Engineering protein kinases with distinct nucleotide specificities and inhibitor sensitivities by mutation of a single amino acid

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A major goal of signal transduction research is to identify the substrates and roles of the many protein kinases. The task might be simplified by the discovery that the mutation of a single amino acid dramatically alters the nucleotide specificity of protein kinases and their inhibition by a particular class of anti-inflammatory drug.

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The reversible phosphorylation of proteins, catalysed by protein kinases and protein phosphatases, regulates most aspects of cell life, including the control of gene transcription, cell growth and differentiation, the control of metabolism and secretion, the immune response, fertilisation, and even the acquisition of memory. The phosphorylation of proteins at serine, threonine and tyrosine residues alters their shape, and hence their ability to function, but it can also affect other parameters, such as the rate at which proteins are degraded and the ability of the proteins to migrate from one cellular location to another. The simplicity, flexibility and reversibility of protein phosphorylation probably explains why this covalent modification has been adopted by eukaryotic cells as their principal control mechanism.

One of the long-term goals of signal transduction research is to identify the intracellular substrates and the physiological roles of each protein kinase and protein phosphatase, but this is a major challenge because about one third of all mammalian proteins, some 30,000 proteins, contain covalently bound phosphate. Moreover, the human genome encodes 1000–2000 protein kinases (the largest single gene family) and 300–500 protein phosphatases. Thus, the 'average' protein kinase phosphorylates 15–30 substrates and the 'average' protein phosphatase dephosphorylates 50–100 intracellular proteins. The problem is made even harder by the tremendous redundancy and overlapping substrate specificities of many protein kinases.

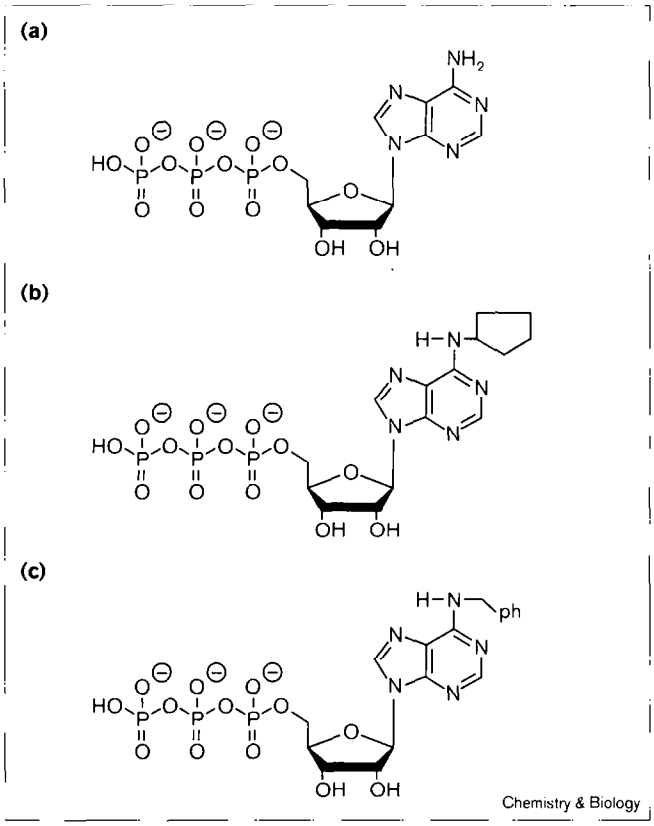
The development of new and improved methods for identifying the intracellular substrates of protein kinases and phosphatases is critical. Here, we review two independent lines of investigation that recently introduced novel approaches that might be helpful in tackling this problem. One approach involves engineering protein kinases with altered nucleotide specificities, whereas the other creates protein kinases that are sensitive to inhibition by a particular class of anti-inflammatory drug. Remarkably, both approaches are based on mutagenesis of the same amino acid in the kinase catalytic domain.

Engineering protein kinases with novel nucleotide specificities

In 1997, Kevan Shokat and his colleagues [1] embarked on a strategy aimed at changing the nucleotide specificities of protein kinases. Their goal was to produce a mutant protein kinase with two key properties. Firstly, the kinase had to be able to accept with high catalytic efficiency an N^6-substituted ATP that the wild-type enzyme (or any other intracellular protein kinase) would not accept. Secondly, the mutant kinase had to use ATP with a lower catalytic efficiency than the wild-type enzyme, so that the N^6-substituted ATP would be used preferentially. If this could be achieved, it might be possible to identify the substrates of a protein kinase simply by introducing the mutant protein kinase and the ^{32}P-labelled N^6-substituted ATP into cell lysates or intact cells, because the substrates of other protein kinases should not be radiolabelled under these conditions [1].

Inspection of the ATP-binding pockets of cyclic AMP-dependent protein kinase (PKA) and cyclin-dependent protein kinase 2 (cdk2) revealed that only two amino acid sidechains were within a 5 Å sphere of the N^6 amino group of the bound ATP (Figure 1a). Shokat and colleagues [1] therefore mutated the equivalent residues (Val323 and Ile338) in the oncogenic protein tyrosine kinase v-Src to alanine, the rationale being that a much smaller sidechain might create an additional 'pocket' in the nucleotide-binding site that would allow particular N^6-substituted ATP derivatives to interact with the mutant, but not the wild-type, enzyme. These studies led to the discovery that the V323A/I338A double mutant (but not the wild-type v-Src) was able to utilise N^6-(cyclopentyl) ATP (Figure 1b) as a substrate. Moreover, no protein became phosphorylated when a lymphocyte extract was incubated with N^6-(cyclopentyl) ATP, implying that no other cellular protein kinase was able to utilise this N^6-substituted ATP. The V323A/I338A double mutant also had a lower affinity for ATP than did the wild-type v-Src [1].

Figure 1



Structures of **(a)** ATP, **(b)** N^6-(cyclopentyl) ATP and **(c)** N^6-(benzyl) ATP.

Figure 2

MAP kinase family member	Sequence surrounding Thr106
SAPK2a/p38	FNDVYLV**T**HLMGADL
SAPK2b/p38β2	FSEVYLV**T**TLMGADL
SAPK3/p38γ	FTDFYLV**M**PFMGTDL
SAPK4/p38δ	FYDFYLV**M**PFMQTDL
SAPK1γ/JNK1	FQDVYLV**M**ELMDANL
SAPK1β/JNK2β	FQDVYLV**M**ELMDANL
MAPK2/ERK2	MKDVYIV**Q**DLMETDL
Other protein kinases mentioned in this review	
v-Src	EEPIYIV**I**EYMSKGS
Fyn	EEPIYIV**T**EYMNKGS
Lck	QEPIYII**T**EYMENGS
Type II TGFβ receptor	GKQVWLI**T**AFHAKGN
Type I TGFβ receptor	WTQLWLV**S**DYHEHGS
Some protein kinases with a bulky hydrophobic residue at the position equivalent to Thr106	
Protein kinase A	NSNLYMV**M**EYVPGGE
Protein kinase C	VDRLYFV**M**EYVNGGD
CaM-dependent protein kinase II	EGHIYLI**F**DLVTGGE
Cyclin-dependent kinase 2	ENKIYLV**F**EFLHQDL
Glycogen synthase kinase-3α	ELYLNLV**L**EYVPETV

Chemistry & Biology

Amino acid sequences surrounding the residue equivalent to the threonine in SAPK2a/p38 and SAPK2b/p38β2 that confers sensitivity to SB 203580. Residues in the position equivalent to Thr106 in SAPK2a/p38 are shown in bold.

The generation of a v-Src mutant with a novel nucleotide specificity (but with a similar substrate specificity) was encouraging, but the k_{cat}/K_m value of the mutant with N^6-(cyclopentyl) ATP was still 50-fold lower than that of the wild-type enzyme with ATP [1]. This led to the search for an 'improved' mutant and, in a recent issue of *Chemistry & Biology*, Shokat and coworkers [2] have achieved this feat. They show that the key residue in engineering a novel nucleotide specificity is Ile338 (Figure 2, shown in bold in v-Src), and find that N^6-(benzyl) ATP (Figure 1c) is a better substrate for I338A or I338G v-Src than N^6-(cyclopentyl) ATP. The k_{cat}/K_m of I338G v-Src with N^6-(benzyl) ATP as a substrate is only fourfold lower than that of wild-type v-Src with ATP. The catalytic domain of the Fyn tyrosine kinase is 85% identical to that of Src. Mutation of the equivalent residue, Thr339, in Fyn to glycine (Figure 2) produced a mutant whose k_{cat}/K_m with N^6-(benzyl) ATP was just as high as that of wild-type Fyn with ATP [2]. As expected, N^6-(benzyl) ATP and N^6-(cyclopentyl)ATP were potent inhibitors of the ATP-dependent activity of I338G v-Src or T339G Fyn, but did not inhibit wild-type v-Src or wild-type Fyn [2].

In summary, the presence of a bulky residue at position 338 of v-Src or position 339 of Fyn prevents these Src family members from utilising N^6-substituted ATP derivatives. The ability to produce mutant protein kinases that utilise N^6-(cyclopentyl) ATP and N^6-(benzyl) ATP derivatives potentially forms the basis of a method for 'tagging' the physiological substrates of these protein kinases in the presence of every other cellular protein kinase.

Engineering a specific cell-permeable inhibitor of mutant I338G v-Src that does not affect wild-type v-Src

In a further recent paper [3], Shokat and colleagues have extended these studies by identifying a cell-permeable inhibitor of I338G v-Src that does not inhibit wild-type v-Src. This was achieved by making derivatives of a pyrazolo[3,4-*d*]pyrimidine (Figure 3a) known to be an inhibitor of Src family members. It was reasoned that this compound was likely to bind in a similar manner to quercetin, an inhibitor of many protein kinases whose interaction with the Src family member Hck has been solved by X-ray crystallography [4]. In this case, the N^4 position of pyrazolo[3,4-*d*]pyrimidine corresponds to the N^6 position of ATP and a panel of N^4-substituted analogues were therefore synthesised. One of these, the N^4(*p-tert*-butylphenyl) derivative (Figure 3b) was found to inhibit I338G v-Src ($IC_{50} = 430$ nM) or T339G Fyn ($IC_{50} = 830$ nM) without inhibiting the wild-type enzymes, even at 500–1000-fold higher concentrations than those required to inhibit the mutants. Interestingly,

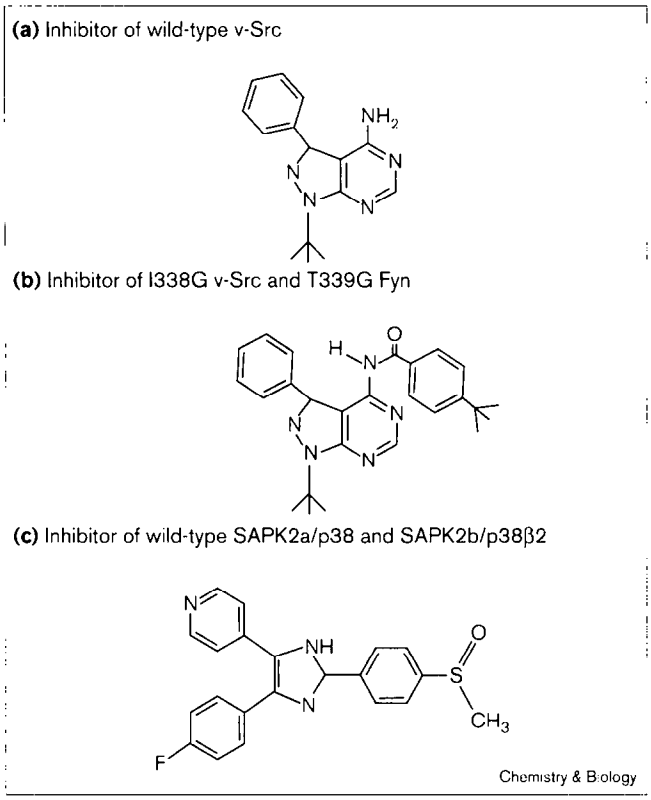
this compound (unlike the unmodified pyrazolo[3,4-*d*]pyrimidine compound) did not affect tyrosine phosphorylation induced by cross-linking the B-cell receptor, establishing that the derivative does not inhibit any of the intracellular protein tyrosine kinases that lie on this signalling pathway. Excitingly, the N^4(*p-tert*-butylphenyl) derivative suppressed both the phosphorylation of p36 (a putative v-Src substrate) and cell transformation induced by expression of I338G v-Src. In contrast, p36 phosphorylation and cell transformation induced by wild-type v-Src were unaffected [3].

Basis for the specificity of the anti-inflammatory drug SB 203580

A class of pyridinyl imidazoles was identified by John Lee and his colleagues at SmithKline Beecham [5] that suppresses the synthesis (and some of the actions) of pro-inflammatory cytokines and that shows promise for the treatment of rheumatoid arthritis and other chronic inflammatory conditions [6]. One of these compounds is SB 203580 (Figure 3c), which has been shown to be a remarkably specific inhibitor of stress-activated protein (SAP) kinase 2a (SAPK2a, also called p38) and SAP kinase-2b (SAPK2b, also called p38β2) [5,7,8]. SB 203580 has been used to identify some of the physiological substrates and cellular functions of SAPK2a/p38 and SAPK2b/p38β2 (reviewed in [9]).

SAPK2a/p38 and SAPK2b/p38β2, which show 74% amino acid sequence identity, are members of the mitogen-activated protein (MAP) kinase family. SAPK3/p38γ and SAPK4/p38δ are two other members of the same protein family, with 60% sequence identity to SAPK2a/p38 and SAPK2b/p38β2. Unlike SAPK2a/p38 and SAPK2b/p38β2, however, SAPK3/p38γ and SAPK4/p38δ are not inhibited by SB 203580 [8], thus raising the question of the mechanism underlying the specificity of this pyridinyl imidazole compound. SB 203580 binds to its protein kinase targets competitively with ATP [10] and elucidation of the three-dimensional structure of SAPK2a/p38 in complex with closely related pyridinyl imidazoles has established that these drugs are inserted into the ATP-binding pocket of SAPK2a/p38 [11,12]. The 4-fluorophenyl ring of the drug does not make contact with residues that interact with ATP, however. One residue near the 4-fluorophenyl ring is Thr106, which is conserved in SAPK2b/p38β2 but is replaced by methionine in SAPK3/p38γ and SAPK4/p38δ (Figure 2). This suggested that the resistance of these protein kinases to SB 203580 might be explained by the presence of a large sidechain at this position, and two recent mutagenesis studies have revealed that this is indeed the case [13,14]. Thus, SAPK3/p38γ and SAPK4/p38δ become sensitive to SB 203580, when the methionine is changed to threonine whereas, conversely, SAPK2a/p38 and SAPK2b/p38β2 become resistant to the drug when Thr106 is changed to methionine [13,14]. Further mutagenesis

Figure 3



Structures of **(a)** pyrazolo[3,4-*d*]pyrimidine, **(b)** the N^4(p-*tert*-butylphenyl) derivative of pyrazolo[3,4-*d*]pyrimidine and **(c)** the pyridinyl imidazole inhibitor SB 203580.

revealed that SAPK3/p38γ becomes more sensitive to the drug (IC_{50} = 10–50 nM) when the methionine is replaced by even smaller residues, such as serine, alanine or glycine. SAPK2a/p38, SAPK2b/p38β2 and SAPK4/p38δ also become most sensitive to SB 203580 (IC_{50} = 15–30 nM), when glycine or alanine is present at this position [13]. In addition to Thr106 of SAPK2a/p38, His107 and Leu108 also contribute to the sensitivity of SAPK2a/p38 towards SB 203580 [14]. These residues are proline and phenylalanine in SAPK3/p38γ and SAPK4/p38δ. When the residues are replaced by histidine and leucine, M109T SAPK3/p38γ and M107T SAPK4/p38δ become more sensitive to inhibition by SB 203580 [14].

SAPK1/JNK, another MAP kinase family member, is only 40% identical to SAPK2a/p38 and SAPK2b/p38β2. Nevertheless, SAPK1/JNK can also be converted to a SB 203580-sensitive form by mutation of Met108 (Figure 2) to a small amino acid. To optimise sensitivity to the drug, however, it was also necessary to change Ile106 to the leucine residue present at the equivalent position in other MAP kinase family members. The I106L/M108A double mutant was inhibited with an IC_{50} value of 30 nM [13]. M108 in SAPK1/JNK is followed by

glutamate. M108T/E109H SAPK1/JNK is inhibited by SB 203580 with an IC_{50} value of 1 μM, as compared to an IC_{50} value of 10 μM for M108T SAPK1/JNK [14]. It will be interesting to study the inhibition of I106L/M108A/E109H SAPK1/JNK by SB 203580.

Most known mammalian protein kinases have a large, hydrophobic residue at the position equivalent to Thr106 of SAPK2a/p38 (Figure 2). Very few protein kinases have threonine at this position and only one (the type I TGFβ receptor) has serine (Figure 2). No known protein kinase has glycine or alanine at this position. Two protein kinases with threonine at this position, the type II TGFβ receptor and the tyrosine protein kinase Lck (Figure 2), were shown to be sensitive to inhibition by SB 203580, although the IC_{50} values were 400–800 times higher than the IC_{50} value for SAPK2a/p38. Nevertheless, sensitivity of the type II TGFβ receptor to SB 203580 was abolished by mutagenesis to methionine, but enhanced by mutagenesis to alanine. The type I TGFβ receptor was inhibited slightly more potently by SB 203580, consistent with the presence of the smaller serine residue at this position [13].

In summary, the size of the residue at the position equivalent to Thr106 of SAPK2a/p38 is critical and diagnostic for determining whether a protein kinase will be sensitive to SB 203580. It will be interesting to see how many of the reported effects of SB 203580 in mammalian cells are abolished by transfection with a drug-insensitive form of SAPK2a/p38. It will also be interesting to see whether SB 203580 can be used to study the physiological roles of other protein kinases by replacing the drug-insensitive wild-type enzymes with SB 203580-sensitive mutants.

Conclusions

Thr106 of SAPK2a/p38 and Ile338 of v-Src are equivalent residues in the kinase catalytic domain. It is remarkable that two independent lines of research have identified this single amino acid as being critical for the ability to accept N^6-substituted ATP derivatives, as well as for the sensitivity to inhibition by the anti-inflammatory drug SB 203580. For a protein kinase to be sensitive to SB 203580, the sidechain of this residue must be no larger than that of threonine, the sensitivity being enhanced when the size of the sidechain is even smaller. The high degree of specificity of SB 203580 is explained by the presence of a large sidechain at this position in nearly all known protein kinases. For a protein kinase to accept N^6-(cyclopentyl) ATP or N^6-(benzyl) ATP, however, the sidechain must be glycine or alanine. Intriguingly, no protein kinase in the public databases has glycine or alanine at this position, which presumably explains why no protein kinases have been detected in cell lysates that are capable of utilising N^6-(cyclopentyl) ATP or N^6-(benzyl) ATP. As stable cell lines expressing a mutant protein kinase and even 'knock-in' mice can be generated quite rapidly, the replacement of a wild-type protein kinase by a mutant enzyme that has acquired a novel nucleotide specificity or sensitivity to a particular drug offers a new approach for identifying the physiological substrates and roles of protein kinases. It remains to be seen how useful this approach will turn out to be, but there is more than a glimmer of hope that it will considerably simplify this daunting problem.

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