BI-D1870 is a specific inhibitor of the p90 RSK (ribosomal S6 kinase) isoforms *in vitro* and *in vivo*

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Hormones and growth factors induce the activation of a number of protein kinases that belong to the AGC subfamily, including isoforms of PKA, protein kinase B (also known as Akt), PKC, S6K p70 (ribosomal S6 kinase), RSK (p90 ribosomal S6 kinase) and MSK (mitogen- and stress-activated protein kinase), which then mediate many of the physiological processes that are regulated by these extracellular agonists. It can be difficult to assess the individual functions of each AGC kinase because their substrate specificities are similar. Here we describe the small molecule BI-D1870, which inhibits RSK1, RSK2, RSK3 and RSK4 *in vitro* with an IC₅₀ of 10–30 nM, but does not significantly inhibit ten other AGC kinase members and over 40 other protein kinases tested at 100-fold higher concentrations. BI-D1870 is cell permeant and prevents the RSK-mediated phorbol

ester- and EGF (epidermal growth factor)-induced phosphorylation of glycogen synthase kinase-3 β and LKB1 in human embryonic kidney 293 cells and Rat-2 cells. In contrast, BI-D1870 does not affect the agonist-triggered phosphorylation of substrates for six other AGC kinases. Moreover, BI-D1870 does not suppress the phorbol ester- or EGF-induced phosphorylation of CREB (cAMP-response-element-binding protein), consistent with the genetic evidence indicating that MSK, and not RSK, isoforms mediate the mitogen-induced phosphorylation of this transcription factor.

Key words: BI-D1870, kinase-specific inhibitor, mitogen-activated protein kinase (MAPK), p90 ribosomal S6 kinase (RSK), phosphorylation.

INTRODUCTION

Members of the AGC [PKA (cAMP-dependent protein kinase)/protein kinase G/PKC (protein kinase C)] subfamily of protein kinases, including isoforms of PKA, PKB (protein kinase B), S6K (p70 ribosomal S6 kinase), PKC, RSK (p90 ribosomal S6 kinase) and MSK (mitogen- and stress-activated protein kinase), mediate many of the cellular effects of extracellular agonists by phosphorylation of key regulatory proteins. These protein kinases possess catalytic domains that are approx. 40 % identical with each other, but contain distinct non-catalytic domains, and participate in diverse signalling pathways. AGC kinases are also activated by different mechanisms. For example, agonists that stimulate adenylate cyclase induce the activation of PKA [1], whereas those that stimulate PI3K (phosphoinositide 3-kinase) induce the activation of PKB, S6K and certain isoforms of PKC [2–4]. RSK isoforms are activated by the MAPK (mitogen-activated protein kinase) family members ERK1 (extracellular-signal-regulated kinase 1) and ERK2 in response to growth factors, phorbol esters and other agonists [5,6], whereas MSK isoforms are activated *in vivo* by two different MAPK family members, namely ERK1/ERK2 and the stress and cytokine-activated p38 MAP kinase [7]. RSK and MSK isoforms are unusual in that they possess two catalytic domains in a single polypeptide. The N-

terminal kinase domain is an AGC family member and catalyses the phosphorylation of all known substrates of these enzymes. The C-terminal kinase domain, which does not belong to the AGC family, is required for the activation of the N-terminal kinase domain (reviewed in [8]).

In order to study the physiological roles of AGC kinases, a commonly used approach has been to over-express the active forms in cells. However, due to the overlapping substrate specificities of many AGC kinases, it is likely that the over-expression of one member of this kinase subfamily will result in the phosphorylation of substrates that are normally phosphorylated by another AGC kinase. Another strategy has been to over-express catalytically inactive ‘dominant negative’ mutants of AGC kinases in cells. However, such mutants are likely to interact with and inhibit the upstream protein kinase(s) that they are activated by, and thus prevent the ‘upstream’ kinase(s) from phosphorylation of other cellular substrates. For example, a dominant negative RSK may interact with ERK1/ERK2 preventing the activation of MSK isoforms and hence the phosphorylation of CREB (cAMP-response-element-binding protein) [9]. Furthermore, in *Saccharomyces cerevisiae*, over-expression of catalytically inactive Rck2p, a kinase that binds to and is activated by the Hog1P MAPK, sequestered the substrate-docking site of the Hog1P kinase, thereby preventing Hog1P from interacting with other substrates

Abbreviations used: AGC, cAMP-dependent protein kinase/protein kinase G/protein kinase C; CDK2–A, cyclin-dependent kinase 2 complexed with cyclin A; CK1, casein kinase-1; CREB, cAMP-response-element-binding protein; DYRK1a, dual-specificity tyrosine-phosphorylated and -regulated kinase 1a; EGF, epidermal growth factor; ERK1, extracellular-signal-regulated kinase 1; FBS, foetal bovine serum; FKHR, forkhead in rhabdosarcoma transcription factor; GSK3 α , glycogen synthase kinase 3 α ; GST, glutathione S-transferase; HEK-293, human embryonic kidney 293; JNK, c-Jun N-terminal kinase; Lck, lymphocyte kinase; MAPK, mitogen-activated protein kinase; MKK1, MAPK kinase-1; MES, mouse embryonic stem; MSK, mitogen- and stress-activated protein kinase; PDK1, phosphoinositide-dependent kinase 1; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PLK1, polo-like kinase 1; PKA, cAMP-dependent protein kinase A; PKB, protein kinase B; PKC, protein kinase C; ROCK, Rho-dependent protein kinase; RSK, p90 ribosomal S6 kinase; S6K, p70 ribosomal S6 kinase; SGK1, serum- and glucocorticoid-induced kinase-1.

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[10]. Thus catalytically inactive Rck2P is acting as a dominant negative mutant of Hog1P and not Rck2P.

A few small cell-permeant molecules have been developed that inhibit the activation of AGC kinase members. For example, several compounds that prevent the activation of MKK-1 (MAPK kinase-1) have been described, which potentially inhibit the activation of ERK1/ERK2 and hence the activation of RSK and MSK isoforms [11,12]. Inhibitors of PI3K have been employed to inhibit the activation of PKB and S6K [13], whereas rapamycin, a drug that inhibits the protein kinase mTOR (mammalian target of rapamycin), prevents the activation of S6K [14]. Although these compounds have been valuable in providing evidence for or against a role of some AGC kinases in the regulation of particular cellular processes, the development of compounds that inhibit individual AGC kinase members specifically would be a major advance. However, thus far, mainly rather non-specific AGC kinase inhibitors have been deployed for this purpose. For example, the use of Ro318220 has been described in over 500 publications to inhibit conventional PKC isoforms, but it also inhibits RSK and S6K with similar potency [15], as well as other kinases that are not AGC-family members [16]. H89, a compound originally identified as a PKA inhibitor, also inhibits MSK1, S6K and ROCK (Rho-dependent protein kinase) with a similar IC_{50} value to PKA [16,17]. Here, we demonstrate that BI-D1870 is a remarkably specific inhibitor of RSK isoforms, with a >500-fold greater selectivity over nine other AGC kinases tested. We also demonstrate that BI-D1870 inhibits RSK activity relatively specifically in cells.

MATERIALS AND METHODS

Materials

BI-D1870 was synthesized as a racemate at Boehringer Ingelheim Pharma GmbH & Co. PD 184352 was obtained by custom synthesis, LY 294002 was from Calbiochem, EGF (epidermal growth factor) and microcystin-LR were from Life Technologies Inc (Paisley, Scotland, U.K.), FBS (foetal bovine serum) and other tissue culture reagents were from BioWhittaker, Complete™ protease-inhibitor cocktail tablets were from Roche. Forskolin, PMA, DMSO, antimycotic/antibiotic solution and dimethyl pimelimidate were from Sigma, while the precast 4–12 % Bis/Tris gradient SDS/PAGE gels were from Invitrogen. GST (glutathione S-transferase)–ERK2 was expressed in *Escherichia coli* and activated with MKK1 [18] and His₆–PDK1 (phosphoinositide-dependent kinase 1) was expressed in *Drosophila* Sf21 cells [19]. Apart from RSK3 (#14–462) and RSK4 (#14–702) that were purchased from Upstate, the protein kinases employed in Table 1 were generated in the Division of Signal Transduction Therapy, University of Dundee. The protocols utilised to assay the 54 protein kinases listed in Table 1 are described in the Supplementary section (see <http://www.BiochemJ.org/bj/401/bj4010029add.htm>).

Buffers

The following buffers were used: Lysis Buffer [50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 % (w/v) Triton-X 100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 μ M microcystin-LR, 0.1 % 2-mercaptoethanol and Complete™ proteinase inhibitor cocktail (one tablet per 25 ml)], Buffer A (50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA and 0.1 % 2-mercaptoethanol), Buffer B (50 mM Tris/HCl, pH 7.5, and 0.1 mM EGTA) and SDS-sample buffer [50 mM Tris/HCl, pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol and 1 % (v/v) 2-mercaptoethanol].

Table 1 Effect of BI-D1870 on 54 protein kinases

Results are presented as percentage of kinase activity relative to control assays (without BI-D1870). Protein kinases were assayed as described in the Supplementary section (at <http://www.BiochemJ.org/bj/401/bj4010029add.htm>), in the absence or presence of the indicated amount of BI-D1870. As ATP concentrations can affect the apparent IC_{50} value of ATP-competitive inhibitors, assays were undertaken at the approximate K_m concentration of ATP, namely 5 μ M for p38 δ , PKB α , GSK3 β , p38 γ , CK2, ERK8, MKK1, PIM2, IKK β , PRK2, MARK3, EF2K and PLK1; 20 μ M for JNK1, PRAK, ROCK-II, P38 β 2, CDK2, CHK1, CHK2, MSK1, CSK, S6K1, PKA, CK1, MAPKAP-K2, SGK1, PKC α , PDK1, NEK7, MAPKAP-K3, Aurora B and MST2; and 50 μ M for p38 α , Lck, AMPK, ERK2, DYRK1a, PHK, RSK1, RSK2, NEK6, NEK2a, CaMK-1, PKD1, SRPK-1, JNK3, MNK1, MNK2, Src, PKB β and smMLCK. Results are means $\pm$ S.D. of triplicates. Abbreviations not defined in main text: AMPK, AMP-activated protein kinase; CaMK-1, Ca²⁺/calmodulin-dependent kinase 1; CHK1, cell cycle checkpoint kinase-1; CSK, C-terminal Src kinase; EF2K, elongation factor 2 kinase; MARK3, microtubule affinity regulating kinase 3; M-K2, MAPK-activated protein kinase-2; MNK, MAPK interacting kinase; MST2, mammalian sterile 20-like 2; NEK, NIMA-related protein kinase; PHK, phosphorylase kinase; PKD1, protein kinase D1; PRAK, p38-regulated/activated protein kinase; PRK2, PKC-related kinase-2; smMLCK, smooth-muscle light-chain kinase; SRPK1, serine/arginine protein kinase 1. Kinase families are defined as in [42].

Protein kinase	Kinase family	Remaining kinase activity (%)		
		BI-D1870		
		10 μ M	0.1 μ M	10 nM
RSK1	AGC	3 $\pm$ 0	1 $\pm$ 1	15 $\pm$ 3
RSK2	AGC	2 $\pm$ 1	2 $\pm$ 0	32 $\pm$ 3
RSK3	AGC	1 $\pm$ 1	13 $\pm$ 2	65 $\pm$ 6
RSK4	AGC	1 $\pm$ 1	12 $\pm$ 3	55 $\pm$ 5
MSK1	AGC	71 $\pm$ 7	87 $\pm$ 2	95 $\pm$ 5
PKB α	AGC	95 $\pm$ 8	116 $\pm$ 12	110 $\pm$ 5
PKB β	AGC	95 $\pm$ 7	97 $\pm$ 1	100 $\pm$ 4
S6K1	AGC	54 $\pm$ 2	68 $\pm$ 1	83 $\pm$ 4
SGK1	AGC	67 $\pm$ 11	83 $\pm$ 4	99 $\pm$ 3
PKC α	AGC	37 $\pm$ 6	42 $\pm$ 2	103 $\pm$ 5
PKA	AGC	66 $\pm$ 6	94 $\pm$ 2	91 $\pm$ 4
PDK1	AGC	79 $\pm$ 7	86 $\pm$ 1	99 $\pm$ 4
ROCKII	AGC	70 $\pm$ 4	80 $\pm$ 0	104 $\pm$ 2
PRK2	AGC	68 $\pm$ 6	72 $\pm$ 1	80 $\pm$ 6
MKK1	STE	49 $\pm$ 1	76 $\pm$ 2	103 $\pm$ 4
MST2	STE	13 $\pm$ 1	49 $\pm$ 4	65 $\pm$ 8
JNK1	CMGC	83 $\pm$ 9	87 $\pm$ 6	98 $\pm$ 5
JNK3	CMGC	72 $\pm$ 1	76 $\pm$ 0	95 $\pm$ 5
p38 α	CMGC	77 $\pm$ 11	86 $\pm$ 6	89 $\pm$ 3
p38 β 2	CMGC	91 $\pm$ 9	86 $\pm$ 2	90 $\pm$ 0
p38 γ	CMGC	89 $\pm$ 4	93 $\pm$ 5	99 $\pm$ 9
p38 δ	CMGC	89 $\pm$ 0	86 $\pm$ 6	89 $\pm$ 1
ERK2	CMGC	74 $\pm$ 0	86 $\pm$ 1	96 $\pm$ 2
ERK8	CMGC	54 $\pm$ 8	110 $\pm$ 4	94 $\pm$ 1
GSK3 β	CMGC	21 $\pm$ 3	29 $\pm$ 3	84 $\pm$ 0
CDK2–A	CMGC	30 $\pm$ 7	38 $\pm$ 10	89 $\pm$ 5
DYRK1a	CMGC	46 $\pm$ 6	57 $\pm$ 5	91 $\pm$ 3
SRPK1	CMGC	80 $\pm$ 10	87 $\pm$ 3	98 $\pm$ 2
M-K2	CaMK	90 $\pm$ 8	98 $\pm$ 10	93 $\pm$ 6
M-K3	CaMK	92 $\pm$ 4	99 $\pm$ 1	58 $\pm$ 5
CaMK-1	CaMK	60 $\pm$ 9	67 $\pm$ 4	94 $\pm$ 5
smMLCK	CaMK	46 $\pm$ 6	67 $\pm$ 1	106 $\pm$ 4
MARK3	CaMK	37 $\pm$ 2	39 $\pm$ 4	101 $\pm$ 1
PKD1	CaMK	53 $\pm$ 5	49 $\pm$ 3	90 $\pm$ 6
AMPK	CaMK	68 $\pm$ 2	70 $\pm$ 2	101 $\pm$ 3
CHK1	CaMK	74 $\pm$ 7	88 $\pm$ 9	99 $\pm$ 8
CHK2	CaMK	65 $\pm$ 2	79 $\pm$ 2	97 $\pm$ 9
PHK	CaMK	100 $\pm$ 5	104 $\pm$ 2	77 $\pm$ 2
MNK1	CaMK	76 $\pm$ 0	80 $\pm$ 0	98 $\pm$ 6
MNK2	CaMK	86 $\pm$ 4	92 $\pm$ 1	122 $\pm$ 4
PRAK	CaMK	74 $\pm$ 5	74 $\pm$ 5	85 $\pm$ 8
PIM2	CaMK	61 $\pm$ 4	71 $\pm$ 0	99 $\pm$ 5
CK1	CK1	32 $\pm$ 0	51 $\pm$ 3	94 $\pm$ 0
CK2	CK1	100 $\pm$ 3	95 $\pm$ 0	101 $\pm$ 3
EF2K	atypical	96 $\pm$ 1	101 $\pm$ 0	108 $\pm$ 3
NEK2a	NEK	81 $\pm$ 0	87 $\pm$ 0	94 $\pm$ 5
NEK6	NEK	92 $\pm$ 1	100 $\pm$ 1	89 $\pm$ 4
NEK7	NEK	76 $\pm$ 4	83 $\pm$ 12	107 $\pm$ 9
IKK β	IKK	75 $\pm$ 4	83 $\pm$ 6	95 $\pm$ 5
Aurora B	Aurora	5 $\pm$ 0	42 $\pm$ 4	54 $\pm$ 6
PLK1	PLK	15 $\pm$ 2	17 $\pm$ 1	33 $\pm$ 1
Lck	TK	35 $\pm$ 2	45 $\pm$ 1	90 $\pm$ 3
CSK	TK	81 $\pm$ 12	92 $\pm$ 7	91 $\pm$ 2
Src	TK	60 $\pm$ 4	69 $\pm$ 0	98 $\pm$ 2

Expression, purification and activation of RSK1 and RSK2

cDNA clones encoding rat *RSK1* were provided kindly by J. Avruch (Department of Molecular Biology, Massachusetts General Hospital, MA, U.S.A.) and the human *RSK2* cDNA was described previously [20]. The rat *RSK1* sequence contained an HA-tag positioned N-terminal to the initiating methionine residue and differed from the Genbank entry M99169, at three residues E551G, S637N and G697A. The human *RSK2* sequence differed from the Genbank entry NM_004586 at one residue, V45G. In order to generate transfer vectors for baculovirus production, the *RSK1* cDNA was cloned into the EcoRI site of pFastBAC-Hta vector (Life Technologies Inc), the resulting construct encodes *RSK1* with an N-terminal His₆-tag. The *RSK2* cDNA was cloned into the BamHI site of a modified pFastBAC1 vector resulting in the residues MAHHHHHHGS being added N-terminally to Pro² of the native sequence. The resulting constructs were then used to generate recombinant baculovirus using the Bac-to-Bac[®] system (Life Technologies Inc) following the manufacturer's protocol. The resulting baculovirus were used to infect Sf21 cells (1.5×10^6 /ml) at a multiplicity of infection of 5. The infected cells were harvested 72 h post infection and His₆-tagged protein purified as described previously for His₆-PDK1 [19]. *RSK1* and *RSK2* were purified with yields of approx. 10 mg/l of infected cells, and were greater than 85 % homogeneous as judged by densitometric scanning of Coomassie Blue-stained SDS/PAGE gels. The purified *RSK1* and *RSK2* isolated in this manner possessed a low basal activity of approx. 5 units/mg when assayed using Crosstide as a substrate [7], but could be activated by ERK2 to a specific activity of >300 units/mg [21]. In order to activate the purified *RSK1* and *RSK2*, a reaction was set up (2.0 ml total volume) in Buffer A containing *RSK1* or *RSK2* (0.2 mg/ml), 5 units/ml active GST-ERK2 and 3 µg/ml His₆-PDK1, 10 mM magnesium acetate and 0.1 mM ATP. Following incubation for 1 h at 30 °C, the specific activity of the *RSK1* and *RSK2* enzymes had increased to 150 and 370 units/mg for *RSK1* and *RSK2* respectively. The reaction was terminated by adding EDTA to a concentration of 20 mM, and passed through a 0.5 ml glutathione-agarose column, equilibrated with Buffer A to remove GST-ERK2. To the eluate from this column, which contained *RSK1* and *RSK2*, NaCl was added to a final concentration of 0.4 M and then the solution was passed over a 0.5 ml heparin-Sepharose column equilibrated in Buffer A containing 0.4 M NaCl to remove PDK1, which interacts with heparin-Sepharose under these conditions [22]. The eluate containing activated *RSK1* and *RSK2* was dialysed into 50 mM Tris/HCl (pH 7.5), 270 mM sucrose, 150 mM NaCl, 0.1 mM EGTA, 0.1 % 2-mercaptoethanol, 0.03 % Brij-35, 1 mM benzamidine and 0.2 mM PMSF, and aliquots were snap frozen in liquid nitrogen and stored at -80 °C prior to use.

The GST-*RSK2*^{1-389:S381E} was expressed in *E. coli* and purified using glutathione-Sepharose as described previously [6]. In order to activate this fragment of *RSK2*, a reaction was set up (1.0 ml total volume) in Buffer A containing 0.4 mg/ml GST-*RSK2*^{1-389:S381E}, 50 µg/ml His₆-PDK1, 10 mM magnesium acetate and 0.1 mM ATP. Following incubation for 1 h at 30 °C the specific activity of the GST-*RSK2*^{1-389:S381E} was 54 units/mg. The reaction was terminated by addition of EDTA to 20 mM, and then 0.27 M sucrose was added, and aliquots of the activated GST-*RSK2*^{1-389:S381E} were snap frozen in liquid nitrogen and stored at -80 °C prior to use.

Antibodies

Antibodies recognizing LKB1 phosphorylated at Ser⁴³¹ were raised in sheep [23]. The antibodies that we raised previously against mouse LKB1 protein [23] did not immunoprecipitate hu-

man LKB1 and we therefore raised a further antibody against human LKB1 expressed in *E. coli* with an N-terminal GST-tag, using a cDNA construct provided generously to us by Dr Nicoletta Resta (Medical Genetics Unit, Department of Biomedicine of Evolutive Age, University of Bari, Bari, Italy). The antibodies were affinity purified on CH-Sepharose coupled covalently to human GST-LKB1 and passed through a column of CH-Sepharose coupled to GST to remove anti-GST antibodies. The antibody that did not bind was selected. This antibody immunoprecipitates human LKB1 efficiently and was used to immunoprecipitate LKB1 from HEK-293 (human embryonic kidney 293) cells. Antisera recognizing the phosphorylated forms of ribosomal S6 protein (Ser²³⁵; available from Upstate) were raised in sheep using the peptide A²²⁹KRRRLSpSLRAST²⁴², where Sp indicates phospho-serine. The antibody was affinity purified on CH-Sepharose that had been coupled covalently to the phosphorylated peptide, then passed over a column of CH-Sepharose coupled to the non-phosphorylated peptide. The flow-through fractions were collected.

The antibody used to immunoprecipitate and immunoblot RSK isoforms, was raised against the peptide RNQSPVLEPVGRS-TLAQRRGIKK (corresponding to residues 712 to 734 of human *RSK2*), whilst the antibody used to immunoprecipitate *MSK1* was raised against the peptide F³⁸⁴KRNAVIDPLQFHM⁴⁰²VER. The following antibodies were from Cell Signalling Technology with the catalogue numbers indicated in parentheses: antibodies for immunoblotting ERK1/ERK2 (#9102), ribosomal S6 protein (#2212s) and CREB (#9192), as well as the phospho-specific antibodies recognising the phosphorylated forms of ERK1/ERK2 (#9106S) and GSK3α (glycogen synthase kinase 3α)/GSK3β (#9331S). The monoclonal antibody recognising the PH (pleckstrin homology) domain of PKB (#05-591), as well as the phospho-specific antibody recognising CREB phosphorylated at Ser¹³³ (#06-519) and the phospho-specific antibody recognising the FKHR (forkhead in rhabdomyosarcoma transcription factor) phosphorylated at Thr²⁴ (#07-162), were from Upstate Inc. In order to immunoblot GSK3α and GSK3β, we combined antibodies raised against the C-terminal residues of rat GSK3α (Q⁴⁷¹APDATP-TLTNSS⁴⁸³) and an antibody raised against the GSK3β protein. The antibody used to immunoprecipitate FKHR has been described previously [24]. Secondary antibodies coupled to horseradish peroxidase for use in immunoblotting were from Pierce.

Protein kinase assays

Purified His₆-*RSK1*, His₆-*RSK2* or GST-*RSK2*^{1-389:S381E} (1–2 units/ml) were assayed for 10 min at 30 °C in a 50 µl assay mixture in Buffer A containing 30 µM substrate peptide (KEAKEKRQEIQIAKRRRLSSLRASTSKSGGSQK), 10 mM magnesium acetate and 100 µM of [γ -³²P]ATP. Reactions were terminated and analysed as described previously [25]. The amount of enzyme that catalysed the phosphorylation of 1 nmol of substrate peptide in 1 min was termed one unit.

In order to assay RSK and *MSK1* in HEK-293 or Rat-2 cell lysates, these kinases were immunoprecipitated from the cell lysates (0.1 mg of lysate protein for RSK and 0.3 mg for *MSK1*) and assayed as described previously [23], except that for RSK assays the immunoprecipitates were washed twice with Buffer A containing 1 mM ATP and twice with Buffer A prior to the assay, as a precaution to ensure dissociation of BI-D1870 from the RSK isoforms.

Cell culture, stimulation and cell lysis

The rat embryo fibroblast cell line, Rat-2, was obtained from the European Collection of Cell Cultures. Rat-2 cells were cultured on

Figure 1 Effect of BI-D1870 on RSK activity *in vitro*

(A) The ATP-competitive kinase inhibitor, BI-D1870, derived from a novel chemical series (WO 03/020722), potentially inhibits RSK1/2. Activated His₆-RSK1 (B) or His₆-RSK2 (C) were assayed in the absence or presence of the indicated concentration of BI-D1870 with 100 μ M ATP (●) or 10 μ M ATP (○). The results are presented as percentage kinase activity relative to the control measured in the absence of BI-D1870. (D) As in (B) and (C), except that an active fragment of RSK comprising the isolated N-terminal catalytic domain (RSK^{1-389:S381E}) activated by phosphorylation with PDK1, as described in the Materials and methods section, was assayed in the absence or presence of BI-D1870 with 100 μ M ATP. Results are presented as the average for two experiments with each determination carried out in triplicate. The error for each data point is less than 5%.

10 cm-diameter dishes in Dulbecco's Modified Eagle's medium supplemented with 10% (v/v) FBS. HEK-293 cells were cultured on 10 cm-diameter dishes in Dulbecco's Modified Eagle's medium supplemented with 10% FBS and 1 $\times$ antimycotic/antibiotic solution. Prior to stimulation, cells were cultured in the absence of serum for 16 h. Inhibitors were dissolved in DMSO at a 1000-fold higher concentration than they were used at. These inhibitors, or the equivalent volume of DMSO as a control, were added to the tissue culture medium 30 min prior to stimulation, unless indicated otherwise. The final concentration of DMSO in the culture medium was 0.1% and had no effect on agonist-induced activation or phosphorylation of any of the substrates examined. The cells were stimulated with the indicated agonists and lysed in 1 ml of ice-cold Lysis Buffer, and centrifuged at 16000 *g* at 4°C for 5 min. The supernatants were frozen in liquid nitrogen and stored at -80°C until use. Protein concentrations were determined using the Bradford method with BSA as the standard.

Immunoprecipitation of LKB1 and FKHR

Polyclonal antibodies (1 mg) raised against human LKB1 or mouse LKB1 or FKHR were coupled covalently to protein G-Sepharose (1 ml) using dimethyl pimelimidate [26]. Rat-2 (0.5 mg) or HEK-293 (1 mg) cell lysate proteins were incubated for 60 min at 4°C with the anti-LKB1- or anti-FKHR-Protein G-Sepharose conjugates (5 μ l). The immunoprecipitates were washed twice with 1 ml of Lysis Buffer containing 0.5 M NaCl, and washed twice with Buffer B. For immunoblot analysis, the beads were resuspended in SDS-Sample Buffer that did not contain 2-mercaptoethanol.

Immunoblotting

For blots of total cell lysates, 20 μ g of lysate protein was used. All samples were subjected to SDS/PAGE and transferred on to

nitrocellulose. For experiments in which LKB1, RSK2, FKHR, GSK3 α /GSK3 β isoforms and the phospho-ribosomal protein S6 and phospho-LKB1 were immunoblotted, the membranes were incubated in 50 mM Tris/HCl (pH 7.5), 0.15 M NaCl, 0.5% Tween 20 and 10% (w/v) dried skimmed milk for 7 h at 4°C in the presence of 1 μ g/ml antibody. Prior to immunoblotting with the phospho-Ser⁴³¹ LKB1-specific antibody or the phospho-ribosomal protein S6 antibody, the antibodies were incubated with the non-phosphorylated forms of the peptide antigen (10 μ g/ml) used to raise the antibodies. All the other antibodies obtained commercially were used a 1000-fold dilution of the stock and 5% (w/v) BSA was used in place of dried skimmed milk as the blocking agent. Proteins were detected using horseradish peroxidase-conjugated secondary antibodies and ECL[®] reagent (Amersham Pharmacia Biotech).

RESULTS

BI-D1870 is a potent inhibitor of RSK isoforms

BI-D1870, derived from a novel series of dihydropteridinones (Figure 1A), was identified in a kinase selectivity screen performed in the Division of Signal Transduction Therapy in the University of Dundee, as a potent inhibitor of RSK1. We found that BI-D1870 inhibited RSK1 and RSK2 with IC₅₀ values of 10 nM (Figure 1B) and 20 nM (Figure 1C) respectively, when the kinase assays were performed with 100 μ M ATP. When the assays were performed at a 10-fold lower ATP concentration, the IC₅₀ of BI-D1870 was reduced to 5 nM for RSK1 and 10 nM for RSK2. These observations indicate that BI-D1870 is a potent ATP-competitive inhibitor of RSK isoforms. An active mutant of RSK2 lacking the C-terminal kinase catalytic domain (RSK2^{1-389:S381E} [20]), was inhibited by BI-D1870, with an IC₅₀ of approx. 30 nM (Figure 1D), indicating that BI-D1870 inhibits the N-terminal

Table 2 IC_{50} values of D1870 for kinases inhibited by BI-D1870

The indicated kinases were assayed in the presence of concentrations of BI-D1870 ranging from 1 nM to 100 μ M and IC_{50} values were extrapolated from this data. RSK isoforms were all assayed with 100 μ M ATP, as the K_m for ATP for RSK3 and RSK4 have not been determined. Other kinases were assayed at the approximate K_m concentration of ATP. Protein kinases were assayed as described in the Supplementary section (at <http://www.BiochemJ.org/bj/401/bj4010029add.htm>) and abbreviations are defined in the legend to Table 1. The results are the means $\pm$ S.E.M. of two separate experiments with each assay condition performed in triplicate.

Protein kinase	Kinase family	ATP concentration in assay (μ M)	IC_{50} (μ M)
RSK1	AGC	100	0.031 ± 0.005
RSK2	AGC	100	0.024 ± 0.005
RSK3	AGC	100	0.018 ± 0.003
RSK4	AGC	100	0.015 ± 0.007
MST2	STE	20	0.86 ± 0.028
GSK3 β	CMGC	5	1.59 ± 0.021
MARK3	CaMK	5	2.20 ± 0.247
CK1	CK1	20	0.45 ± 0.042
Aurora B	Aurora	20	0.34 ± 0.141
PLK1	PLK	50	0.10 ± 0.029

AGC kinase domain. BI-D1870 also inhibited RSK3 and RSK4 with similar potency to RSK1 and RSK2 (Tables 1 and 2).

BI-D1870 is a specific inhibitor of RSK isoforms

In order to investigate the specificity of BI-D1870, we studied the effect of 10 μ M, 0.1 μ M and 0.01 μ M BI-D1870 at ATP concentrations which approximate the K_m constant for ATP, towards a panel of 54 protein kinases which included 14 AGC kinase members [RSK1, RSK2, RSK3, RSK4, MSK1, PKB α , PKB β , S6K1, SGK1 (serum and glucocorticoid-induced kinase-1), PKC α , PDK1, PKA, PRK2 (PKC-related kinase 2) and ROCK-II], as well as three tyrosine kinases (Table 1). At 0.1 μ M BI-D1870, a concentration that inhibited the four RSK isoforms by over 98 %, most enzymes on the panel were not affected significantly, apart from PLK1 (polo-like kinase 1) whose activity was reduced by 83 %, and Aurora B, DYRK1a (dual-specificity tyrosine-phosphorylated and -regulated kinase 1a), CDK2-A (cyclin-dependent kinase 2 complexed with cyclin A), Lck (lymphocyte kinase), CK1 (casein kinase-1) and GSK3 β , whose activities were reduced by 40–70 % (Table 1). PLK1 was inhibited by BI-D1870 with an IC_{50} of 100 nM, whilst the IC_{50} values for Aurora B, DYRK1a, CDK2-A, Lck, CK1 and GSK3 β were 10- to 100-fold higher than that of the RSK isoforms (Table 2). Importantly, the activity of MSK1, the kinase most closely related to RSK, was not inhibited significantly by 0.1 μ M BI-D1870.

Evidence that BI-D1870 inhibits RSK activity specifically in HEK-293 cells

To examine whether BI-D1870 could inhibit RSK activity in cells, we investigated the effect of BI-D1870 on the RSK-catalysed phosphorylation of its known substrates in HEK-293 cells, using PMA as an agonist to activate ERK1/ERK2 and RSK isoforms. Cells were incubated with 10 μ M BI-D1870, for periods of 15 min for a total of 4 h prior to stimulation with PMA, the phosphorylation of GSK3 α at Ser²¹ and GSK3 β at Ser⁹, which is mediated by RSK under these conditions [27], was investigated. These experiments demonstrated that incubation of cells with BI-D1870 greatly inhibited the PMA-induced phosphorylation of GSK3 α and GSK3 β (Figure 2A). In contrast, BI-D1870 had little effect at any time point on the PMA-induced activation of ERK1/ERK2,

Figure 2 BI-D1870 inhibits RSK activity *in vivo*

(A) HEK-293 cells that had been deprived of serum for 16 h, were incubated in the absence or presence of 10 μ M BI-D1870 for the indicated times, then stimulated with 400 ng/ml PMA (TPA) for 20 min. The cells were lysed and the lysates were immunoblotted with the indicated antibodies as described in the Materials and methods section. (B) As in (A), except that cells were incubated for 30 min with the indicated concentrations of BI-D1870 prior to stimulation with PMA. Similar results were obtained in two separate experiments. Phosphorylation of GSK3 isoforms was quantitated by densitometric analysis of autorads.

which is catalysed by Raf and MKK1, or the phosphorylation of CREB at Ser¹³³, which is largely catalysed by MSK1 and MSK2 isoforms in fibroblasts [9]. We next studied the effect of varying the concentration of BI-D1870 on the PMA-induced phosphorylation of GSK3 isoforms and another RSK substrate, the protein kinase LKB1 (which is phosphorylated by RSK at Ser⁴³¹ [23]) in HEK-293 cells. These experiments showed that the phosphorylation of both proteins was inhibited in HEK-293 cells with an IC_{50} of approx. 1 μ M (Figure 2B).

Stimulation of HEK-293 cells with PMA also activates S6K, whose principal cellular substrate is thought to be the ribosomal protein S6 [2]. Incubation of cells for up to 4 h with 10 μ M BI-D1870 did not affect the PMA-induced phosphorylation of the

Figure 3 Effect of BI-D1870 on activation of RSK and MSK1

(A) HEK-293 cells that had been deprived of serum for 16 h, were incubated in the absence or presence of 10 μ M BI-D1870 or 2 μ M PD 184352 as indicated. After 30 min the cells were stimulated with PMA (TPA) for 20 min, then the cells were lysed and the RSK isoforms immunoprecipitated with an antibody that recognized both RSK1 and RSK2. The immunoprecipitates were washed to remove BI-D1870 and assayed in the absence of BI-D1870. (B) As in (A) except that the cell lysates were immunoblotted with the indicated antibodies, as described in the Materials and methods section. (C) As in (A), except that MSK1 was immunoprecipitated from the lysates and assayed. In (A) and (C) the results are presented as means $\pm$ S.E.M. for two separate experiments with each determination carried out in triplicate.

ribosomal S6 protein (Figure 2), indicating that BI-D1870 does not inhibit the activation or activity of S6K in cells. In HEK-293 cells, PMA also induced a weak activation of p38 MAPK, which was also unaffected by BI-D1870 (Figure 2B).

In order to test whether BI-D1870 affected the activation of RSK isoforms, HEK-293 cells were stimulated with PMA in the absence or presence of BI-D1870. RSK1 and RSK2 isoforms were immunoprecipitated with an antibody that recognizes both forms of RSK, the immunoprecipitates were washed and assayed for activity in the absence of BI-D1870. Treatment of the cells with BI-D1870 did not markedly affect the large PMA-induced activation of ERK1/ERK2, RSK isoforms or the phosphorylation of RSK at Ser³⁸¹ and Thr⁵⁷⁴, two phosphorylation sites required for the activation of RSK [5] (Figures 3A and 3B). BI-D1870 also did not affect the PMA-induced activation of MSK1 in HEK-293 cells (Figure 3C).

We also compared the effectiveness of BI-D1870 to inhibit the phosphorylation of GSK3 isoforms with that of PD 184352, a

Figure 4 Effect of BI-D1870 on EGF-stimulated Rat-2 cells

Serum-deprived Rat-2 cells were incubated for 30 min in the presence of the indicated concentrations of BI-D1870. The cells were then stimulated with 100 ng/ml EGF for 10 min, lysed and immunoblotted with the indicated antibodies.

potent inhibitor of the activation of MKK1 [12] (Figures 3A and 3B). BI-D1870 (10 μ M) inhibited the PMA-induced phosphorylation of GSK3 isoforms to the same extent as PD 184352 (2 μ M). As expected, and in contrast with BI-D1870, PD 184352 also prevented the phosphorylation of ERK1/ERK2 and the phosphorylation and activation of RSK and MSK isoforms as well as the phosphorylation of CREB.

Further evidence that BI-D1870 is a specific RSK inhibitor

We next assessed the effectiveness of BI-D1870 as an inhibitor of RSK activity in EGF-stimulated Rat-2 cells. As observed in HEK-293 cells, BI-D1870 inhibited the EGF-induced phosphorylation of LKB1 at Ser⁴³¹ with an IC₅₀ of approx. 1 μ M. Furthermore, BI-D1870 did not affect the activation of ERK1/ERK2 and MSK1, nor did it inhibit the phosphorylation of CREB (Figure 4).

In contrast with PMA, EGF not only activates the isoforms of RSK, MSK, S6K [23], but also triggers the activation of PKB as judged by using phospho-specific antibodies that recognize PKB phosphorylated at Thr³⁰⁸ and Ser⁴⁷³ (Figure 4). Since PKB phosphorylates GSK3 α and GSK3 β at the same residues as RSK *in vivo* (reviewed in [27]), it would be expected that, as shown previously in Swiss 3T3 cells [28], blockade of PKB activation as well as blockade of RSK activity would be needed to prevent the EGF-induced phosphorylation of GSK3 isoforms. Consistent with this notion, treatment of Rat-2 cells with BI-D1870 alone or with the PI3K inhibitor LY294002 alone did not affect the EGF-induced phosphorylation of GSK3 isoforms, but when added to Rat-2 cells together, the EGF-induced phosphorylation of GSK3 α or GSK3 β was blocked completely (Figure 4 and Figure 5). Similarly, addition of both PD 184352 and LY 294002, to inhibit the activation of RSK and PKB respectively, prevented EGF-induced phosphorylation of GSK3, whereas either inhibitor alone or a combination of PD 184352 with BI-D1870 did not (Figure 5). As expected, LY 294002 inhibited the activation of PKB and the phosphorylation of two of its substrates, FKHR and FKHL1, which are not phosphorylated by RSK [29]. BI-D1870 did not affect the activation of PKB or the phosphorylation of the FKHR isoforms in EGF-stimulated cells (Figure 5), indicating that this compound does not affect the PKB pathway.

Figure 5 Effect of BI-D1870, PD 184352 and LY294002 on signalling pathways in Rat-2 cells

Serum-deprived Rat-2 cells were incubated for 30 min in the absence or presence of 10 μ M BI-D1870, 2 μ M PD 184352 or 100 μ M LY 294002 as indicated. The cells were then stimulated with 100 ng/ml EGF for 10 min, lysed and the cell lysates were immunoblotted with indicated antibodies as described in the Materials and methods section. Similar results were obtained in two separate experiments.

BI-D1870 does not inhibit PKA activity *in vivo*

PKA also phosphorylates GSK3 α and GSK3 β at Ser²¹ and Ser⁹ [30], and LKB1 at Ser⁴³¹ [23,31], in the presence of agonists that elevate cAMP. In order to verify that BI-D1870 does not inhibit the activity of PKA *in vivo*, we stimulated HEK-293 and Rat-2 cells with forskolin, an activator of adenylate cyclase, which elevates the level of cAMP and hence the activity of PKA in the presence or absence of 10 μ M BI-D1870. We then measured the phosphorylation of GSK3 α , GSK3 β and LKB1 at the residues that are phosphorylated by PKA. BI-D1870 (10 μ M) did not inhibit the forskolin-induced phosphorylation of the GSK3 isoforms or LKB1 significantly (Figure 6).

BI-D1870 induces the activation of ERK1/ERK2 in Rat-2 cells, but not in HEK-293 cells

Interestingly, the incubation of Rat-2 cells with BI-D1870 in the absence of EGF induced a time- (Figure 7A) and dose-dependent (Figure 7B) phosphorylation of ERK1/ERK2. ERK1/ERK2 phosphorylation was induced 5 min after treatment of cells with 10 μ M BI-D1870, with maximal activation observed between 20 and 80 min, and a slow decline thereafter (Figure 7A). Incubation of Rat-2 cells with 10 μ M BI-D1870 for 30 min resulted in the phosphorylation of ERK1 and ERK2 to approx. 50 % of the level

Figure 6 BI-D1870 does not inhibit PKA *in vivo*

Serum-deprived Rat-2 cells or HEK-293 cells were incubated for 30 min in the absence or presence of 10 μ M BI-D1870. The cells were either left unstimulated or stimulated with 20 μ M forskolin for 10 min. The cells were lysed and the lysates immunoblotted with the indicated antibodies. Similar results were obtained in two separate experiments.

Figure 7 BI-D1870 activates ERKs in Rat-2 cells

(A) Serum-deprived Rat-2 cells were incubated for the indicated times in the presence of 10 μ M BI-D1870 in the absence of EGF. The cells were lysed and the lysates immunoblotted with the indicated antibodies. (B) As in (A), except that cells were incubated with the indicated concentrations of BI-D1870 for 30 min and then either left unstimulated or stimulated with 100 ng/ml EGF for 10 min. For the immunoblotting studies similar results were obtained in two separate experiments.

that is observed following stimulation with EGF (Figure 7B). Consistent with a significant activation of ERK and hence MSK1, BI-D1870 induced the phosphorylation of CREB at Ser¹³³. In contrast, 10 μ M BI-D1870 did not stimulate a detectable increase in ERK phosphorylation in HEK-293 cells (Figure 2B). These results are considered further in the Discussion.

DISCUSSION

The results in the present study indicate that BI-D1870 is a highly specific cell-permeant inhibitor of RSK, which acts as an ATP-competitive inhibitor of the N-terminal AGC domain of RSK (Figure 1). BI-D1870 is cell-permeant because at 10 μ M it inhibits the phosphorylation of three known RSK substrates (GSK3 α , GSK3 β and LKB1) in response to agonists that induce the activation of RSK. BI-D1870 is stable in tissue culture medium as it inhibited the phosphorylation of RSK substrates efficiently even after 4 h, without affecting the PMA-induced phosphorylation of ERK1/ERK2 (Figure 2A). BI-D1870 inhibited all RSK

isoforms, with IC_{50} values of 10–40 nM in the presence of 20–100 μ M ATP (Figure 1 and Tables 1 and 2). However, our *in vivo* results indicate that to inhibit the phosphorylation of RSK substrates completely in cells, a concentration of 10 μ M BI-D1870 is required (Figures 2B and 4A). The requirement for higher compound concentration to suppress the activity of a protein kinase *in vivo* compared with those required *in vitro* is frequently observed with many ATP-competitive inhibitors of protein kinases. One reason is the far higher mM intracellular concentration of ATP in cells compared with the *in vitro* assay. A second reason is that there may be a barrier to the penetration of cells by BI-D1870. A third possibility is that RSK is a relatively abundant and active protein kinase, and inhibiting its activity partially may be insufficient to prevent the phosphorylation of substrates. A fourth possibility is that the concentrations of PMA and EGF employed in this study induced maximal activation of ERK1/ERK2 and RSK isoforms; that is, lower levels of BI-D1870 may inhibit RSK more effectively after stimulation with lower agonist concentrations.

A remarkable feature of BI-D1870 is its very high specificity for RSK isoforms. BI-D1870 inhibited RSK isoforms >500-fold more potently than the ten other related AGC kinases that were tested *in vitro*. We have also verified that BI-D1870 is a highly specific RSK inhibitor *in vivo*, because treatment of cells with 10 μ M BI-D1870 did not significantly inhibit the phosphorylation of the ribosomal S6 protein by S6K, the phosphorylation of GSK3 isoforms or FKHR1/FKHL1 by PKB, the phosphorylation of CREB by MSK1, the phosphorylation of GSK3 isoforms or LKB1 by PKA, the PDK1-mediated activation of S6K1 and PKB α , or the PKC-mediated PMA-induced activation of ERK1/ERK2. It will be important in future work to perform a detailed structural analysis of BI-D1870 bound to RSK to understand the basis for the high specificity of BI-D1870. Understanding how BI-D1870 inhibits RSK activity at the structural level may provide important insights into how specific inhibitors of other AGC kinases could be developed.

It has been reported recently, that an acetylated flavonol glycoside isolated from the tropical plant *Forsteronia refracta* termed SL0101 inhibited RSK2 with an IC_{50} of 90 nM [32]. The selectivity of this compound is uncertain, as it has not yet been tested against a large panel of other kinases, but was reported not to inhibit MSK1, S6K1, PKA and the FAK (focal adhesion kinase) kinase significantly. Employing an elegant structure-based design strategy, Taunton et al. [33] have synthesized an inhibitor, fmk, which interacts covalently with and modifies covalently the C-terminal kinase domain of RSK isoforms. In cell extracts, fmk formed a specific covalent interaction with RSK isoforms and also inhibited the phosphorylation of histone H3 at Ser¹⁰ in cells that over-express RSK2. However, the specificity of fmk towards a panel of protein kinases was not assessed [33] and whether it is really a specific inhibitor of RSK has yet to be rigorously evaluated.

As discussed in the Introduction, it was originally shown that a dominant negative mutant of RSK inhibited the growth factor-induced phosphorylation of the transcription factor CREB at Ser¹³³ and that the over-expression of RSK in cells induced the phosphorylation of CREB at this residue [34]. This was originally interpreted to indicate that RSK was catalysing the phosphorylation and, hence, the activation of CREB. However, the finding that CREB was a vastly more efficient substrate for MSK1 than RSK *in vitro* [7] and that the compound H89, at concentrations that inhibit MSK isoforms but not RSK, prevents growth-factor-induced CREB phosphorylation [23,35], indicated that MSK1, rather than RSK, may mediate the phosphorylation of CREB in cells. The finding that the growth factor and phorbol ester induced phosphorylation of CREB was greatly reduced, whereas

phosphorylation of GSK3 or LKB1 [23] mediated by RSK was unaffected in MES (mouse embryonic stem) cells lacking MSK1 [36] and mouse embryonic fibroblast cells lacking both MSK1 and MSK2 [9], strongly support the conclusion that MSK1/MSK2 mediate the phosphorylation of CREB. Interestingly, trace residual growth-factor-induced phosphorylation of CREB that occurs in the fibroblast cell lines lacking MSK1 and MSK2 is not prevented by 10 μ M BI-D1870, indicating that this is not catalysed by RSK isoforms [9]. The finding in the present study, that the RSK inhibitor BI-D1870 does not inhibit the phosphorylation of CREB detectably in cells, but completely inhibits the phosphorylation of GSK3 isoforms and LKB1 (Figures 2 and 4), provides further evidence that RSK is not rate limiting for the phosphorylation of CREB in the cells, under the conditions that we have examined. Moreover, the observation that addition of BI-D1870 to Rat-2 cells (in the absence of any other agonist) stimulates ERK1/ERK2 phosphorylation, resulting in the activation of MSK1 and CREB phosphorylation, but not the phosphorylation of the GSK3 isoforms (Figure 7), also strongly supports the conclusion that MSK1/MSK2 (rather than RSK isoforms) mediates the phosphorylation of CREB.

The observation that BI-D1870 alone significantly activated the ERK1/ERK2 pathway in Rat-2 cells (Figure 7) indicates that RSK activity controls a negative-feedback loop that regulates ERK1/ERK2 activation in certain cell types. This conclusion is also supported by the finding that, in MES cells lacking PDK1 and in which RSK is therefore inactive, ERK1/ERK2 phosphorylation in unstimulated cells is elevated significantly [37]. It is therefore possible that in unstimulated Rat-2 cells, basal levels of RSK activity suppress the activity of a protein that stimulates the ERK1/ERK2 pathway. The inhibition of RSK that occurs when cells are incubated with BI-D1870 may switch off this negative-feedback loop. Douville and Downward [38] reported that in PC12 cells, RSK phosphorylates and inactivates mSOS, the guanine nucleotide exchange factor for Ras. More recently RSK has been shown to inhibit ERK/MAPK signalling during *Drosophila* development, although the precise mechanism by which RSK inhibited ERK in this system was not defined [39]. The role of RSK in regulating the activity of ERK1/ERK2 is likely to be cell specific, as the addition of 10 μ M BI-D1870 to unstimulated HEK-293 cells did not induce detectable activation of ERK1/ERK2, p38/MAPK (Figure 2B) or JNK (c-Jun N-terminal kinase; results not shown). RSK is thought to play an important role in stimulating cell growth and promoting cell survival [8,40] and is therefore a potential target for an anti-cancer drug. However, the observation that inhibition of RSK in some cells promotes the activation of ERK1/ERK2, which might therefore stimulate rather than inhibit cell growth, is potentially an adverse side effect. Furthermore, the discovery that mutation of the RSK2 gene in humans results in Coffin–Lowry syndrome, characterized by mental retardation, facial dysmorphisms and skeletal deformations [41], also indicates that drugs that inhibit RSK isoforms could have undesired effects.

RSK has been suggested to phosphorylate a large number of cellular substrates and to play an important role in promoting cell growth and survival (reviewed in [8,40]). However, the evidence for an involvement of RSK in regulating these processes in many cases is based largely on the over-expression of constitutively active and dominant negative RSK mutants in cells, which, as discussed above, can lead to erroneous conclusions. The activation of RSK also requires its phosphorylation at one site by PDK1. Thus a dominant negative mutant of RSK could also interact with PDK1, preventing PDK1 from activating a number of other AGC kinase members. Small cell-permeant inhibitors of RSK, such as BI-D1870, should not suffer from these problems and have

the potential to facilitate the investigation of the cellular roles of RSK in the same way that inhibitors of MKK-1 activation (PD 98059, U0126 and PD 184352) and the p38 MAPK inhibitor (SB 203580) have enabled many roles of particular MAPK pathways to be defined. In order to determine whether a substrate is phosphorylated by RSK in cells, it will be necessary in the future to demonstrate that it is inhibited by BI-D1870, as well as by inhibitors of the activation of MKK-1. Furthermore, the phosphorylation of the substrate should not occur in cells that lack PDK1, which also lack RSK activity [37]. Thus far, the only RSK substrates that have met all of these criteria are GSK3 α , GSK3 β and LKB1. It will be important to deploy BI-D1870 to re-evaluate the role that RSK plays in mediating phosphorylation of the myriad of other substrates [8,40] that have been suggested for this protein kinase.

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REFERENCES

- Scott, J. D. (1991) Cyclic nucleotide-dependent protein kinases. *Pharmacol. Therapeut.* **50**, 123–145
- Avruch, J., Belham, C., Weng, Q., Hara, K. and Yonezawa, K. (2001) The p70 S6 kinase integrates nutrient and growth signals to control translational capacity. *Prog. Mol. Subcell. Biol.* **26**, 115–154
- Parekh, D. B., Ziegler, W. and Parker, P. J. (2000) Multiple pathways control protein kinase C phosphorylation. *EMBO J.* **19**, 496–503
- Vanhaesebroeck, B. and Alessi, D. R. (2000) The PI3K-PDK1 connection: more than just a road to PKB. *Biochem. J.* **346**, 561–576
- Dalby, K. N., Morrice, N., Caudwell, F. B., Avruch, J. and Cohen, P. (1998) Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90^{sk} that are inducible by MAPK. *J. Biol. Chem.* **273**, 1496–1505
- Frodin, M., Jensen, C. J., Merienne, K. and Gammeltoft, S. (2000) A phosphoserine-regulated docking site in the protein kinase RSK2 that recruits and activates PDK1. *EMBO J.* **19**, 2924–2934
- Deak, M., Clifton, A. D., Lucocq, L. M. and Alessi, D. R. (1998) Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *EMBO J.* **17**, 4426–4441
- Hauge, C. and Frodin, M. (2006) RSK and MSK in MAP kinase signalling. *J. Cell Sci.* **119**, 3021–3023
- Wiggin, G. R., Soloaga, A., Foster, J. M., Murray-Tait, V., Cohen, P. and Arthur, J. S. (2002) MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts. *Mol. Cell. Biol.* **22**, 2871–2881
- Jiang, L., Niu, S., Clines, K. L., Burke, D. J. and Sturgill, T. W. (2004) Analyses of the effects of Rck2p mutants on Pbs2pDD-induced toxicity in *Saccharomyces cerevisiae* identify a MAP kinase docking motif, and unexpected functional inactivation due to acidic substitution of T379. *Mol. Gen. Genet.* **271**, 208–219
- Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) PD 98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.* **270**, 27489–27494
- Sebolt-Leopold, J. S., Dudley, D. T., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R. C., Tecle, H., Barrett, S. D., Bridges, A., Przybranowski, S. et al. (1999) Blockade of the MAP kinase pathway suppresses growth of colon tumors *in vivo*. *Nat. Med.* **5**, 810–816
- Vanhaesebroeck, B., Leever, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J. and Waterfield, M. D. (2001) Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* **70**, 535–602
- Chung, J., Kuo, C. J., Crabtree, G. R. and Blenis, J. (1992) Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* **69**, 1227–1236
- Alessi, D. R. (1997) The protein kinase C inhibitors Ro 318220 and GF 109203X are equally potent inhibitors of MAPKAP kinase-1 β (Rsk-2) and p70 S6 kinase. *FEBS Lett.* **402**, 121–123
- Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95–105
- Thomson, S., Clayton, A. L., Hazzalin, C. A., Rose, S., Barratt, M. J. and Mahadevan, L. C. (1999) The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase. *EMBO J.* **18**, 4779–4793
- Alessi, D. R., Smythe, C. and Keyse, S. M. (1993) The human CL100 gene encodes a Tyr/Thr-protein phosphatase which potently and specifically inactivates MAP kinase and suppresses its activation by oncogenic ras in *Xenopus* oocyte extracts. *Oncogene* **8**, 2015–2020
- Balendran, A., Casamayor, A., Deak, M., Paterson, A., Gaffney, P., Currie, R., Downes, C. P. and Alessi, D. R. (1999) PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. *Curr. Biol.* **9**, 393–404
- Jensen, C. J., Buch, M. B., Krag, T. O., Hemmings, B. A., Gammeltoft, S. and Frodin, M. (1999) 90-kDa ribosomal S6 kinase is phosphorylated and activated by 3-phosphoinositide-dependent protein kinase-1. *J. Biol. Chem.* **274**, 27168–27176
- Sutherland, C., Campbell, D. G. and Cohen, P. (1993) Identification of insulin-stimulated protein kinase-1 as the rabbit equivalent of rskm-2. Identification of two threonines phosphorylated during activation by mitogen-activated protein kinase. *Eur. J. Biochem.* **212**, 581–588
- Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B. and Cohen, P. (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B α . *Curr. Biol.* **7**, 261–269
- Sapkota, G. P., Kieloch, A., Lizcano, J. M., Lain, S., Arthur, J. S., Williams, M. R., Morrice, N., Deak, M. and Alessi, D. R. (2001) Phosphorylation of the protein kinase mutated in Peutz-Jeghers cancer syndrome, LKB1/STK11, at Ser⁴³¹ by p90^{RSK} and cAMP-dependent protein kinase, but not its farnesylation at Cys⁴³³, is essential for LKB1 to suppress cell growth. *J. Biol. Chem.* **276**, 19469–19482
- Woods, Y. L., Rena, G., Morrice, N., Barthel, A., Becker, W., Guo, S., Unterman, T. G. and Cohen, P. (2001) The kinase DYRK1A phosphorylates the transcription factor FKHR at Ser³²⁹ *in vitro*, a novel *in vivo* phosphorylation site. *Biochem. J.* **355**, 597–607
- Alessi, D. R., Cohen, P., Ashworth, A., Cowley, S., Leever, S. J. and Marshall, C. J. (1995) Assay and expression of mitogen-activated protein kinase, MAP kinase kinase, and Raf. *Methods Enzymol.* **255**, 279–290
- Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York
- Frame, S. and Cohen, P. (2001) GSK3 takes centre stage more than 20 years after its discovery. *Biochem. J.* **359**, 1–16
- Shaw, M. and Cohen, P. (1999) Role of protein kinase B and the MAP kinase cascade in mediating the EGF-dependent inhibition of glycogen synthase kinase 3 in Swiss 3T3 cells. *FEBS Lett.* **461**, 120–124
- Rena, G., Guo, S., Cichy, S. C., Unterman, T. G. and Cohen, P. (1999) Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B. *J. Biol. Chem.* **274**, 17179–17183
- Fang, X., Yu, S. X., Lu, Y., Bast, R. C., Jr., Woodgett, J. R. and Mills, G. B. (2000) Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 11960–11965
- Collins, S. P., Reoma, J. L., Gamm, D. M. and Uhler, M. D. (2000) LKB1, a novel serine/threonine protein kinase and potential tumour suppressor, is phosphorylated by cAMP-dependent protein kinase (PKA) and prenylated *in vivo*. *Biochem. J.* **345**, 673–680
- Smith, J. A., Poteet-Smith, C. E., Xu, Y., Errington, T. M., Hecht, S. M. and Lannigan, D. A. (2005) Identification of the first specific inhibitor of p90 ribosomal S6 kinase (RSK) reveals an unexpected role for RSK in cancer cell proliferation. *Cancer Res.* **65**, 1027–1034
- Cohen, M. S., Zhang, C., Shokat, K. M. and Taunton, J. (2005) Structural bioinformatics-based design of selective, irreversible kinase inhibitors. *Science* **308**, 1318–1321
- Xing, J., Ginty, D. D. and Greenberg, M. E. (1996) Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science* **273**, 959–963
- Caivano, M. and Cohen, P. (2000) Role of mitogen-activated protein kinase cascades in mediating lipopolysaccharide-stimulated induction of cyclooxygenase-2 and IL-1 β in RAW264 macrophages. *J. Immunol.* **164**, 3018–3025
- Arthur, J. S. and Cohen, P. (2000) MSK1 is required for CREB phosphorylation in response to mitogens in mouse embryonic stem cells. *FEBS Lett.* **482**, 44–48

- 37 Williams, M. R., Arthur, J. S., Balendran, A., van der Kaay, J., Poli, V., Cohen, P. and Alessi, D. R. (2000) The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr. Biol.* **10**, 439–448
- 38 Douville, E. and Downward, J. (1997) EGF induced SOS phosphorylation in PC12 cells involves P90 RSK-2. *Oncogene* **15**, 373–383
- 39 Kim, M., Lee, J. H., Koh, H., Lee, S. Y., Jang, C., Chung, C. J., Sung, J. H., Blenis, J. and Chung, J. (2006) Inhibition of ERK-MAP kinase signaling by RSK during *Drosophila* development. *EMBO J.* **25**, 3056–3067
- 40 Nebreda, A. R. and Gavin, A. C. (1999) Perspectives: signal transduction. Cell survival demands some Rsk. *Science* **286**, 1309–1310
- 41 Trivier, E., De Cesare, D., Jacquot, S., Pannetier, S., Zackai, E., Young, I., Mandel, J. L., Sassone-Corsi, P. and Hanauer, A. (1996) Mutations in the kinase Rsk-2 associated with Coffin–Lowry syndrome. *Nature* **384**, 567–570
- 42 Manning, G., Whyte, D. B., Martinez, R., Hunter, T. and Sudarsanam, S. (2002) The protein kinase complement of the human genome. *Science* **298**, 1912–1934

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