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Development of photo-crosslinking reagents for protein kinase–substrate interactions

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Abstract The identification of relevant protein kinase–protein substrate partners remains a serious challenge on a genome-wide scale. The design and synthesis of a photo-activatable nucleotide reagent to crosslink protein kinases with their substrates is described in which an azido group is appended to the γ-phosphoryl and purine moieties of ATP. In the absence of UV, compounds of this class were shown to act as competitive inhibitors versus ATP and non-competitive inhibitors versus peptide substrate for the protein tyrosine kinase Csk, suggesting that they can form a ternary complex with kinase and protein substrate. In vitro experiments with protein kinases indicate the bifunctional reagent can induce covalent protein–protein crosslinking that is dependent on UV irradiation. That significant kinase–substrate crosslinking occurs is suggested by the fact that this crosslinking is competitively inhibited by ATP. The crosslinked adducts can be readily cleaved by phosphodiesterase which supports the model for crosslinking and provides a simple method to deconvolute the linked protein partners. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Protein kinase; Csk; Src; Nucleotide; Photo-crosslinking

1. Introduction

The protein kinases are one of the largest protein superfamilies in eukaryotic genomes and have attracted considerable scientific interest because of their roles in cell physiology and pathophysiology [1]. For most kinases the true protein substrates are unknown. Likewise for most phosphorylated proteins, the protein kinase(s) responsible are not determined. With roughly 600 protein kinases and thousands of phosphorylated proteins (many on multiple sites) in humans, connecting substrates and kinases represents a complex proteomics problem that will likely require many independent approaches to reach resolution [2]. Protein crosslinking reagents have provided a rich source of information about protein–protein interactions in various biological systems. However, among the available reagents there are none developed which selectively link protein kinase–protein substrate pairs. We considered the possibility that a photo-activatable reagent that was designed with the ATP moiety as the tether would allow for kinase–substrate-directed crosslinking (see Fig. 1). Protein kinases follow ternary complex mechanisms and bind both ATP and protein substrate in close proximity [3]. In high resolution structures of protein kinases, the adenine moiety is buried in the active site of the kinase and the γ-phosphate protrudes toward the kinase surface where the protein substrate is thought to bind [4]. Thus protein kinase–protein substrate crosslinking reagents of the general form in Fig. 1 were proposed. In this report, we describe the design, synthesis, and in vitro evaluation of a novel protein kinase–protein substrate crosslinking agent.

2. Materials and methods

2.1. Synthesis of compounds

Compounds **1–3** were prepared from ATP (purchased from Sigma) and the corresponding amine (aniline, azidoaniline, phenylhydrazine purchased from Sigma or Aldrich) using a coupling route described previously [5,6]. For example, the synthesis of **1** was performed as follows. Adenosine 5′-triphosphate sodium salt (15 mg, 27.3 μmol) was dissolved in water (5 ml) and the pH of the solution was brought to 7 (pH paper) by titrating with sodium hydroxide (1 M). *N*-Cyclohexyl-*N*′-(2-morpholiniethyl)carbodiimide methyl-*p*-toluene sulfonate (purchased from Aldrich) (382 mg, 0.90 mmol) was added to the reaction mixture with another 1 ml water. The pH of solution was brought down to 5.6 with HCl (1 M) and maintained in a pH range between 5.6 and 5.8 throughout the reaction as determined by pH paper. Aniline hydrochloride (82 mg, 63.3 mmol) was added to the solution and the reaction mixture was titrated back to pH 5.6–5.8 with sodium hydroxide (1 M). The reaction was left stirring for an additional 4 h and then treated with triethylamine to reach pH 8.5. The mixture was purified on a DEAE Sephadex-A25 anion exchange column with pH 8 triethylammonium bicarbonate (TEAB) buffer. Briefly, a Sephadex DEAE A25 (3 g) was swelled in water, placed into a column, washed with water (100 ml), 1 M TEAB (100 ml) and again water (175 ml). A gradient system was used for purifications (A = water, B = TEAB 1 M) using a flow rate of 2 ml/min and fraction sizes of 6 ml at 4°C; gradient: 0–60 ml, 0% B; 60–260 ml, 0–40% B, 260–360 ml, 40–100% B. Compound **1** was eluted in 61% TEAB (triethylammonium salt, 14.3 mg, 77%). Compound **4** was synthesized from ATPγS (purchased from Boehringer) and the commercially available 4-azido-bromoacetanilide (purchased from Sigma) following a procedure previously described [3]. Compound **5** was prepared analogous to compounds **1–3** except that 8-azido-ATP was employed in place of ATP. Compounds were purified analogously to compound **1** and then lyophilized to dryness. Each compound showed appropriate ^{1}H nuclear magnetic resonance (NMR), ^{31}P NMR, and electrospray mass spectrometry (MS) data for structural assignments and purity (>90%). Wherever possible, light exposure was limited. These compounds were stored in darkness in frozen aqueous solution at neutral pH at −80°C and shown to be stable for several months. Final con-

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centrations were determined from the UV absorbance at 260 nm and normalized based on the sum of the component acetanilide and ATP-related moieties. Spectroscopic data for compound **5**: ^{1}H NMR (D_2O) δ 8.14 (s, H-2, 1H), 6.96 (dd, H-aromatic, 2H), 6.71 (dd, H-aromatic, 2H,), 5.87 (d, H-1′, 1H), 4.92 (ddd, H-2′, 1H,), 4.42 (ddd, H-3′, 1H), 4.15–4.30 (m, H-4′, 1H), 4.01–4.15 (m, H-5′, 2H). ^{31}P NMR (D_2O) δ −6.53 (d, P_γ), −7.92 (d, P_α), −19.5 (t, P_β). Negative ion EIMS of **5** (calcd. for $C_{16}H_{18}N_{12}O_{12}P_3$; $M-H^+$, *m/z* 663).

2.2. Csk kinase assays

Recombinant human Csk was prepared as described previously [7]. Kinetic assays were performed as described using radiolabelled ATP and the random copolymer peptide substrate 4:1 poly(Glu,Tyr) as reported previously [7]. In these experiments, radiolabelled ATP is separated from phosphorylated peptide using 10% SDS–PAGE. Assay conditions employed fixed concentrations of either poly(Glu,Tyr) (200 μg/ml) or ATP (10 μM) with varying concentration of the other substrate. Under these conditions, K_m for ATP was found to be ~10 μM, K_m for poly(Glu,Tyr) ~30 μg/ml, and k_{cat} ~25 min^{-1}. The data for each assay were globally fit to either linear competitive, non-competitive, or uncompetitive models using the commercially available software KinetAsyst II and the selection of fit was based on minimization of standard error and visual inspection. The competitive inhibition plots showed no significant improvement (less than two-fold increased standard error) by incorporating a K_i-intercept term, whereas the non-competitive inhibition plots showed at least a two-fold improvement by incorporating a K_i-intercept term. The plotted lines displayed in Fig. 3 are calculated based on a global fit of the data in each case.

2.3. Protein crosslinking

Recombinant human Csk (full-length) and recombinant chicken Src (83–533) were expressed and purified to near homogeneity as described previously [8]. An open 0.6 ml plastic tube (Eppendorf) containing 25 μl of buffered solution (50 mM Tris–HCl, 4 mM $MnCl_2$, pH 7.4) at room temperature along with Csk (0.5 μM), Src (5 μM), in the presence or absence of **5** (25 or 100 μM), was exposed to long wavelength (broad spectrum centered around 365 nm) UV (Spectroline, hand-held lamp) for 45 min. In experiments where ATP was added, 2 mM (Fig. 4A) or 8 mM (Fig. 4C) ATP (final) was included. In experiments where phosphodiesterase was added, after UV exposure, 0.029 units of phosphodiesterase I (bovine intestinal mucosa, Sigma) was employed and the mixture was allowed to stand another 10 min. Reactions were then run out on 10% SDS–PAGE, transferred to nitrocellulose, and stained with anti-Src or anti-Csk antibodies (Upstate Biological) and imaged by autoradiography. Monomeric molecular weights for recombinant Csk and Src are both ~50 kDa. Each experiment was performed at least twice and representative results are shown.

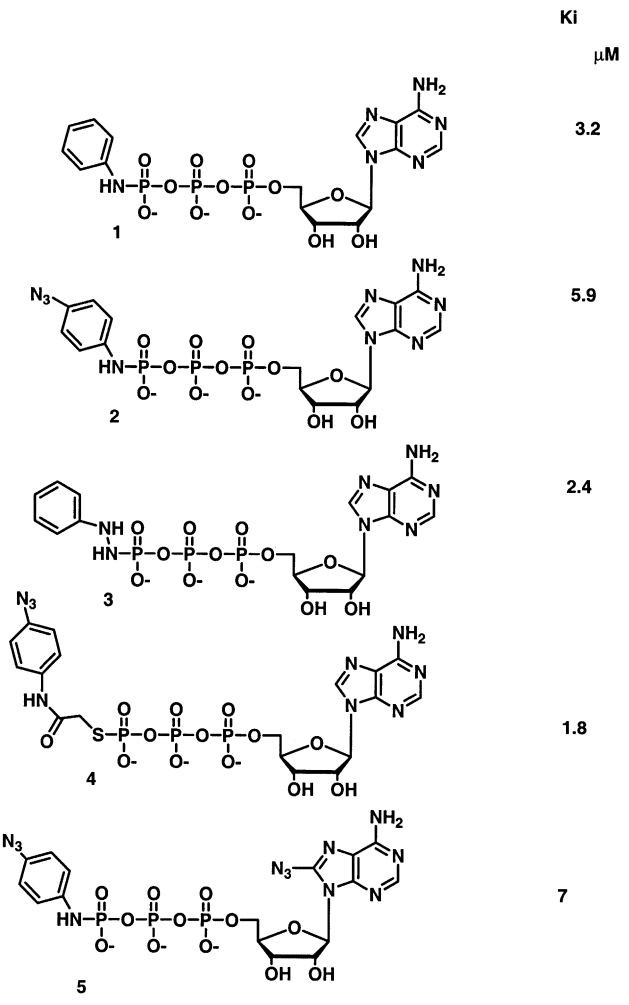

Fig. 2. Synthetic ATP analogs and K_i values for inhibition of protein tyrosine kinase Csk. Values represent the K_i for a competitive inhibition model versus ATP at saturating 4:1 poly(Glu,Tyr) concentration. The standard errors for these measurements are ±20%. See Section 2 for synthetic procedures and kinase assays.

3. Results and discussion

3.1. Effects of γ-substitution of ATP on kinase inhibition

Two potential concerns about the design for a crosslinking reagent shown in Fig. 1 were: (1) whether substitutions on the γ-phosphate would prevent or greatly reduce the ability of the peptide substrate from binding, and (2) whether the azide moiety on the adenine would prohibit its high affinity binding with the active site of the protein kinase. To initiate an investigation of these issues, a series of ATP analogs derivatized in the γ-position were synthesized (Fig. 2, **1–4**) [5,6]. These were then tested as inhibitors of the protein tyrosine kinase Csk, a useful protein kinase for enzymatic studies [7]. We were gratified to find that these compounds were fairly potent inhibitors of kinase activity with K_i 2–6 μM (Fig. 2), slightly lower than the K_m of ATP (10 μM) under these conditions. Moreover, as exemplified by compound **2** (Fig. 3), each of these compounds showed a linear competitive pattern of inhibition versus the substrate ATP but proved to be non-competitive versus the tyrosine peptide substrate (poly(Glu,Tyr)). This pattern of inhibition suggests that these ATP analogs block ATP binding but do not prevent appreciably peptide substrate binding. This is the case because non-competitive inhibitors can bind to a form of the enzyme that still has the (peptide) substrate bound. Thus, it is likely that these ATP analogs can form a ternary complex with protein kinases and protein substrates.

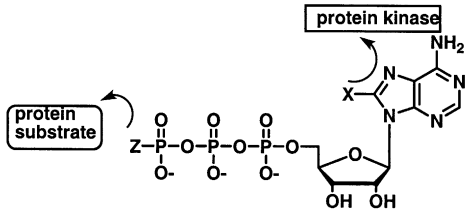

Fig. 1. Strategy for protein kinase–protein substrate crosslinking.

3.2. Protein crosslinking by compound 5

Our next goal was to construct the bifunctional compound **5** which was prepared by condensing 8-azido-ATP and azidoaniline. As a Csk inhibitor **5** displayed a K_i that was suffi-

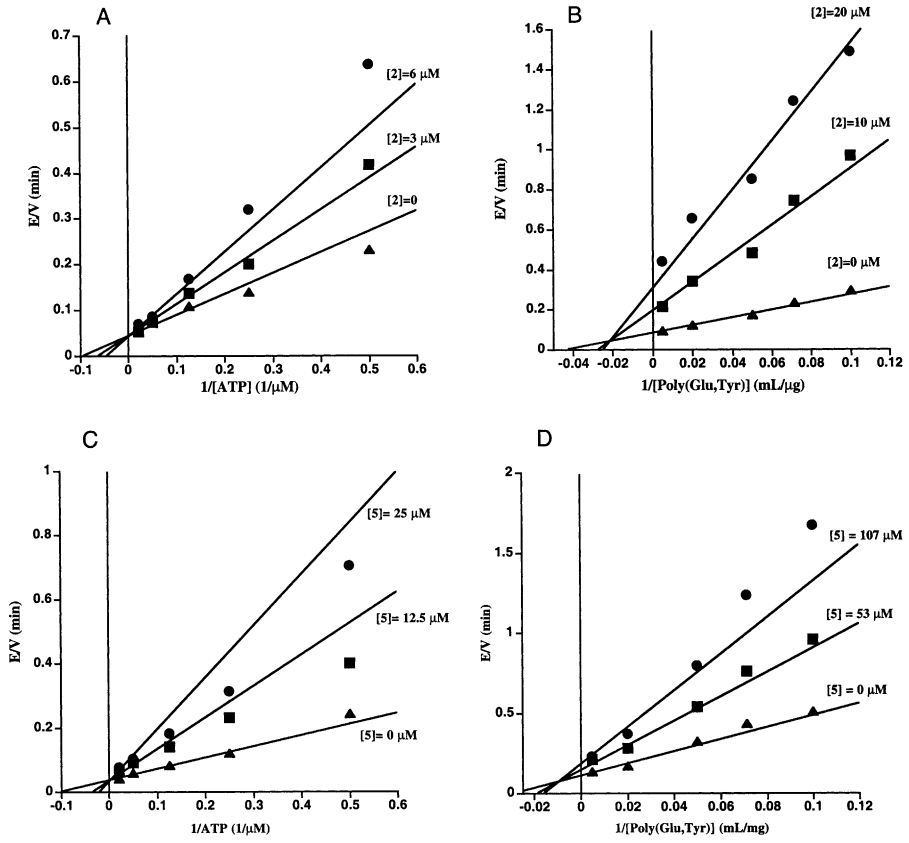

Fig. 3. Pattern of inhibition of Csk by ATP analogs **2** and **5**. A,C: Lineweaver–Burk plot of $1/V$ vs. 1/ATP with varying concentration of compound **2** or **5** shows linear competitive inhibition. B,D: Lineweaver–Burk plot of $1/V$ vs. 1/poly(Glu,Tyr) with varying concentration of compound **2** or **5** shows linear non-competitive inhibition. Kinase assays and fits were carried out as described in Section 2.

ciently low to be attractive (7 μM) and was also competitive versus ATP and non-competitive versus peptide (see Fig. 3). Consequently, we designed a set of experiments to assess the possibility of protein photo-crosslinking. We used mixtures that had the proteins Src, a protein tyrosine kinase that can phosphorylate itself, and Csk, a protein tyrosine kinase that can phosphorylate Src [8,9]. The resultant mixtures were then analyzed by Western blot with an anti-Src antibody (see Fig. 4A). We observed several high molecular weight bands (100 kDa and above) that were likely to be covalent complexes containing Src based on the fact that the bands were stained using anti-Src antibody (Fig. 4A, lane 5). That these bands were actually covalent adducts is likely because a denaturing gel was used and furthermore, the formation of the adducts depended on UV light and compound **5** (Fig. 4A, lanes 2 and 3, Fig. 4B, lane 3). Moreover, 8-azido-ATP failed to induce crosslinking, establishing that the γ-phosphate-azido substitution was critical (data not shown). Since Csk can phosphorylate Src and Src can autophosphorylate intermolecularly [8,9], it should be possible to observe a variety of crosslinked adducts, consistent with the heterogeneous pattern observed in Fig. 4. Moreover, it has recently been shown that Csk phosphorylation of Src is more efficient when Src is dimerized or oligomerized [8], suggesting that multiple crosslinked adducts of these proteins would be favored. In the absence of Csk, decreased crosslinking is observed (Fig. 4B, compare lanes 1 and 2), consistent with the idea that Csk–Src crosslinks are present in the mixture. Immunoblotting with anti-Csk antibody also suggests the presence of Csk in the crosslinks, although this experiment is not definitive since some cross-reactivity between Src protein and anti-Csk is observed (Fig. 4C).

Having established that compound **5** could induce protein crosslinking, we next investigated whether the model shown in Fig. 1 was likely. It would be predicted from this model that ATP when present in excess could reduce or eliminate crosslinking by **5** since it would compete for the same nucleotide binding site. When excess ATP was co-mixed with the crosslinking reaction, the intensity of the crosslinked Src bands was significantly diminished, suggesting that the majority of the observed crosslinking involved the protein kinase ATP binding site as designed (Fig. 4A, lane 4; Fig. 4C, lane 2). That the crosslinking was not completely eliminated by ATP competition could suggest some non-specific crosslinking involving other sites on the Src or Csk proteins. If so, enhanced crosslinking specificity might be achievable by reducing crosslinking time, adjusting the geometry of the crosslinker, as well as by understanding the optimal concentration of protein partners, and these will be addressed in future investigations.

One important step in dissecting the information content present in the crosslinked complexes is to be able to have a reliable and gentle method to decompose the complexes into their constituent protein components. For example, after crosslinking in a crude cell extract system, it would be advantageous to immunopurify a crosslinked protein of interest and then separate it away from its unknown partner(s) to aid in

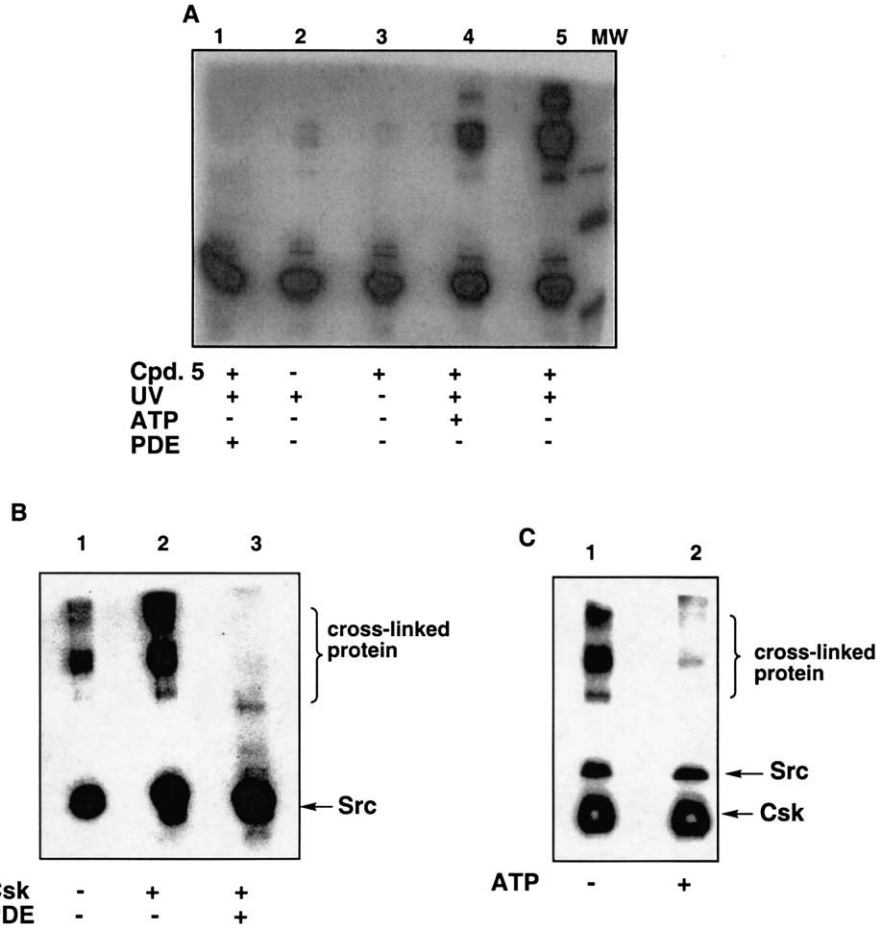

Fig. 4. Western blot of in vitro protein crosslinking by compound **5**. A: These reaction mixtures contained Csk (0.5 μM), Src (5 μM), ±**5** (25 μM) and were immunoblotted with anti-Src antibody. Molecular weight markers from top to bottom, 116, 66, 45 kDa. B: These reaction mixtures contained **5** (100 μM), Src (5 μM), ±Csk (0.5 μM) and were immunoblotted with anti-Src antibody. C: These reaction mixtures contained **5** (100 μM), Csk (0.5 μM), Src (5 μM) and were immunoblotted with anti-Csk antibody. For other details, see Section 2.

identification by MS or N-terminal sequencing. Since the linker contains a triphosphate species, it might be possible to cleave it enzymatically using a non-specific phosphodiesterase at neutral pH, liberating the component proteins. After the crosslinked reaction mixture was treated with UV light and **5**, a non-specific phosphodiesterase was added for 10 min prior to Western blot analysis. Impressively, the bands for crosslinked Src-containing complexes disappeared, indicating that the triphosphate linkages in **5** were critical in holding the crosslinks together (Fig. 4A, lane 1; Fig. 4B, lane 3). This not only adds chemical proof of the nature of the crosslink but also provides a simple route to decomposing the crosslink, which as mentioned will ultimately be helpful in target analysis. It also suggests that the phosphate diesters are sufficiently exposed to allow access to other enzymes.

4. Conclusions

In summary, these studies describe the synthesis of several γ-phosphate-tagged nucleotide analogs and explores their potential as kinase inhibitors with the hope of creating protein kinase–substrate crosslinking reagents. It was shown that γ-substitution still allows high affinity interaction with a kinase and likely does not prevent the peptide substrate from binding to the protein tyrosine kinase Csk. The bifunctional crosslinking agent **5** led to easily observable protein crosslinking that was likely to be covalent. Competition with ATP suggests that much of the protein crosslinking was nucleotide site-mediated validating the design. A simple enzymatic method was shown to efficiently cleave the linkages. It is expected that immunoprecipitation or other affinity purification procedures when combined with this crosslinking method will allow exploitation of the reagent in cell lysates to identify novel kinase–substrate interactions. Ultimately, replacement of the phosphates, to allow cell permeability, and the adenosine with high affinity nucleoside analogs may also increase the specificity and utility of the approach. We believe however that the approach described here provides a new direction in the proteomics analysis of protein kinase–substrate interactions.

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