Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase

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Abstract The substrate specificity of protein kinase-Bα (PKBα, also known as RAC kinase or Akt) was investigated using synthetic peptide substrates related to the sequence surrounding the phosphorylation site on glycogen synthase kinase-3 (GSK3). The minimum sequence motif required for efficient phosphorylation was Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Xaa is any amino acid, Yaa and Zaa are small residues other than glycine and Hyd is a bulky hydrophobic residue (Phe, Leu). The most effective substrate, Arg-Pro-Arg-Thr-Ser-Ser-Phe, was phosphorylated with a K_m of 5 μM and V_{max} of 260 U/mg. PKBα phosphorylated histone H2B (K_m 5 μM, V_{max} 68 U/mg) specifically at Ser-36 which also lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd motif. The peptide Arg-Pro-Arg-Ala-Ala-Thr-Phe may be a relatively specific substrate for PKBα because, unlike other substrates, it is not phosphorylated by p70 S6 kinase or MAP kinase activated protein (MAPKAP) kinase-1.

Key words: Protein kinase B; Akt or RAC kinase; Insulin; PI 3-kinase; Kinase specificity; Kinase substrate; S6 kinase

1 Introduction

Protein kinase Bα (PKBα) [1], also known as RACα [2] and c-Akt [3], is the cellular homologue of v-Akt, a protein encoded in the genome of the AKT-8 acute transforming retrovirus isolated from a rodent T-cell lymphoma [4]. Two other isoforms of PKB, termed PKBβ [5] and PKBγ [6], have been identified. PKBβ, also known as RACβ and Akt-2, is overexpressed in a significant number of ovarian [5] and pancreatic [7] cancers, while PKBα is overexpressed in the breast cancer epithelial cell line MCF7 [2]. All forms of PKB possess an N-terminal pleckstrin homology (PH) domain, followed by a catalytic domain and a short C-terminal tail. The catalytic domain is most similar to cyclic AMP-dependent protein kinase (PKA, 65% similarity) and to protein kinase C (PKC, ~5% similarity), findings that gave rise to two of its names, namely PKB (i.e. between PKA and PKC) and RAC (**R**elated to **A** and **C** kinase).

PKBα is activated within 1 min following stimulation of cells with insulin [8,9]. The activation of PKBα by insulin or IGF-1 is accompanied by its phosphorylation at Thr-308 and Ser-473, and phosphorylation of both of these residues is required to achieve a high level of activity [10]. Like the activation of PKBα [11,12], the phosphorylation of Thr-308 and Ser-473 is prevented by wortmannin, an inhibitor of phosphoinositide (PI) 3-kinase [10].

The only physiological substrate for PKBα to have been identified to date is the protein kinase glycogen synthase kinase-3 (GSK3). GSK3 is inhibited in response to insulin with a half-time of 2 min, slightly slower than that for activation of PKBα (1 min) and inhibition results from its phosphorylation at the same serine residue (Ser-21 in GSK3α and Ser-9 in GSK3β) which is targetted by PKBα in vitro. Like the activation of PKBα, the inhibition of GSK3 by insulin is prevented by the PI 3-kinase inhibitors wortmannin and LY 294002.

GSK3α and GSK3β are phosphorylated at Ser-21 and Ser-9, respectively, by two other insulin-stimulated protein kinases, namely p70 S6 kinase and MAP kinase-activated protein kinase-1 (MAPKAP-K1, also known as p90 S6 kinase). However, these enzymes are not rate-limiting for the inhibition of GSK3 by insulin in L6 myotubes because specific inhibitors of their activation (rapamycin-p70 S6 kinase; PD 98059-MAPKAP kinase-1) have no effect [9]. The inhibition of GSK3 is thought to contribute to the stimulation of glycogen synthesis [9] and protein synthesis [13] by insulin.

The activation of PI 3-kinase is essential for many of the effects of insulin and growth factors, including the stimulation of glucose transport, fatty acid synthesis and DNA synthesis, protection of cells against apoptosis and actin cytoskeletal rearrangements (reviewed in [14]). These observations raise the question of whether PKBα mediates any of these events by phosphorylating other proteins. To address this issue we have characterised the substrate specificity requirements of PKBα. We find that the optimal consensus sequence for phosphorylation by PKBα is the motif Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Yaa and Zaa are small amino acids and Hyd is a large hydrophobic residue (Phe, Leu). We also demonstrate that PKBα phosphorylates histone H2B (a substrate frequently used to assay PKBα in vitro) at Ser-36 which lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd motif. These studies identified a further PKBα substrate (Arg-Pro-Arg-Ala-Ala-Thr-Phe) that, unlike other peptides, is not phosphorylated to a significant extent by either p70 S6 kinase or MAPKAP-K1.

2. Materials and methods

2.1. Materials

All peptides used to assay PKBα, and TTYADFIASGRTGRRNAIHD (the specific peptide inhibitor of cyclic AMP dependent pro-

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Abbreviations: PKB, protein kinase B; PI 3-kinase, phosphonositide 3-kinase; MAPKAP-kinase-1, MAP kinase activated protein kinase-1; PH, pleckstrin homology; HA, haemagglutinin; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C

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tein kinase – PKI) were synthesised at Dundee on an Applied Biosystems 431A peptide synthesiser. Their purity (>95%) was established by high-performance liquid chromatography and electrospray mass spectrometry, and their concentrations were determined by quantitative amino acid analysis. Reagents for tissue culture, myelin basic protein (MBP) and IGF-1 were purchased from Life Technologies (Paisley, UK), and monoclonal antibodies recognising the haemagglutinin (HA) epitope sequence YPYDVPDYA, Protein G-Sepharose and histone H2B from Boehringer (Lewes, UK). MAPKAP kinase-1β [15] and p70 S6 kinases [16] were purified from rabbit skeletal muscle and rat liver, respectively, by Dr. Nick Morrice and Sue Dale at the MRC Protein Phosphorylation Unit.

2.2. Preparation and assay of PKBα

The construction of cytomegalovirus vectors (pCMV5) of the human haemagglutinin epitope-tagged wild type (HA-PKBα) was described previously [10]. 293 cells grown on 10-cm dishes were transfected with a DNA construct expressing HA-PKBα using a modified calcium phosphate procedure [10]. The cells were deprived of serum for 16 h prior to lysis and, where indicated, were stimulated for 10 min in the presence of 50 ng/ml IGF-1 to activate PKBα. The cells were lysed in 1.0 ml ice-cold buffer A (50 mM Tris-HCl pH 7.5, 1 mM EDTA 1 mM EGTA, 1% (by vol.) Triton X-100, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1 μM Microcystin-LR, 0.27 M sucrose, 1 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, and 0.1% (by vol.) 2-mercaptoethanol), the lysate centrifuged at 4°C for 10 min at 13 000×*g* and the supernatant obtained from one 10 cm dish of cells (2–3 mg protein) was incubated for 60 min on a shaking platform with 20 μl of protein G-Sepharose coupled to 10 μg of HA monoclonal antibody. The suspension was centrifuged for 1 min at 13 000×*g*, the Protein G-Sepharose-antibody-HA-PKBα complex washed twice with 1.0 ml of buffer A containing 0.5 M NaCl, and twice with buffer B (50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 0.01% (by vol.) Brij 35 and 0.1% (by vol.) 2-mercaptoethanol). The PKBα immunoprecipitates were diluted in buffer B to an activity of 2.0 U/ml towards the peptide GRPRTSSFAEG, termed Crosstide [9] and 0.1 ml aliquots snap frozen in liquid nitrogen and stored at −80°C. No significant loss of PKBα activity occurred upon thawing the PKBα immunoprecipitates or during storage at −80°C for up to 3 months. The standard PKBα assay (50 μl) contained: 50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 0.1% (by vol.) 2-mercaptoethanol, 2.5 μM PKI, 0.2 U/ml PKBα, Crosstide (30 μM), 10 mM magnesium acetate and 0.1 mM [γ-^{32}P]ATP (100–200 cpm/pmol). The assays were carried out for 15 min at 30°C, the assay tubes being agitated continuously to keep the immunoprecipitate in suspension, then terminated and analysed as described [17]. One unit of activity was that amount of enzyme which catalysed the phosphorylation of 1 nmol of Crosstide in 1 min. The phosphorylation of other peptides, histone H2B and MBP was carried out in an identical manner. All the Crosstide kinase activity in HA-PKBα immunoprecipitates is catalysed by PKBα (see Section 3) and the PKBα concentration in the immunoprecipitates was estimated by densitometric scanning of Coomassie blue-stained polyacrylamide gels, using bovine serum albumin as a standard. Protein concentrations were determined by the method of Bradford using bovine serum albumin as standard [18]. Michaelis constants (K_m) and V_{max} values were determined from double-reciprocal plots of $1/V$ against $1/S$, where V is the initial rate of phosphorylation, and S is the substrate concentration. The standard errors for all reported kinetic constants were within < ±20% (S.E.M.), and the data are reported as mean values for 3 independent determinations.

2.3. Tryptic digestion of histone 2B phosphorylated by PKBα

Histone H2B (30 μM) was phosphorylated with 0.2 U/ml HA-PKBα. After 60 min, 0.2 vol. of 100% (by mass) trichloroacetic acid was added, and the sample incubated for 1 h on ice. The suspension was centrifuged for 10 min at 13 000×*g*, the supernatant discarded and the pellet washed five times with 0.2 ml of ice-cold acetone. The pellet was resuspended in 0.3 ml of 50 mM Tris-HCl pH 8.0, 0.1% (by vol.) reduced Triton X-100 containing 2 μg of alkylated trypsin and, after incubation for 16 h at 30°C, the digest was centrifuged for 5 min at 13 000×*g*. The supernatant, containing 95% of the ^{32}P radioactivity, was chromatographed on a Vydac C18 column as described in the legend to Fig. 2.

3. Results

3.1. Preparation of protein kinase Bα

In order to examine the substrate specificity of PKBα, it was first necessary to obtain a kinase preparation that was not contaminated with any other protein kinase activities. 293 cells were therefore transiently transfected with a DNA construct expressing haemagglutinin-tagged PKBα (HA-PKBα), stimulated with IGF-1 and the HA-PKBα immunoprecipitated from the lysates (see Section 2). IGF-1 stimulation resulted in 38-fold activation of PKBα (Fig. 1) and analysis of the immunoprecipitates by SDS-polyacrylamide gel electrophoresis revealed that the 60 kDa PKBα was the major protein staining with Coomassie Blue apart from the heavy and light chains of the haemagglutinin monoclonal antibody (Fig. 1, lanes 2,3). The minor contaminants were present in control immunoprecipitates derived from 293 cells transfected with an empty pCMV5 vector but lacked HA-PKB activity (Fig. 1, lane 4). Furthermore, a catalytically inactive mutant HA-PKBα immunoprecipitated from the lysates of IGF-1-stimulated 293 cells had no Crosstide kinase activity [10]. Thus, all the Crosstide kinase activity in HA-PKB immunoprecipitates is catalysed by PKBα.

Table 1
Molecular basis for the substrate specificity of PKBα

A	Peptides	K_m (μM)	V_{max} (U/mg)	V (0.1 mM)
1	GRPRTS**S**FAEG	4	260	100
2	RPRTS**S**FA	8	305	109
3	GRPRTS**S**F	8	385	129
4	RPRTS**S**F	5	260	105
5	RPRTS**T**F	30	243	78
6	RPRTS**A**F	–	0	
7	PRTS**S**F	–	0	
8	RPRTS**S**	>500	ND	2
9	KPRTS**S**F	>500	ND	4
10	RPKTS**S**F	>500	ND	2
B				
1	RPRTS**S**F	5	260	105
2	RPRTS**S**L	8	278	104
3	RPRTS**S**V	21	300	102
4	RPRTS**S**A	250	265	30
5	RPRTS**S**K	80	308	67
6	RPRTS**S**E	>500	ND	9
7	RPRTS**S**PA[a]	–	0	
C				
1	RPRTS**S**F	5	260	105
2	RPRA**S**F	12	230	89
3	RPRV**S**F	25	273	77
4	RPRG**S**F	60	163	37
5	RPRN**S**F	>500	ND	21
6	RPRTA**S**F	20	213	83
7	RPRTG**S**F	25	233	77
8	RPRTV**S**F	30	365	89
9	RPRTN**S**F	30	300	81
10	RPRAA**S**F	25	215	77
11	RPRGG**S**F	105	345	55
12	RPRGA**S**F	105	160	37
13	RPRAG**S**F	49	114	70

The phosphorylated residue is shown in bold-face type; the altered residue is underlined. V (0.1 mM) is the relative rate of phosphorylation at 0.1 mM peptide relative to peptide 1. ND, not determined.
[a]An alanine residue was added to the C-terminal of the peptide RPRTSSP, since we have experienced difficulty in synthesising peptides terminating in proline.

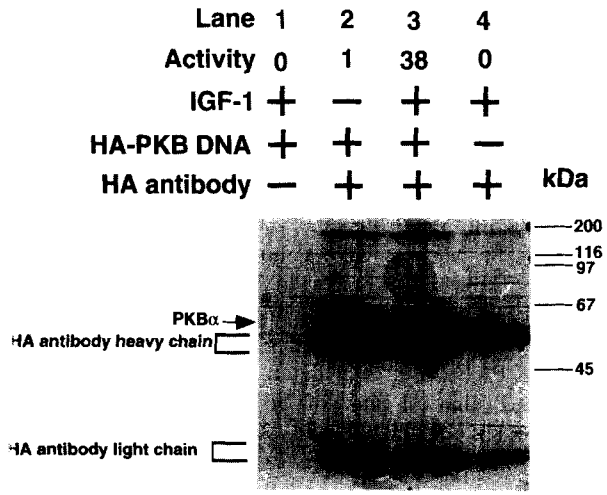

Fig. 1. Characterisation of PKBα. 293 cells grown on 10 cm dishes were transiently transfected with either the pCMV5 DNA constructs expressing wild-type PKBα or the empty pCMV5 cloning vector. After stimulating the cells for 10 min with 50 ng/ml IGF-1 or buffer as a control, the cells were lysed and immunoprecipitations carried out from each dish using the HA monoclonal antibody (Section 2.2). Aliquots of each immunoprecipitate (2% of the sample) were used for each assay. The activities are reported relative to those obtained for PKBα derived from unstimulated cells. The remainder of the immunoprecipitate was applied to a polyacrylamide gel, which was then stained with Coomassie blue. The positions of the molecular mass markers myosin (200 kDa), β-galactosidase (115 kDa), glycogen phosphorylase (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (43 kDa) are marked.

3.2. Identification of the residues in histone H2B phosphorylated by PKBα

Currently, three substrates are used to assay PKBα activity in different laboratories, histone H2B, MBP and Crosstide. The last mentioned is the synthetic peptide GRPRTSSFAEG very closely related to residues 3–13 of GSK3β, the serine underlined (Ser-9) being the residue phosphorylated by PKBα [9]. PKBα phosphorylated Crosstide with a K_m of 4 μM and a V_{max} of 260 U/mg (Table 1A, peptide 1), histone H2B with a K_m of 5 μM and a V_{max} of 68 U/mg, and MBP with a K_m of 5 μM and a V_{max} of 25 U/mg. Thus, the V_{max} values of histone H2B and MBP are 4- and 10-fold lower than for Crosstide, respectively. In order to identify the residue(s) in histone H2B phosphorylated by PKBα, ^{32}P-labelled histone H2B was digested with trypsin (see Section 2) and the resulting peptides chromatographed on a C_{18} column at pH 1.9. Only one major ^{32}P-labelled peptide (termed T1) eluting at 19.5% acetonitrile was observed (Fig. 2A). The peptide contained phosphoserine (data not shown), its sequence commenced at residue 34 of histone H2B and a single burst of radioactivity occurred after the third cycle of Edman degradation (Fig. 2B), demonstrating that PKBα phosphorylates histone H2B at Ser-36 within the sequence Arg-Ser-Arg-Lys-Glu-Ser-Tyr. Thus, like the serine phosphorylated in Crosstide, Ser-36 of histone H2B lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd motif (where Hyd is a bulky hydrophobic residue -Phe in Crosstide, Tyr in H2B).

3.3. Molecular basis for the substrate specificity of PKBα

To characterise further the substrate specificity requirements for PKBα, we first determined the minimum sequence phosphorylated efficiently by PKBα by removing residues sequentially from the C-terminal and N-terminal end of Crosstide. Removal of the N-terminal glycine and up to three residues from the C-terminus had little effect on the kinetics of phosphorylation by PKBα (Table 1A, compare peptides 1 and 4). However, any further truncation of either the N- or C-terminus virtually abolished phosphorylation (Table 1A, peptides 7 and 8). The minimum peptide phosphorylated efficiently by PKBα (Arg-Pro-Arg-Thr-Ser-Ser-Phe) was found to be phosphorylated exclusively at the second serine residue as expected (data not shown). Consistent with this finding, a peptide in which this serine was changed to alanine was not phosphorylated by PKBα (Table 1A, peptide 6). All further studies were therefore carried out using variants of peptide 4 in Table 1A (see below).

A peptide in which the second serine of peptide 4 (Table 1A) was replaced by threonine was phosphorylated with a K_m of 30 μM and an unchanged V_{max} (Table 1A, peptide 5). All the ^{32}P radioactivity incorporated was present as phosphothreonine and solid-phase sequencing revealed that the peptide was only phosphorylated at the second threonine residue, as expected (data not shown). Thus, PKBα is capable of phosphorylating threonine as well as serine residues, but has a preference for serine.

We next changed either of the two arginine residues in peptide 4 in Table 1A to lysine. These substitutions drastically decreased the rate of phosphorylation by PKBα (Table 1A,

Table 2
Comparison of the substrate specificities of PKBα, MAPKAP kinase-1, and p70 S6 kinase

	Peptide	Protein kinase Bα		MAPKAP kinase-1		p70 S6 kinase	
		K_m (μM)	V_{max} (U/mg)	K_m (μM)	V_{max} (U/mg)	K_m (μM)	V_{max} (U/mg)
A							
1	KKKNRTLSVA	185	270	0.2[a]	1 550[a]	3.3[a]	890[a]
2	KKRNRTLSVA	80	300	0.7[a]	1 800[a]	1.5[a]	1 520[a]
3	KKRNKTLSVA	> 500	ND	17[a]	840[a]	34[a]	760[a]
4	KKRNRTLTV	388	330	40[a]	270[a]	4.8[a]	1 470[a]
B							
1	GRPRTSSFAEG	4	250	2	790	3	1 270
2	RPRTSSF	5	260	12	840	125	705
3	RPRTSTF	30	240	> 500	ND	211	590
4	RPRAASF	25	215	20	1 020	> 500	ND
5	RPRAATF	25	230	> 500	ND	> 500	ND

Peptides 1 and 2 are very good substrates for MAPKAP kinase-1 and p70 S6 kinase, and peptide 3 is a relatively specific substrate for p70 S6 kinase [16].
[a]Data reported previously [16]; ND, not determined.

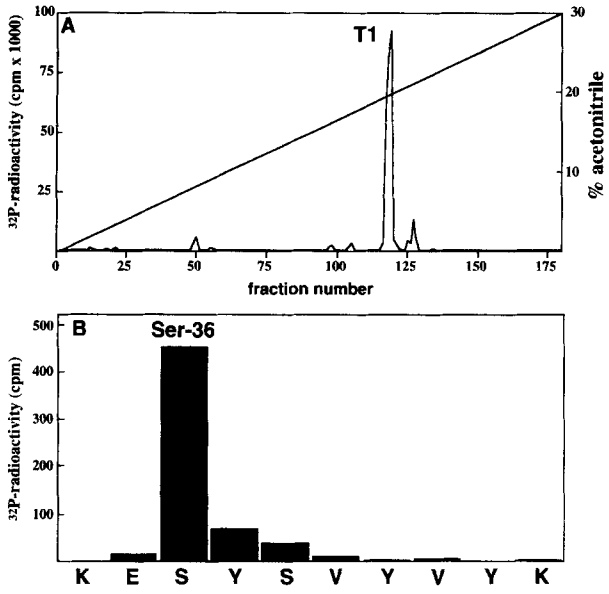

Fig. 2. PKBα phosphorylates Ser-36 of histone 2B. Histone 2B that had been phosphorylated with PKBα using [γ-^{32}P]ATP (Section 2.3) was digested with trypsin and chromatographed on a Vydac 218TP54 C18 column (Separations Group, Hesperia, CA) equilibrated with 0.1% (by vol.) trifluoroacetic acid (TFA) in water. The column was developed with a linear acetonitrile gradient (diagonal line) at a flow rate of 0.8 ml/min and fractions of 0.4 ml were collected. (A) Tryptic peptide map of ^{32}P-labelled histone H2B. 70% of the radioactivity applied to the column was recovered from the major ^{32}P-peptide eluting at 19.5% acetonitrile. (B) A portion of the major ^{32}P-peptide (50 pmol) was analysed on an Applied Biosystems 476A sequencer, and the phenylthiohydantoin (Pth) amino acids identified after each cycle of Edman degradation are shown using the single-letter code for amino acids. A portion of the major ^{32}P-peptide (1000 cpm) was then coupled covalently to a Sequelon arylamine membrane and analysed on an Applied Biosystems 470A sequencer using the modified programme described in [23]. ^{32}P radioactivity was measured after each cycle of Edman degradation.

peptides 9 and 10) demonstrating a strict requirement for arginine (and not simply any basic residue) at both positions.

We then examined the effect of substituting the residues situated immediately C-terminal to the phosphorylated serine in peptide 4 (Table 1B). The data clearly demonstrate that the presence of a large hydrophobic residue at this position is critical for efficient phosphorylation, with the K_m increasing progressively with decreasing hydrophobicity of the residue at this position (Table 1B, peptides 1–4). Replacement of the C-terminal residue with Lys increased the K_m 18-fold and a substitution at this position with either Glu or Pro almost abolished phosphorylation (Table 1B, peptides 5–7).

Replacement of the Thr situated two residues N-terminal to the phosphorylated serine increased the K_m with any amino acid tested (Table 1C). Substitution with Ala only increased K_m by 2–3-fold, but substitution with other residues was more deleterious and with Asn (a residue of similar size and hydrophilicity to Thr) phosphorylation was almost abolished (Table 1C). Replacement of the Ser situated one residue N-terminal to the phosphorylated serine also increased the K_m with any amino acid tested, but the effects were less severe than at position $n-2$ (Table 1C). When residues $n-2$ and $n-1$ were both changed to Ala, the resulting peptide RPRAASF was phosphorylated by PKBα with a K_m only 5-fold higher than that of RPRTSSF. In contrast, the peptides RPRGGSF, RPRAGSF, and RPRGASF were phosphorylated less efficiently (Table 1C).

3.4. Comparison of the substrate specificity of PKBα with MAPKAP kinase-1, and p70 S6 kinase

Since MAPKAP-K1 and p70 S6 kinase phosphorylate the same residue in GSK3 phosphorylated by PKBα, and studies with synthetic peptides have established that MAPKAP-K1 and p70 S6 kinase also preferentially phosphorylate peptides in which basic residues are present at positions $n-3$ and $n-5$ [16], we compared the specificities of MAPKAP-K1, p70 S6 kinase and PKBα in greater detail.

MAPKAP kinase-1 and p70 S6 kinase phosphorylate the peptides KKKNRTLSVA and KKRNRTLSVA with extremely low K_m values of 0.2–3.3 μM, respectively (Table 2). However, these peptides were phosphorylated by PKBα with 50–900-fold higher K_m values (Table 2A, peptides 1 and 2). The peptide KKRNRTLTV, which is a relatively specific substrate for p70 S6 kinase [16] was also phosphorylated very poorly by PKBα (Table 2A, peptide 4).

Crosstide is phosphorylated by p70 S6 kinase and MAPKAP kinase-1 with similar efficiency to PKBα ([16]; Table 2B, peptide 1). However, truncation of Crosstide to generate the peptide RPRTSSF was deleterious for phosphorylation by MAPKAP-K1 and even worse for p70 S6 kinase (Table 2B, peptides 1 and 2). Moreover, changing the phosphorylated serine in RPRTSSF to threonine increased the K_m for phosphorylation by p70 S6 kinase much more than for PKBα and almost abolished phosphorylation by MAPKAP-K1 (Table 2B, peptide 3). The peptide RPRAASF was phosphorylated by MAPKAP-K1 with essentially identical kinetics to that of PKBα; however, phosphorylation by p70 S6 kinase was virtually abolished (Table 2B, peptide 4). Based on these observations we synthesized the peptide RPRAATF. This peptide was phosphorylated by PKBα with a K_m of 25 μM and similar V_{max} to RPRTSSF, but was not phosphorylated to a significant extent by either MAPKAP-K1 or p70 S6 kinase (Table 2B, peptide 5).

4. Discussion

Many protein kinases phosphorylate serine and threonine residues that lie in particular amino acid sequence motifs. For example, PKA phosphorylates serine residues that lie in Arg-Arg-Xaa-Ser sequences, while casein kinase-2 phosphorylates serine or threonine residues that are followed by clusters of acidic residues [19]. In this paper, we establish that the minimum consensus sequence for efficient phosphorylation by PKBα is Arg-Xaa-Arg-Yaa-Zaa-Ser-Hyd, where Xaa is any amino acid, Yaa and Zaa are small amino acid other than glycine (Ser, Thr, Ala) and Hyd is a bulky hydrophobic residue (Phe, Leu) (Table 1). The heptapeptide with the lowest K_m value was RPRTSSF, its K_m of 5 μM being comparable to many of the best peptide substrates identified for other protein kinases. The V_{max} for this peptide (260 nmol min^{-1} mg^{-1}) may be an underestimate because the PKBα was obtained by immunoprecipitation from extracts of IGF-1-stimulated 293 cells overexpressing this protein kinase, and a significant proportion of the PKBα may not have been activated by IGF-1 treatment.

The requirement for arginine residues at positions $n-3$ and

$n-5$ (where n is the site of phosphorylation) is very stringent, because substituting either residue with lysine decreases phosphorylation drastically. Serine and threonine residues were preferred at positions $n-1$ and $n-2$, although the K_m value was only increased about 5-fold when both of these residues were changed to Ala. Serine was preferred at position n, since changing it to threonine caused a 6-fold increase in the K_m. The peptide RPRAATF, which was phosphorylated with a K_m of 25 μM and similar V_{max} to RPRTSSF, may therefore be a better substrate for assaying PKBα in partially purified preparations, because unlike Crosstide, it contains only one phosphorylatable residue and is not phosphorylated significantly by MAPKAP-K1 or p70 S6 kinase (Table 2, and see below).

The proline at position $n-4$ was not altered in this study because it was already clear that this residue was not critical for the specificity of PKBα. Residue $n-4$ is proline in GSK3β but alanine in GSK3α. Both GSK3 isoforms are equally good substrates for PKBα in vitro [9], and the peptide GRARTSSFA (corresponding to the sequence in GSK3α) is phosphorylated by PKBα with a K_m of 10 μM and V_{max} of 230 U/mg (data not shown). Moreover, in histone H2B, the residue located four amino acids N-terminal to the PKBα phosphorylation site is serine (Fig. 2). The presence of Glu and Lys at positions $n-1$ and $n-2$ may explain why histone H2B is phosphorylated by PKBα with a 4-fold lower V_{max} than the peptide RPRTSSF.

Two other protein kinases which are activated by insulin and other growth factors, p70 S6 kinase and MAPKAP-K1, require basic residues at positions $n-3$ and $n-5$ [16], explaining why they also phosphorylate and inactivate GSK3 in vitro [10]. Indeed, there is evidence that MAPKAP-K1 plays a role in the inhibition of GSK3 by EGF because, unlike inhibition by insulin which is prevented by inhibitors of PI 3-kinase, the inhibition of GSK3 by EGF is only suppressed partially by inhibitors of PI 3-kinase (D.A.E Cross, Y. Saito and P. Cohen, unpublished work). Moreover, in NIH 3T3 cells, the inhibition of GSK3α and GSK3β by EGF is largely prevented by expression of a dominant negative mutant of MAP kinase kinase-1 [21]. In contrast, p70 S6 kinase is not rate limiting for the inhibition of GSK3 in the cells that have been examined so far because rapamycin, which prevents the activation of p70 S6 kinase by EGF or insulin, has no effect on the inhibition of GSK3 by these agonists [9,22].

Additional similarities between p70 S6 kinase, MAPKAP-K1 and PKBα include the failure to phosphorylate peptides containing Pro at position $n+1$ and dislike of a lysine at the same position. This suggests that, in vivo, these kinases are unlikely to phosphorylate the same residues as MAP kinases (which phosphorylates Ser/Thr-Pro motifs) or protein kinase C (which prefers basic residues C-terminal to the site of phosphorylation). However, the present work has also revealed significant differences in the specificities of these enzymes. In particular, MAPKAP-K1 and (to a lesser extent) p70 S6 kinase can tolerate substitution of the Arg at position $n-5$ by lysine whereas PKBα cannot (see Table 1A, Table 2A and [16]). MAPKAP-K1 and p70 S6 kinase can also tolerate, to some extent, substitution of Arg at position $n-3$ by Lys. For example, the peptide KKRNKTLSVA is phosphorylated by MAPKAP-K1 and p70 S6 kinase with K_m values of 17 and 34 μM, respectively, as compared to K_m values of 0.7 and 1.5 μM for the peptide KKRNRTLSVA (Table 2A). In contrast, PKBα does not phosphorylate the peptide KKRNKTLSVA (Table 2A) or any other peptide that lacks Arg at position $n-3$. PKBα and p70 S6 kinase, but not MAPKAP-K1, phosphorylate Thr as well as Ser (Table 1A) and can phosphorylate peptides lacking any residue at position $n+2$ ([16] and Table 2A), while PKBα and MAPKAP-K1, but not p70 S6 kinase, can tolerate substitution of both the $n-1$ and $n-2$ positions of the peptide RPRTSSF with Ala (Table 2B). These differences explain why the peptide RPRAATF is a relatively specific substrate for PKBα.

One of the best peptide substrates for MAPKAP-K1 and p70 S6 kinase (KKRNRTLSVA) was a very poor substrate for PKBα (Table 2A, peptide 2), despite the presence of Arg at positions $n-3$ and $n-5$. The presence of Leu at position $n-1$ and Val at position $n+1$ are likely to explain the high K_m for phosphorylation, because PKBα prefers a small hydrophilic residue at the former position and a larger hydrophobic residue at the latter position (Tables 1 and 2).

Finally, it is important to emphasise that GSK3 is unlikely to be the only physiological substrate for PKBα. The identification of a consensus sequence for phosphorylation by PKBα reported in this paper should therefore prove useful in pinpointing other physiological targets for this protein kinase which is likely to play important roles in mediating a number of the intracellular actions of insulin, IGF-1 and other growth factors.

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