Identification and characterization of serine/threonine protein kinase activity intrinsic to the L protein of vesicular stomatitis virus New Jersey

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A photoaffinity analogue of ATP, 8-azido-adenosine 5'-triphosphate (8-N3ATP), was used to probe ATP-binding sites in native transcription complexes of vesicular stomatitis virus (VSV) (New Jersey serotype). The analogue was found to be a substrate for a serine/threonine protein kinase that phosphorylated both the NS and L proteins of native complexes. The analogue failed to interact with the RNA polymerase, another ATP-utilizing activity associated with the transcription complex. Kinetic analyses of both ATP and 8-N3ATP utilization by the protein kinase yielded biphasic saturation curves. Photolysis of 8-N3ATP in the presence of VSV transcription complexes resulted in selective labelling of the L protein. The photolabelling of L was saturable and apparently biphasic. Photolabelling of the L protein was significantly reduced by competition with ATP whereas other nucleoside triphosphates (GTP, UTP and CTP) were ineffective competitors. The stoichiometry of photolabelling was 0.2 at 10 μM-8N3ATP and 1.3 at 100 μM-ATP. These data provide chemical evidence for a virus-encoded serine/threonine protein kinase which resides on the L protein.

Introduction

The rhabdovirus vesicular stomatitis virus (VSV) packages and delivers to its host cell a nucleoprotein complex through which it directs the event in the chain of biosynthetic processes that starts its multiplication cycle, transcription of the five genes encoded in the non-segmented, negative strand RNA genome (Wagner, 1987). The transcription complex is composed of the viral genomic RNA and three viral proteins. These are the nucleoprotein N which associates tightly with the genomic RNA to form the template for all viral RNA synthesis, L and the highly phosphorylated viral NS protein; the latter two constitute the viral transcriptase (Banerjee, 1987; Emerson, 1987).

In addition to its roles in generating 5' capped and methylated, 3' polyadenylated mRNAs, the transcription complex participates in replication of the viral genome (Wertz et al., 1987). We are probing the functional topology of this complex through photoaffinity labelling of native transcription complexes prepared from purified VSV (New Jersey serotype) virions. In the accompanying paper (Hammond et al., 1992) we have demonstrated that the photoactive analogue of UTP, 8-azido-adenosine 5'-triphosphate (5-N3UTP), serves as a substrate for the viral RNA-dependent RNA polymerase, and that it specifically and selectively photolabels the L protein of native transcription complexes. In this paper we present analyses of the interactions of the photoactive analogue, 8-N3ATP, with ATP-binding sites within the complex. Three enzymic activities which utilize ATP as a substrate are expressed by purified transcription complexes: RNA-dependent RNA polymerase, poly(A) polymerase and protein kinase. The photoactive nucleotide 8-N3ATP interacted with only one of these three potential targets, a protein kinase. This kinase phosphorylated serine and threonine residues on both the L and NS proteins.

Although the polymerase (Hunt et al., 1976) and poly(A) polymerase (Hunt et al., 1984) activities have been demonstrated to be encoded in the viral genome, the origin of the transcription complex-associated protein kinase activity has been controversial (Leader & Katan, 1988). Results of several investigations suggested that protein kinase activity could be separated from the transcription complex (Imblum & Wagner, 1974; Harmon et al., 1983) and from the L protein (Massey et al., 1990). However, protein kinase activity has also been reported to co-purify with the Indiana L protein, and purified L protein was photolabelled with azido ATP (Sánchez et al., 1985). In probing the native transcription...
complex of VSV New Jersey with a photoactive analogue of ATP we have generated chemical evidence that a protein kinase is encoded in the VSV genome and that it resides on the native L protein.

**Methods**

**Materials.** Non-radioactive 8-N3ATP and [γ-32P]8-N3ATP (sp. act. 5 to 20 Ci/mmol) were prepared as described previously (Potter & Haley, 1982). The nucleotide [γ-32P]ATP (7000 Ci/mmol, adjusted to 5 to 20 Ci/mmol prior to use) was purchased from ICN, [α-32P]CTP (800 Ci/mmol) was obtained from NEN Research Products. Nucleoside triphosphates were purchased from Calbiochem. All other chemicals used were of the highest quality available from commercial sources. The source and preparation of VSV (New Jersey serotype) transcription complexes is described in the accompanying paper (Hammond et al., 1992).

**Analysis of protein kinase activity.** Protein kinase reactions contained 10 mM-HEPES-KOH pH 8.0, 50 mM-NaCl, 10% glycerol, 1 mM-2-mercaptoethanol, transcription complex (300 μg protein/ml), [γ-32P]ATP or [γ-32P]8-N3ATP, and 5 mM-MgCl2. The reactions were incubated for 30 min at 31°C. To minimize non-specific occlusion of unbound [γ-32P]ATP or [γ-32P]8-N3ATP during protein concentration, the samples were treated with 10 μl of a solution containing 650 mM-NaCl, 10% 2-mercaptoethanol and 3 mM-ATP. The proteins were then precipitated with 7% HClO4 as described in the accompanying paper (Hammond et al., 1992). Electrophoretic analyses of the samples on SDS-polyacrylamide gels, detection of protein bands by staining and autoradiography, and quantification of the data by laser densitometry were as described (Hammond et al., 1992).

**Phosphoamino acid analysis.** Transcription complexes were phosphorylated in standard kinase reactions containing 40 μM-[γ-32P]ATP and 5 mM-MgCl2. The samples were then treated with 7% HClO4 as described above. The rinsed pellets were suspended in 6 M-HCl and transferred to Pyrex tubes (18 x 150 mm). The tubes were sealed under vacuum and the samples heated at 110°C for 90 min. Following removal of the HCl in vacuo, the hydrolysates were suspended in water and re-lyophilized. Samples were then suspended in 100 μl of 25 mM-KH2PO4 pH 5.0 and combined with 5 μl of phosphoamino acid standards (approximately 0.5 absorbance units each of phosphoserine, phosphothreonine and phosphotyrosine). The mixtures were filtered through a 0.22 μm nylon membrane and analysed by HPLC on a Partisil 10-SAX column with a 20 mM to 45 mM gradient of KH2PO4 pH 5.0. Fractions collected at 18 s intervals (600 μl) were combined with 5 ml Liquisint (National Diagnostics) scintillation cocktail and assayed for radioactivity utilizing an H# quench correction program on a Beckman LS 3801 liquid scintillation counting system. To correct for the differential rates of chemical destruction of phosphoserine, phosphothreonine and phosphotyrosine, the half-life for each species was used for the conditions employed (6 M-HCl, 110°C). The values used were 6-75 h, 1-75 h and 1-0 h for phosphothreonine, phosphoserine and phosphotyrosine respectively (T. Vanaman, personal communication).

**In vitro transcription.** Transcription reactions containing 15 mM-Tris-HCl pH 8.0, 50 mM-NaCl, 5 mM-MgCl2, 500 μM-2-mercaptoethanol, 10% glycerol, 100 μM-UTP, 100 μM-GTP, 50μM-[γ-32P]CTP, transcription complex (300 μg protein/ml) and various concentrations of either ATP or 8-N3ATP were incubated at 31°C for the times indicated. Polymerization was monitored as described in the accompanying paper (Hammond et al., 1992).

**Results**

**Characterization of protein kinase activity associated with native transcription complexes of VSV New Jersey**

In order to permit future correlation of structural with functional analyses of the transcription complex, the studies presented herein were focused on native transcription complexes which were prepared from purified virions under conditions that maintained the competence of the complexes to transcribe mature VSV mRNA (Hammond & Lesnaw, 1987). The complexes contained, **Fig. 1. Phosphorylation of transcription complex-associated proteins by endogenous protein kinase activity. Standard in vitro protein kinase reactions, described in Methods, contained 20 μM-[γ-32P]ATP and 5 mM-MgCl2. Incubation, perchloric acid precipitation and subsequent SDS-PAGE analysis were performed as described in Methods. The positions of the L, N, NS and X proteins are indicated to the left of the Coomassie blue-stained gel (lane 1). 32P-radiolabelled proteins were visualized by autoradiography (lane 2).**
in addition to the viral genomic RNA, the viral L, N, NS and X proteins (Fig. 1, lane 1). The X protein has been previously demonstrated to be a differently phosphorylated form of the viral phosphoprotein NS (Lesnaw et al., 1979). Purified transcription complexes were assayed for the presence of protein kinase activity by incubation with [γ-32P]ATP under reaction conditions that support transcription. In the presence of MgCl₂, the L, NS and X proteins, but not the predominant N protein, were radiolabelled (Fig. 1, lane 2). No radioactivity was detected in reactions in which MgCl₂ was substituted with CaCl₂ or EDTA (data not shown). These results indicated the presence in the transcription complex, of a Mg²⁺-dependent ATP-utilizing protein kinase which selectively phosphorylates the endogenous viral proteins, L and NS(X).

The amino acid substrate specificity of the endogenous protein kinase activity was determined by subjecting the in vitro phosphorylated transcription complexes to acid hydrolysis, and analysing the radiolabelled amino acids by HPLC. Phosphoserine and phosphothreonine, but not phosphotyrosine, were detected (Fig. 2).

The kinetics of ATP utilization by the transcription complex-associated protein kinase activity were determined for both the L and NS(X) endogenous protein substrates. This was accomplished by SDS-PAGE and autoradiographic analysis of transcription complexes phosphorylated in multiple standard in vitro protein kinase reactions containing several concentrations of [γ-32P]ATP. Quantification of the data obtained using either of the endogenous protein substrates, L or NS(X), revealed that ATP binding saturated between 0.2 μM and 30 μM. Moreover, the saturation profiles suggested the existence of two saturation events. Kₘ values of approximately 2 μM and 17 μM were extrapolated from the data obtained with the L protein (Fig. 3).

Substrate utilization of 8-N₃ATP by the endogenous protein kinase

The ability of 8-N₃ATP to serve as the phosphate donor for the endogenous protein kinase activity was assessed by standard in vitro reactions in which [γ-32P]ATP was substituted with [γ-32P]8-N₃ATP. The pattern of phosphorylated proteins displayed in the autoradiogram resulting from SDS-PAGE analysis of the reaction
Fig. 3. Kinetic analysis of the transcription complex-associated protein kinase using ATP as the variable substrate. Standard *in vitro* protein kinase reactions containing 5 mM-MgCl₂ and different amounts of [γ-³²P]ATP were incubated and analysed by SDS-PAGE and autoradiography. The data obtained for the L protein (○) and the NS(X) proteins (■) were quantified by laser densitometry as described in Methods.

Fig. 4. Substrate utilization of 8-N₃ATP by the transcription complex-associated protein kinase. A standard *in vitro* protein kinase reaction containing 5 mM-MgCl₂ and 20 μM-[γ-³²P]8-N₃ATP was incubated and analysed by SDS-PAGE as described in Methods. Shown are photographs of the Coomassie blue-stained protein bands (lane 1) and the corresponding autoradiogram (lane 2).

Fig. 5. Kinetic analysis of 8-N₃ATP substrate utilization by the transcription complex-associated protein kinase. The components of *in vitro* protein kinase reactions were as described in the legend to Fig. 3 except that [γ-³²P]8-N₃ATP was substituted for [γ-³²P]ATP. Reaction conditions, SDS-PAGE, autoradiography and quantification of the data obtained for phosphorylation of the L protein (○) and NS/X proteins (■) were as described in Methods.

...products (Fig. 4) was identical to that obtained from reactions containing [γ-³²P]ATP as the phosphate donor (Fig. 1); both the L and NS(X) proteins, but not the N protein, were radiolabelled.

Kinetic analyses of the endogenous protein kinase-mediated phosphorylation of L and NS(X) proteins in the presence of different concentrations of [γ-³²P]8-N₃ATP indicated two distinct saturation events for each protein substrate (Fig. 5). The *Kₘ* values for [γ-³²P]8-N₃ATP utilization extrapolated from the data obtained for the L protein substrate, 19 μM and 74 μM, were in close agreement with those derived from the NS(X) protein substrate data, 18 μM and 70 μM (Fig. 5).

8-N₃ATP does not interact with the active site of the RNA polymerase

The potential interaction of 8-N₃ATP with the nucleotide-binding pocket of the RNA polymerase active site was assessed by its ability to serve as an alternative substrate for the polymerase. Although 8-N₃ATP was a good substrate for the protein kinase, it failed to serve as a substrate for the viral RNA polymerase. No incorporation of [α-³²P]CTP into product RNA was detected in standard *in vitro* transcription reactions in which ATP was substituted with 8-N₃ATP (Fig. 6a). Moreover, no detectable inhibition of polymerase activity was observed in standard reactions which additionally contained high concentrations of 8-N₃ATP (Fig. 6b). The failure of 8-N₃ATP to serve as a substrate for, or to inhibit, polymerase activity indicated that the analogue did not bind to the polymerase active site.
VSV-encoded protein kinase

Photoaffinity labelling of native transcription complexes with 8-N3ATP

Photolysis of $[\gamma^{32}P]8$-N$_3$ATP in the presence of native transcription complexes resulted in selective radiolabelling of the L protein (Fig. 7, lane 3). By contrast, no radiolabelling was observed in control reactions not exposed to u.v. light (Fig. 7, lane 2). Light-dependent photolabelling of the L protein was obtained in reactions which contained either Mg$^{2+}$ or Ca$^{2+}$ ions. However, a higher level of photolabelling was observed in reactions containing Ca$^{2+}$ ions (data not shown). This effect probably reflects the inability of Ca$^{2+}$ ions to support catalysis and the resulting increased Ca$^{2+}$ 8-N$_3$ATP retention in the active site. For these reasons, Ca$^{2+}$ ions were employed in all subsequent reactions.

Radiolabelling of the L protein did not increase with increasing concentrations of $[\gamma^{32}P]8$-N$_3$ATP above 100 $\mu$m (data not shown). This saturation phenomenon reflected the specificity of photoincorporation of 8-N$_3$ATP into the L protein. Analysis of the dependence of L protein photolabelling upon $[\gamma^{32}P]8$-N$_3$ATP concentrations below 100 $\mu$m revealed a biphasic saturation curve (Fig. 8). The two binding constants for 8-N$_3$ATP extrapolated from the data (the apparent $K_d$ values), 22 $\mu$m and 70 $\mu$m (Fig. 8), are in close agreement with the two binding constants ($K_m$ values) extrapolated from the kinetic analysis of 8-N$_3$ATP substrate utilization by the protein kinase (Fig. 5).

Additional evidence that 8-N$_3$ATP was specifically bound to the protein kinase domain was obtained from protection experiments in which photolabelling was carried out as before but in the presence of unlabelled nucleoside triphosphates or nucleoside monophosphates. Eight nucleotides were tested for their ability to compete with 8-N$_3$ATP binding (Table 1). The twofold increase in protection of the transcription complex from photolabelling by $[\gamma^{32}P]8$-N$_3$ATP obtained in the presence of the natural substrate for the protein kinase, ATP compared to AMP, reflected the specificity of photoin-
Fig. 8. Saturation of [γ-32P]8-N3ATP photoincorporation into the VSV L protein of native transcription complexes. Photolabelling reactions contained various concentrations of [γ-32P]8-N3ATP. The samples were photolysed and analysed by SDS-PAGE and autoradiography. Relative photoincorporation was determined by quantitative laser densitometry as described in Methods.

corporation of the photoactive analogue. The apparent decrease in photolabelling observed with the monophosphorylated nucleoside was most likely the result of adsorption of U.V. light by these compounds and of their ability to bind weakly at the binding site for ATP. In contrast, no differential protection was obtained with the other three tri- and monophosphorylated nucleosides. These data confirm the specific interaction of 8-N3ATP with a nucleotide-binding site(s) of a protein kinase activity located on the L protein.

The stoichiometry of 8-N3ATP cross-linking was determined using a filter binding assay (Hammond et al., 1992). At low 8-N3ATP (10 μM) concentration, a stoichiometry of 0.2 moles per mole L protein was determined. When the concentration of 8-N3ATP was increased to 100 μM, a stoichiometry of 1:3 was obtained.

Discussion

The initial observation of protein kinase activity associated with purified virions of VSV (Strand & August, 1971) has been followed by numerous studies aimed at identifying the genomic origin, protein substrates and putative functional significance of the activity. Incubation of detergent-disrupted virions with [γ-32P]ATP consistently results in radiolabelling of the endogenous NS and M proteins (Imblum & Wagner, 1974; Moyer & Summers, 1974; Beckes et al., 1989). The predominant amino acid residues phosphorylated are serine and threonine; however, under certain virus growth conditions, phosphotyrosine is also detected (Clinton & Huang, 1981; Clinton et al., 1982). The tyrosine phosphorylating activity and the majority, but not all, of the serine and threonine phosphorylating activities are associated with the membrane fraction of the virion and are most likely to be of cellular origin (Clinton et al., 1982; Harmon et al., 1983; Imblum & Wagner, 1974; Beckes et al., 1989; Moyer & Summers, 1974; Sánchez et al., 1985).

In light of the above data, we preceded our characterization of the interaction of 8-N3ATP with the VSV New Jersey transcription complex with a search for transcription complex-associated protein kinase activity. In order to maintain the physical and functional integrity of all catalytic sites, in all protein kinase analyses, we employed native complexes and monitored phosphorylation of viral proteins endogenous to the complex under buffer conditions that supported transcription of mature mRNA. This strategy was chosen so that data generated in each of our various studies could be correlated to extend our knowledge of interactions between the multiple functional domains within the transcription complex which contribute to the overall process of RNA synthesis. Two interesting observations emerged from these initial assays. First, in addition to phosphorylation of both the NS protein and its electrophoretic variant X, which has been well documented to occur under a variety of conditions as referenced above, significant and reproducible phosphorylation of the L protein was obtained under our assay conditions. Second, variable substrate (ATP) kinetic analyses revealed two distinct saturation events. The absence of tyrosine phosphorylation in these reactions indicated that both saturation events corresponded to the observed serine/threonine phosphorylation.

The native transcription complex presents multiple potential targets to the photoaffinity analogue 8-N3ATP, namely, the nucleotide-binding sites for the RNA-dependent RNA polymerase, poly(A) polymerase and protein kinase activities. Interaction of 8-N3ATP with the active site(s) of the protein kinase activity was indicated by its ability to mimic ATP in serving as an efficient phosphate donor for the selective phosphorylation of the L, NS and X proteins. Moreover, the biphasic nature of the saturation curve obtained from a variable substrate (8-N3ATP) kinetic analysis, coupled with the close agreement between the two Km values derived from the L protein phosphorylation data with the corresponding values for NS(X) protein phosphorylation, mimicked the results obtained with ATP as the phosphate donor.

The analogue 8-N3ATP does not appear to interact with the active site of the RNA polymerase; it failed to serve as a substrate for, and failed to inhibit, RNA polymerase activity. Similar results have also been reported for the interaction of 8-N3ATP with the RNA polymerase of the VSV Indiana serotype (Sánchez et al.,...
Table 1. Natural substrate-dependent protection of the VSV L protein from photolabelling by [γ-32P]β-N3ATP

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Additional nucleotide</th>
<th>Relative relative radioactivity</th>
<th>Normalized radioactivity</th>
<th>Comparative photolabelling</th>
<th>NMP/NTP ratio**</th>
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<tr>
<td>1</td>
<td>–</td>
<td>0.127</td>
<td>1.062</td>
<td>8.36</td>
<td>100</td>
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<tr>
<td>2</td>
<td>AMP</td>
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<td>3</td>
<td>ATP</td>
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<tr>
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<td>0.740</td>
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<tr>
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<td>0.148</td>
<td>0.794</td>
<td>5.36</td>
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* Photolabelling was carried out with 15 μM [γ-32P]β-N3ATP as described in Methods.
† Present at a concentration of 100 μM.
‡ Derived from integration of densitometric scans of the Coomassie blue-stained bands.
§ Derived from integration of densitometric scans of an autoradiogram prepared from the dried gel. Corrected for light-independent radiolabelling.
¶ Relative radioactivity divided by relative protein.
** Ratio of photolabelling in the presence of a monophosphate versus a triphosphate form of the nucleoside.

1985). Whether 3' polyadenylation of VSV mRNAs is a function of the RNA polymerase triggered into a chattering mode by the conserved U7 sequences present at the 5' ends of the five VSV genes (Rose & Schubert, 1987), or reflects an independent active site is not at present known. Because the expression of poly(A) polymerase activity requires concurrent RNA polymerization (Abraham & Banerjee, 1976; Banerjee et al., 1974) and because 8-N3ATP is not a substrate for the RNA polymerase, we could not directly assess the substrate utilization of 8-N3ATP by a putative poly(A) polymerase. However, as discussed below, the correlation between the parameters of 8-N3ATP substrate utilization by the protein kinase and L protein photolabelling by 8-N3ATP suggested that potential ATP-binding sites other than those associated with the protein kinase activity do not serve as targets for the photoactive analogue. The differential interaction of 8-N3ATP with the kinase and polymerase active sites reflects both the shapes of the corresponding nucleotide-binding pockets and differences in the structural conformations of 8-N3ATP and ATP.

The specificity of the selective radiolabelling of the L protein upon photolysis of [γ-32P]β-N3ATP in the presence of native transcription complexes was indicated by the u.v. light dependence and the saturation ability of radiolabelling. Two observations suggested that the photolabelling of the L protein was occurring at the nucleotide-binding site(s) of the protein kinase(s). The two apparent K₅ values extrapolated from the biphasic photolabelling saturation curve and the two apparent Kₘ values extrapolated from the 8-N3ATP substrate utilization data were similar. L protein photolabelling was reduced in the presence of competing substrate ATP, but not in the presence of GTP, CTP or UTP, and to a far lesser extent with NMPs.

The two saturation events characteristic of both ATP and 8-N3ATP substrate utilization by the protein kinase, and photoinsertion of 8-N3ATP into the L protein, could reflect a single kinase active site capable of assuming either of two slightly different conformations. Alternatively, the dual saturation events could reflect two distinct protein kinase active sites on the L protein. Support for the latter model derives from the computer-assisted identification of several putative ATP-binding sites, reminiscent of the G-X-G-X-G-(X)₅₋₁₀-K motif associated with the active sites of certain protein kinases (Kamps et al., 1984) in the predicted amino acid sequence of the L protein. The two regions, residues 754 to 778 and 1332 to 1351, were originally identified in the amino acid sequence of the L protein (Massey & Lenard, 1987) predicted from the nucleotide sequence of the Indiana serotype L gene (Schubert et al., 1984). Expansion of the search for amino acid similarities between the VSV L protein and database entries revealed significant similarities between the VSV L protein (Indiana) residues 725 to 1102 and the catalytic domain of the Ab1 subfamily of tyrosine kinases, as well as similarities between VSV L residues 1291 to 1671 and the catalytic domain of the PDGFR subfamily of tyrosine kinases (McClure & Perrault, 1989). The corresponding regions of the predicted amino acid sequence of the New Jersey L protein (Feldhaus & Lesnaw, 1988) exhibited appreciably lower similarity to these tyrosine kinase domains (McClure & Perrault, 1989). No tyrosine kinase activity was detected in our...
preparations of transcription complexes of either the New Jersey serotype (this paper) or of the Indiana serotype (D. C. Hammond & J. A. Lesnaw, unpublished results). If these tyrosine kinase-like motifs in the VSV L protein correspond to the active sites of the observed serine/threonine kinase activities, functional and sequence divergence of these 'similogs' have co-evolved. An additional region (residues 1651 to 1675 of the L protein) also resembles a motif found in a variety of ATP-binding proteins, and is perfectly conserved in the rabbit, the parainfluenza, Sendai, Newcastle disease and measles paramyxoviruses (S. Enkemann, A. L. Feldhaus, D. C. Hammond & J. A. Lesnaw, unpublished observations). The functional significance of these conserved motifs will become apparent as the enzyme active sites are mapped on the L protein.

The data presented herein constitute chemical evidence for the presence of a virus-encoded protein kinase activity in the VSV New Jersey transcription complex. Further, the photoaffinity data obtained with native transcription complexes indicate that L protein residues contribute to the formation of the ATP-binding domain(s) of this protein kinase. With this work, we have established the optimal conditions for specific photolabelling with 8-N3ATP. This information is essential for our current studies, the aim of which is isolation and sequence analysis of photolabelled L peptides that constitute the protein kinase active site.

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References


VSV-encoded protein kinase


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