A non-radioactive method for the assay of many serine/threonine-specific protein kinases

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The generation of drugs that modulate the activities of particular protein kinases has become a prime focus of the pharmaceutical and biotechnology industry. Consequently, improved methods for the development of high-throughput screening formats for these enzymes is a high priority. In the present study, we have designed three generic peptide substrates that can be used to assay a diverse range of protein kinases. These peptides share a common seven-residue epitope that includes the site

of phosphorylation, and against which we have generated a phospho-specific antibody. Thus a large number of serine/threonine-specific protein kinases can be screened using a simple non-radioactive format.

Key words: drug discovery, high-throughput screening, peptide substrate, phospho-specific antibody, protein kinase inhibitor.

INTRODUCTION

The attachment and removal of phosphate from proteins, catalysed by protein kinases and protein phosphatases, regulates nearly all aspects of cell life. In recent years, it has become apparent that abnormal protein phosphorylation is a cause or consequence of many diseases and pathological conditions, such as arthritis, cancer, diabetes, heart disease, hypertension and stroke. For this reason, the development of drugs that modulate the activities of particular protein kinases and phosphatases has become a priority for the pharmaceutical industry [1]. Several compounds that inhibit a protein kinase or a protein phosphatase have been approved for clinical use. Cyclosporin, the immunosuppressant drug that permitted the widespread use of organ transplantation, inhibits a protein phosphatase (protein phosphatase 2B/calcineurin) [2], whereas its successor, rapamycin, inhibits a protein kinase, termed the ‘mammalian target of rapamycin’ [3]. Moreover, Gleevec (STI 571), an inhibitor of the Abl tyrosine kinase, was approved for clinical use by the U.S. Food and Drug Administration in May 2001. This compound has shown impressive efficacy for the treatment of chronic myelogenous leukaemia, a disease in which Abl is converted to a constitutively active form by a chromosomal rearrangement (reviewed in [4]). Gleevec is the first drug to be developed by targeting a specific protein kinase for inhibition, and has heightened interest in this area considerably.

One of the first steps in the drug-discovery process involves screening compound libraries to identify ‘lead’ inhibitors of particular protein kinases. Traditionally, such ‘high-throughput screening’ for protein-kinase inhibitors has involved strategies that measure the effect of compounds on the incorporation of radiolabelled phosphate (³²P and ³³P) into peptide and protein substrates. However, one of the major drawbacks of such

radioactive assay formats is that compound libraries are becoming extremely large (> 1 million entities), as a consequence of advances in combinatorial chemistry and the integration of compound libraries resulting from mergers and acquisitions. Consequently, high levels of radioactivity are required to run these assays, and the industry has increasingly looked to develop alternative non-radioactive assay formats. One potentially attractive non-radioactive assay is to use phospho-specific antibodies that recognize the product of the kinase reaction. In theory, the simplest approach would be to use phospho-specific antibodies that recognize phosphoserine, phosphothreonine or phosphotyrosine. Indeed, such antibodies have been used with great success for the assay of protein tyrosine kinases. However, the development of equivalent assays for serine/threonine-specific protein kinases has been much slower, because it has proved extremely difficult to develop suitable antibodies that recognize phosphoserine or phosphothreonine. It has therefore been necessary to develop separate phospho-specific antibodies for each phosphorylated substrate.

In the present paper, we introduce three synthetic peptide substrates that are excellent substrates for a large number of serine/threonine-specific protein kinases. These peptides share a common seven-residue epitope that includes the site of phosphorylation, and against which we have generated an effective phospho-specific antibody. Thus many protein kinases can be screened using a common format.

EXPERIMENTAL

Chemicals and peptides

Ro 318220, K252c and rottlerin were purchased from Calbiochem–Novabiochem (La Jolla, CA, U.S.A.). Y 27632 was obtained by chemical synthesis, and UCN01 was a gift from Dr

Abbreviations used: AMPK, AMP-activated protein kinase; CaMKII, calmodulin-dependent protein kinase II; CHK, checkpoint kinase; ERK, extracellular signal-regulated protein kinase; Fmoc, fluorenyl-9-methoxycarbonyl; GST, glutathione S-transferase; MAPKAP-K, mitogen-activated protein kinase-activated protein kinase; MKK, mitogen-activated protein kinase kinase; MSK, mitogen- and stress-activated protein kinase; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PRAK, p38-regulated/activated kinase; pS, phosphoserine; ROCK, Rho-dependent protein kinase; SAPK, stress-activated protein kinase; SGK, serum- and glucocorticoid-induced kinase; S6K, ribosomal S6 kinase.

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Carl Smythe (School of Life Sciences, University of Dundee, Dundee, Scotland, U.K.). Histone H1 was purchased from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). All peptides were synthesized by Dr Graham Bloomberg (Department of Biochemistry, University of Bristol, Bristol, U.K.) or by Mr F. B. Caudwell (Division of Signal Transduction Therapy, School of Life Sciences, University of Dundee, Dundee, Scotland, U.K.). Biotinylated peptides were prepared by first attaching a C6 spacer (Calbiochem) to resin-bound peptide using standard methodology. Fluoren-9-ylmethoxycarbonyl (Fmoc)-C6-OH/2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/di-isopropylethylamine was dissolved in dimethyl formamide in a ratio of 1:1:2, and coupled for 45 min in a PioneerTM automatic peptide synthesizer (Applied Biosystems, Warrington, Cheshire, U.K.), according to the manufacturer's instructions. Biotin was attached by reacting Fmoc deprotected resin-bound peptide for 3 h with biotin-*N*-hydroxysuccinimidyl ester/di-isopropylethylamine (1:1 ratio).

Source and purification of protein kinases (see [5] for further details)

Apart from the AMP-activated protein kinase (AMPK; a gift from Dr S. Hawley and Dr D. G. Hardie, School of Life Sciences, University of Dundee, Dundee, Scotland, U.K.) and the catalytic subunit of cAMP-dependent protein kinase (PKA; a gift from Dr C. Smythe), which were isolated from rat liver and bovine heart respectively, all other protein kinases were expressed as either glutathione S-transferase (GST)-fusion or His-tagged proteins (by Ms C. Clark, Ms F. Douglas, Dr A. Paterson or Ms G. Wiggin, Division of Signal Transduction Therapy, School of Life Sciences, University of Dundee, Dundee, Scotland, U.K.). The expressed proteins corresponded to the human sequences, apart from rat Rho-dependent protein kinase (ROCK)II. GST-fusion proteins were purified on GSH-agarose (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) and His-tagged proteins on Ni²⁺-nitrilotriacetate-agarose (Qiagen) in accordance with the manufacturer's instructions. All the protein kinases are available from Upstate Ltd (www.upstate.com), apart from calmodulin-dependent protein kinase II (CaMKII), mitogen-activated protein kinase-activated protein kinase (MAPKAP-K)3 and cGMP-dependent protein kinase (PKG). The last mentioned was generously given by Dr Susan Lohmann (Institut für Klinische Biochemie und Pathobiochemie, University of Würzburg, Würzburg, Germany).

Protein kinases expressed in *Escherichia coli*

The following were expressed in *E. coli* as GST-fusion proteins: extracellular signal-regulated protein kinase-2 (ERK2), stress-activated protein kinase-2a (SAPK2a, also called p38), MAPKAP-K2, MAPKAP-K3, CaMKII (Calbiochem–Novabiochem) and checkpoint kinase (CHK)2. CHK2 contained an additional His-tag at the extreme C-terminus to aid purification of the full-length product. ERK2 was activated using Raf-activated MAPK kinase 1 (MKK1) and SAPK2a/p38 using a constitutively active mutant of MKK6. MKK1 and MKK6 were also expressed in *E. coli* as GST-fusion proteins.

Protein kinases expressed in Sf21 cells

The following enzymes were expressed in Sf21 cells: CHK1, p38-regulated/activated kinase (PRAK), protein kinase B α (PKB α), serum- and glucocorticoid-induced kinase 1 (SGK1[S422D], where S442D is a Ser⁴²² → Asp substitution, and lacking the N-

terminal 59 residues), mitogen- and stress-activated protein kinase 1 (MSK1), p70 ribosomal S6 kinase 1 (S6K1[T412E], lacking the C-terminal 104 residues), c-Raf, protein kinase C (PKC α ; Calbiochem–Novachem) and rat ROCKII[1–543].

Activation of protein kinases

MAPKAP-K2 and MAPKAP-K3 were activated by incubation with MgATP²⁻ and ERK2; ERK2 with MKK1; MKK1 with Raf; SAPK2a/p38 with a constitutively active mutant of MKK6 in which the residues required for activation were changed to aspartic acid; PRAK with SAPK2a/p38; PKB α , SGK1 and S6K1 with 3-phosphoinositide-dependent protein kinase-1.

Antibodies

Antibodies that recognize the common seven-residue phosphorylated motif (LpSFAEPG; where pS represents phosphoserine) contained within each of the three generic peptide substrates (see the Results section) were raised against the peptide LpSFAEPGC. The C-terminal cysteine residue was added to enable coupling to keyhole-limpet (*Diodora aspera*) haemocyanin. The peptide–protein conjugate was injected into sheep at Diagnostics Scotland (Pennicuik, Lothian, Scotland, U.K.), and the anti-sera purified on protein G–Sepharose (Amersham Pharmacia Biotech) by Dr J. Leitch (Division of Signal Transduction Therapy, School of Life Sciences, University of Dundee, Dundee, Scotland, U.K.). Antibodies were used at a concentration of 2 μ g/ml for immunoblotting and 10–20 μ g/ml for detection of phosphorylated peptides in the modified ELISA format. Rabbit anti-sheep antibodies conjugated to peroxidase were obtained from Pierce and used at a 1 in 10000 dilution.

Kinase assays

All protein kinase assays were linear with respect to time. The assays with standard peptide substrates have been described previously [5]. The assays with the three generic peptides introduced in the present paper were carried out in the same buffers as follows. Unlabelled or biotinylated substrate peptides were assayed for 10 min at 30 °C in 50 μ l incubation volumes, in the presence of 0.1 mM [γ -³²P]ATP or unlabelled ATP. Radioactive assays were terminated by spotting 40 μ l of each incubation on to phosphocellulose paper followed by immersion in 75 mM phosphoric acid. Subsequently, all papers were washed a further three times in phosphoric acid, once in acetone, then dried and the radioactivity was counted. Non-radioactive assays were terminated by the addition of EDTA to a final concentration of 20 mM, then diluted with an equal volume of PBS, containing 0.1% Tween 20, and the biotinylated peptides were captured by incubation for 60 min on pre-washed 96-well streptavidin-coated plates (Equilon A/S, Vedbaek, Denmark). The plates were washed six times (5 min/wash) in PBS, containing 0.1% Tween 20, and were then incubated for 60 min with antibody (10–20 μ g/ml) raised against the generic phospho-specific antibody (see above). The plates were washed six times (5 min/wash) in PBS, containing 0.1% Tween 20, and the binding of the phospho-specific antibody was detected by incubating the plates with peroxidase-conjugated anti-sheep antibodies (Pierce). The plates were washed a further six times as above, and binding was visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech) on a Fuji Film LAS-1000 charge-coupled-device camera. Signals were quantified on LAS-1000IR software (Fuji Film).

RESULTS AND DISCUSSION

Development of generic peptide substrates

Many protein kinases phosphorylate serine residues that lie in particular amino-acid motifs. For example, PKA and PKG phosphorylate serine residues that lie in Arg-Arg-Xaa-Ser-sequences [6], whereas PKB, SGK and S6K1 phosphorylate serines that lie in Arg-Xaa-Arg-Xaa-Xaa-Ser- motifs [7,8]. On the other hand, MAPKAP-K2, MAPKAP-K3 and CaMKII recognize Hyd-Xaa-Arg-Xaa-Xaa-Ser- motifs, where Hyd is a bulky hydrophobic residue [9]. In addition, nearly all of these protein kinases prefer a bulky hydrophobic residue immediately C-terminal to the site of phosphorylation. The requirement of many protein kinases for specific sequences N-terminal to the site of phosphorylation and their less stringent requirements C-terminal to the site of phosphorylation led us to test a number of peptides that share a common C-terminal epitope that might be used to generate a single phospho-specific antibody capable of assaying many protein kinases. These studies resulted in the development of the three generic peptides shown in Table 1,

Table 1 Kinetic parameters for peptide phosphorylation by a number of protein kinases

PKB α , SGK1, MSK1, S6K1, PKA, PKG, ROCKII, MAPKAP-K2, MAPKAP-K3, PRAK, CHK1 and CHK2 were assayed in 50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 0.1% (v/v) β -mercaptoethanol and 0.01% (w/v) Brij-35; AMPK in 50 mM Hepes, pH 7.4, 1 mM dithiothreitol, 0.02% Brij-35 and 0.2 mM AMP; PKC α in 20 mM Hepes, pH 7.4, 0.03% Triton X-100, 0.1 mM CaCl₂, 0.1 mg/ml phosphatidylserine and 10 μ g/ml 1,2-dioleoyl-*sn*-glycerol; and CaMKII in 50 mM Hepes, pH 7.4, 5 mM CaCl₂ and 0.03 mg/ml calmodulin. The $V_{\max}$ values for the three generic peptides developed in this paper are given relative to the standard peptide substrates for each protein kinase. The standard peptide substrates were GRPRTSSFAEG (PKB, SGK1, MSK1 and S6K1), LRRASLG (PKA), KEAKEKRQEIAKRRRLSSLRASTSKSGGSQK (ROCKII and PKG), KKLNRRLTSLVA (MAPKAP-K2 and MAPKAP-K3), KKLRLRLTSLVA (PRAK), KKKVSRSGLYR-SPSPMPENLNRPR (CHK1 and CHK2), HMRSAMSGHLVLRKRR (AMPK), MHRQETVDCLK (CaMKII) and histone H1 (PKC).

A					
Enzyme	Substrate ...	KKLNRTL ^S FAEPG		Standard	
		K_m (μ M)	Relative V_{max} (%)	K_m (μ M)	Relative V_{max} (%)
MAPKAP-K2		5	52	7	100
MAPKAP-K3		30	105	18	100
PRAK		40	40	> 100	100
CHK1		5	117	5	100
CHK2		> 300	221	> 300	100
AMPK		75	114	80	100
CaMKII		300	80	60	100
B					
Enzyme	Substrate ...	RARTLSFAEPG		Standard	
		K_m (μ M)	Relative V_{max} (%)	K_m (μ M)	Relative V_{max} (%)
PKB α		2	100	4	100
SGK1		4	105	4	100
MSK1		2	156	6	100
S6K1		6	124	8	100
C					
Enzyme	Substrate ...	RRRLSFAEPG		Standard	
		K_m (μ M)	Relative V_{max} (%)	K_m (μ M)	Relative V_{max} (%)
PKA		30	161	18	100
PKG		50	122	5	100
ROCKII		> 100	39	5	100
PKC α		> 500	39	2	100

Figure 1 Generation of a phospho-specific antibody that recognizes the phosphorylated epitope LpSFAEPG

Aliquots (10 ng or 100 ng) of the peptide LpSFAEPG conjugated to BSA were spotted on to a nitrocellulose membrane, and were immunoblotted with the phospho-specific antibody raised against the phospho-peptide immunogen in the presence or absence of the peptides (10 μ g/ml) shown on the right-hand side.

which contain a common seven-residue epitope related to the sequence surrounding the serine residue in glycogen synthase kinase 3 that is phosphorylated by PKB [10]. Using the standard radioactive filter-binding assay, one or more of these three peptides was phosphorylated efficiently by the 15 different protein kinases that we tested, which fall into several different kinase subfamilies. Indeed, in many cases, one of these three peptides proved to be phosphorylated as efficiently, and sometimes even more efficiently, than the substrates in routine use for their assay.

Although PKC could be assayed perfectly well using the peptide Arg-Arg-Arg-Leu-Ser-Phe-Ala-Glu-Pro-Gly (Table 1), this substrate was inferior to others that have been used to assay this protein kinase. This is because PKC prefers basic residues C-terminal to the site of phosphorylation, which are not present in the epitope, in addition to basic residues N-terminal to the site of phosphorylation [6].

Generation of a phospho-specific antibody to the common C-terminal epitope

The major purpose of the present study was to simplify high-throughput screening of protein kinases by developing a non-radioactive method applicable to the assay of many protein kinases. In order to do this, we therefore generated a phospho-specific antibody capable of recognizing the phosphorylated epitope (LpSFAEPG) common to the three generic substrates (see the Experimental section). This antibody recognized < 10 ng of the conjugated phospho-peptide antigen (Figure 1), but did not recognize the unphosphorylated form of the peptide

Figure 2 Comparison of the new ELISA-based protein kinase assay with the standard radioactive filter-binding assay

The protein kinases indicated were assayed by the ELISA method (open bars) or standard radioactive assay (filled bars) using the peptide substrates RARTLSFAEPG (**A**), KKLNRTLSFAEPG (**B**) and RRRLSFAEPG (**C**), and in the presence or absence of the kinase inhibitors shown. The results are presented relative to control incubations in the absence of inhibitors, and are expressed as the means $\pm$ S.E.M. for four determinations (two independent experiments). Similar results were obtained in several further experiments.

(results not shown). The ability of the antibody to recognize the three generic substrate peptides in their phosphorylated form was established by competition studies. These experiments showed that the phospho-peptide antibody was not only neutralized by pre-incubation with the seven-residue phospho-peptide antigen, but also by each of the phosphorylated generic peptide substrates. In contrast, the unphosphorylated forms of the same peptides were unable to neutralize the antibody (Figure 1).

Development of a non-radioactive assay for many protein kinases

We then used the three generic peptides, in conjunction with the phospho-specific antibody described above, to set up a simple non-radioactive ELISA-based assay for a subset of the protein

kinases in Table 1 (see the Experimental section). The assays were run in parallel with standard radioactive filter-binding assays under linear rate conditions, where less than 10% of the peptide was phosphorylated (Figure 2). The results showed that the non-radioactive assay was of similar accuracy to the standard radioactive procedure, and a similar degree of inhibition was observed in both assays in the presence of known inhibitors of these protein kinases.

Concluding remarks

In the present paper, we have described a simple non-radioactive assay that is suitable for the high-throughput screening of many protein kinases and is the subject of a patent application. We anticipate that the same three peptides can be used as substrates

by all the closely related isoforms of the protein kinases studied, and probably by most, if not all, of the other members of the same kinase subfamilies. We also anticipate that additional peptides with the same C-terminal epitope may be used to assay other protein kinases that recognize a specific motif that is N-terminal to the site of phosphorylation. For example, CK1 (previously called casein kinase I) phosphorylates serine residues that lie three residues C-terminal to another phosphoserine residue [11]. It may, therefore, phosphorylate peptides of the type Xaa-phosphoSer-Xaa-Leu-Ser-Phe-Ala-Glu-Pro-Gly that contain the C-terminal epitope. We therefore expect that our strategy will be applicable to at least 50 and probably more than 100 protein kinases.

The assay that we have developed is not applicable to protein kinases that have a stringent requirement for a particular motif or residue C-terminal to the site of phosphorylation. For example, MAP kinases and cyclin-dependent protein kinases require a proline residue immediately C-terminal to the site of phosphorylation [6], which is a negative determinant for the protein kinases tested in this paper. Similarly CK2 requires several consecutive acidic residues C-terminal to the site of phosphorylation, whereas DNA-dependent protein kinase requires a C-terminal glutamine [6]. However, one could envisage developing a second series of peptide substrates with a common N-terminal epitope capable of assaying many protein kinases that require a specific C-terminal motif for phosphorylation.

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