from Invitrogen. YOP tyrosine phosphatase was obtained from New England Biolab (Ipswich, MA). Constructs harboring various tyrosine kinases were kindly provided by Dr. Keqiang Ye, Emory University.

**Mass Spectrometry-based Studies**—2–3 × 10^6 293T cells that were transiently transfected with both GST-tagged RSK2 and various tyrosine kinases were lysed 24 h post-transfection. GST-tagged RSK2 was enriched by glutathione-Sepharose 4B beads in a GST pulldown assay and applied to SDS-PAGE, followed by Coomassie Blue staining. RSK2 protein bands were excised and treated with trypsin, followed by analysis using liquid chromatography coupled with mass spectrometry (Cell Signaling Technology). Tandem mass spectra were collected in a data-dependent manner with an LTQ ion trap mass spectrometer (ThermoFinnigan).

<table>
<thead>
<tr>
<th>Sequence containing Tyr(P) (*), Frequency of detection, and Frequency of detection</th>
<th>Frequency of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr-483 DRYV*DDQYYVVFTELMK</td>
<td>1</td>
</tr>
<tr>
<td>Tyr-488 DAVYDDGK*YYVVFTELMK</td>
<td>5</td>
</tr>
<tr>
<td>Tyr-529 TVEY*LHAQQGVRHR</td>
<td>1</td>
</tr>
<tr>
<td>Tyr-644 PLGQG*HSSVGFPAK</td>
<td>1</td>
</tr>
<tr>
<td>Tyr-707 GAMAATY*SALNR</td>
<td>5</td>
</tr>
<tr>
<td>Tyr-470 Y*QHPNTTLK</td>
<td>1</td>
</tr>
<tr>
<td>Tyr-580 AENLMLTPCV*TANFVAPEVLK</td>
<td>2</td>
</tr>
</tbody>
</table>

**In Vitro Reconstitution of RSK2 Tyrosine Phosphorylation by Src**—The in vitro tyrosine phosphorylation reconstitution assay was performed as previously described (14). In brief, 293T cells transiently transfected with GST-tagged RSK2 constructs were lysed and GST-RSK2 variants were pulled down by glutathione-Sepharose 4B beads, followed by protein tyrosine phos-
phatase treatment at 30 °C for 2 h. The beads were washed and applied to the in vitro kinase assay with active Src kinase at 30 °C for 30 min as described above. The treated beads were washed and incubated with 293T cell lysates pretreated with 10 μM U0126 for 2 h, followed by SDS-PAGE and Western blotting.

RESULTS

RSK2 Is Tyrosine-phosphorylated at Tyr-529 and Activated in 293T and COS7 Cells upon EGF Stimulation—We recently reported a novel two-step model that leukemogenic FGFR3 activates RSK2 by both assisting inactive ERK binding via tyrosine phosphorylation of RSK2 at Tyr-529 and activating the MEK/ERK pathway (14). Mutation at Tyr-529 resulted in decreased inactive ERK binding and consequently decreased ERK-dependent phosphorylation and activation of RSK2 (14).

To determine whether tyrosine phosphorylation at Tyr-529 is a common mechanism for ERK-dependent activation of RSK2, we examined the phosphorylation status of RSK2 in response to EGF stimulation. Using a specific phospho-RSK2 antibody (Tyr-529) (14), we observed that upon EGF treatment, RSK2 was tyrosine phosphorylated at Tyr-529 and activated as assessed by Ser-386 phosphorylation, which is an index of RSK2 activation, in 293T cells that do not express FGFR3 (Fig. 1). We also observed that treatment with MEK1 inhibitor U0126 effectively inhibited EGF-induced phosphorylation at RSK2 Ser-386 in 293T cells but had no effects on Tyr-529 phosphorylation (Fig. 1), suggesting that EGF-dependent activation of RSK2 requires ERK activation. Similar results were obtained using COS7 cells (data not shown).

EGF Treatment Promotes Inactive ERK Binding to RSK2—We have demonstrated the phosphorylation at Tyr-529 facilitates inactive ERK binding to RSK2 (14). We next performed a GST pulldown assay in which the bead-bound GST-tagged RSK2 was incubated with U0126-pretreated 293T cell lysates. As shown in Fig. 2, EGF induced Tyr-529 phosphorylation of GST-tagged RSK2 along with increased inactive ERK binding, compared with GST or GST-tagged RSK2 from cells in the absence of EGF treatment. Together, these data suggest that EGF-dependent Tyr-529 phosphorylation may be involved in regulation of ERK binding to RSK2 in 293T and COS7 cells that is independent of FGFR3, suggesting the existence of alternative tyrosine kinase(s).

EGFR Does Not Directly Phosphorylate RSK2 at Tyr-529—Because FGFR3 directly phosphorylates RSK2 at Tyr-529 (14), we hypothesized that EGFR as a receptor tyrosine kinase may similarly phosphorylate RSK2. We performed an in vitro kinase assay in which purified recombinant RSK2 CTD proteins (rRSK2 CTD) and RSK2 Tyr-529 mutant proteins were incubated with recombinant EGFR or FGFR3 (rSrc or rFyn, respectively). We observed that recombinant active rSrc or rFyn efficiently phosphorylated RSK2 at Tyr-529, whereas rEGFR had no effect on RSK2 phosphorylation (Fig. 3). These results suggest that EGFR does not directly phosphorylate RSK2 at Tyr-529.
EGFR was not able to phosphorylate rRSK2 CTD at Tyr-529 in the in vitro kinase assay (Fig. 3). In contrast, active recombinant FGFR3 directly phosphorylates rRSK2 CTD as previously reported (14), and this phosphorylation was abolished in the rRSK2 Y529F mutant.

**EGF-induced Tyr-529 Phosphorylation Is Mediated by Src Family Members Including Src and Fyn**—To identify the potential upstream tyrosine kinases of RSK2, we next performed mass spectrometry-based studies. Plasmids encoding GST-RSK2 and distinct tyrosine kinases, including HA-tagged focal adhesion kinase, HA-tagged active form of Fyn, FynA (Y528F), HA-tagged active form of Src, SrcA (Y527F), Pyk2, or a constitutively activated fusion tyrosine kinase TEL-JAK2, were transiently co-transfected into 293T cells. Tyrosine phosphorylation levels of the bead-bound GST-tagged RSK2 were probed by a pan-phospho-Tyr antibody (pY99). We observed that overexpression of active Src and Fyn resulted in tyrosine phosphorylation of RSK2. In contrast, RSK2 was not significantly tyrosine-phosphorylated in cells co-transfected with focal adhesion kinase, Pyk2, or TEL-JAK2 (Fig. 4A). The RSK2 protein bands were excised from the SDS-PAGE gel and digested with trypsin, followed by mass spectrometry-based analysis. We identified that RSK2 was tyrosine-phosphorylated at a group of tyrosine sites (Table 1), including Tyr-529 (spectra presented in Fig. 4B), due to expression of the constitutively activated Src and Fyn.

We next tested whether Tyr-529 is a major phosphorylation site of Src, along with the other two most frequent phosphorylation sites of RSK2 by Src or Fyn, Tyr-488 and Tyr-707 as controls, which were identified by mass spectrometry-based studies (Table 1). We performed Western blotting experiments to determine whether mutation at Tyr-488, Tyr-529, or Tyr-707 would significantly decrease the tyrosine phosphorylation levels of RSK2 by Src. The results showed that Tyr-488 is a major site of Src but mutations at Tyr-529 or Tyr-707 did not significantly decrease Src-dependent tyrosine phosphorylation of RSK2 (Fig. 4C). However, we have previously characterized the Tyr-488 site that is also phosphorylated by FGFR3 (14), and substitution of Tyr-488 did not affect RSK2 activation. In contrast, mutation at Tyr-529 significantly attenuated RSK2 activation, suggesting that Tyr-529, but not Tyr-488, contributes to regulation of RSK2 activation (14).

**Treatment of PP2, a Specific Inhibitor of Src Family Members, Effectively Attenuates RSK2 Tyrosine Phosphorylation at Tyr-529 and Activation in 293T Cells Stimulated by EGF**—We next tested whether Src is involved in EGF-dependent Tyr-529 phosphorylation of RSK2. Indeed, as shown in Fig. 5, treatment of PP2, an inhibitor of Src family members, effectively decreased the phosphorylation level of RSK2 at Tyr-529 in response to EGF in 293T cells, as well as activation of RSK2 as assessed by the attenuated Ser-386 phosphorylation. Similar results were obtained using CO57 cells (data not shown). Together, these data suggest that Src tyrosine kinase family members are downstream signal transducers of EGF and involved in EGF-dependent tyrosine phosphorylation at Tyr-529 and activation of RSK2.

**Src and Fyn Directly Phosphorylate RSK2 at Tyr-529**—Next we examined whether Src- or Fyn-dependent RSK2 Tyr-529 phosphorylation occurs in cells. By using the specific phospho-RSK2 antibody (Tyr-529), we observed that GST-tagged RSK2 WT, but not Y529F mutant, were specifically tyrosine-phosphorylated at Tyr-529 in 293T cells expressing constitutively activated SrcA or FynA (Fig. 6).

We then determined whether Src or Fyn phosphorylates RSK2 directly at Tyr-529 or indirectly by activating other tyrosine kinases. Purified recombinant rRSK2 CTD or CTD Y529F proteins were incubated with recombinant active Src or Fyn (rSrc or rFyn, respectively) that are constitutively activated (Invitrogen). As shown in Fig. 7, wild-type RSK2 CTD domain was highly tyrosine-phosphorylated at Tyr-529 by rSrc or rFyn,
whereas Tyr-529 phosphorylation was abolished in the RSK2 CTD Y529F mutant.

Phosphorylation of RSK2 at Tyr-529 by Src Facilitates Inactive ERK Binding—We next tested whether the tyrosine phosphorylation of RSK2 at Tyr-529 by Src precedes the inactive ERK binding to RSK2. We performed a GST pulldown assay in which the bead-bound GST-tagged RSK2 WT or Y529F mutant proteins were first dephosphorylated by protein tyrosine phosphatase to remove tyrosine phosphorylation and then treated with recombinant active rSrc, followed by incubation with U0126-treated 293T cell lysates. As shown in Fig. 8A, Tyr-529 phosphorylation was reconstituted in GST-RSK2 along with increased inactive ERK binding upon Src treatment compared with GST-RSK2 in the absence of Src treatment. However, substitution of Tyr-529, but not Tyr-488, abolished Src-dependent Tyr-529 phosphorylation and resulted in decreased ERK binding to Y529F mutant, compared with RSK2 WT and the control Y488F mutant in the presence of Src (Fig. 8B). We have previously demonstrated that the global structure as well as the intrinsic kinase property of the Y529F mutant protein were not altered compared with RSK2 WT and thus the decreased inactive ERK binding of RSK2 Y529F mutant is not due to structural alterations (14).

DISCUSSION

Together, our data support a novel model composed of a series of sequential events by which EGF activates RSK2 and mediates mitogenic signals in mammalian cells. The first event involves tyrosine phosphorylation at Tyr-529 of RSK2 by Src family members including Src or Fyn, but not EGFR, in response to EGF, which facilitates binding of the inactive form of ERK to RSK2 in the initial step of ERK-dependent RSK2 activation (Fig. 9). This binding, which is required for phosphorylation and activation of RSK2 by ERK, in turn promotes the next step where ERK is activated via the Ras/Raf/MEK/MAPK pathway downstream of EGF, leading to ERK-mediated phosphorylation and activation of RSK2. When RSK2 is activated, active ERK readily dissociates from RSK2 (11) (Fig. 9).

Our findings suggest that tyrosine phosphorylation of RSK2 at Tyr-529 in response to EGF is mediated indirectly by Src tyrosine kinase family members, not directly by the activated receptor tyrosine kinase EGFR. In contrast, FGFR3 directly phosphorylates RSK2 at Tyr-529, and this phosphorylation is independent of Src due to our observation that treatment of Src inhibitor PP2 had no effects on FGFR3-dependent tyrosine phosphorylation of RSK2 at Tyr-529 in cells (data not shown).

Thus, tyrosine phosphorylation at Tyr-529 of RSK2 might represent a general mechanism for ERK-dependent activation of RSK2 in response to the mitogenic stimuli, including EGF. Tyrosine phosphorylation at Tyr-529 may provide an additional docking site to promote the binding of inactive ERK to the C terminus of RSK2, or stabilize such binding. Further detailed structural studies are warranted to illuminate this process.

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