Photoaffinity labelling of the ATP-binding site of the epidermal growth factor-dependent protein kinase

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Epidermal growth factor (EGF), after binding to its receptor, activates a tyrosine-specific protein kinase which phosphorylates several substrates, including the EGF receptor itself. The effects of a photoaffinity analogue of ATP, 3'-O-{3-[N-(4-azido-2-nitrophenyl)amino]propionyl}adenosine 5'-triphosphate (arylazido- β -alanyl-ATP) on the EGF-dependent protein kinase in A431 human tumour cell plasma membrane vesicles was investigated. This analogue was capable of inactivating the EGF-receptor kinase in a photodependent manner. Partial inactivation occurred at an analogue concentration of 1 μ M and complete inactivation occurred at 10 μ M when a 2 min light exposure was used. Arylazido- β -alanine at 100 μ M and ATP at 100 μ M were incapable of inactivating the enzyme with 2 min of light exposure. The photo-dependent inactivation of the enzyme by the analogue could be partially blocked by 20 mM-ATP and more effectively blocked by either 20 mM-adenosine 5'-[β -imido]triphosphate or 20 mM-guanosine 5'-[β -imido]triphosphate, indicating nucleotide-binding site specificity. Arylazido- β -alanyl-[α -³²P]ATP was capable of labelling membrane proteins in a photodependent manner. Numerous proteins were labelled, the most prominent of which ran with an apparent M_r of 53 000 on polyacrylamide-gel electrophoresis. A band of minor intensity was seen of M_r corresponding to the EGF receptor (170 000). Immunoprecipitation of affinity-labelled and solubilized membranes with an anti-(EGF receptor) monoclonal antibody demonstrated that the M_r 170 000 receptor protein was photoaffinity labelled by the analogue. The M_r 53 000 peptide was not specifically bound by the anti-receptor antibody. The affinity labelling of the receptor was not enhanced by EGF, suggesting that EGF stimulation of the kinase activity does not result from changes in the affinity of the kinase for ATP. These studies demonstrate that arylazido- β -alanyl-ATP interacts with the ATP-binding site of the EGF-receptor kinase with apparent high affinity and that this analogue is an effective photoaffinity label for the kinase. Furthermore, these studies demonstrate that the EGF receptor, identified by using monoclonal antibodies, contains an ATP-binding site, providing further confirmation that the EGF receptor and EGF-dependent protein kinase are domains of the M_r 170 000 protein.

Abbreviations used: EGF, epidermal growth factor; arylazido- β -alanyl-ATP, 3'-O-{3-[N-(4-azido-2-nitrophenyl)amino]propionyl}adenosine 5'-triphosphate; AMP-PNP, adenosine 5'-[β -imido]triphosphate; GMP-PNP, guanosine 5'-[β -imido]triphosphate; FSBA, *p*-fluorosulphonylbenzoyl-5'-adenosine; SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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EGF, a 53-amino-acid polypeptide, is a potent mitogen (see Carpenter & Cohen, 1979; Schlesinger *et al.*, 1982; Carpenter, 1983 for reviews). In its presence, cells undergo a number of metabolic changes which are ultimately followed by cell proliferation. Among the earlier events are the binding of EGF to its specific plasma membrane receptor and activation of a tyrosine-specific protein kinase (Carpenter *et al.*, 1978, 1979, 1980; Cohen *et al.*, 1980; King *et al.*, 1980; Ushiro &

Cohen, 1980). This protein kinase is believed to mediate the transmembrane signal of EGF binding, thereby initiating the cellular response to the hormone. The kinase is similar to the transforming gene product of the Rous sarcoma virus, p60^{src}, in that they are both tyrosine-specific protein kinases (Hunter & Sefton, 1980; Collett *et al.*, 1980) with overlapping protein substrate specificities *in vitro* (Kudlow *et al.*, 1981; Chinkers & Cohen, 1981; Pike *et al.*, 1982) and *in vivo* (Hunter & Cooper, 1981). Evidence has accumulated that the EGF receptor and the protein kinase are domains of the same molecule. The receptor and the kinase copurify (Cohen *et al.*, 1980), and variants of A431 cells with different quantities of receptors have corresponding alterations in protein kinase activity (Buss *et al.*, 1982). More direct evidence derives from the finding that FSBA, an ATP binding site affinity label, bound to proteins in A431 plasma membranes including an M_r 170000 protein which corresponds in size to the EGF receptor (Buhrow *et al.*, 1982). However, the chemically reactive group in this analogue that covalently binds it to the protein is at a position that corresponds to the triphosphate region of ATP. Since the receptor is itself a substrate for the EGF-dependent protein kinase, it must occupy a position close to the ATP-binding site of the kinase. The reactive group in FSBA therefore could be positioned by the kinase adjacent to its EGF receptor substrate, resulting in covalent linkage of FSBA to the receptor. Such affinity labelling could give the false impression that the kinase and receptor reside within the same molecule. Even if it were true that the receptor and kinase are domains of the same molecule, future studies to determine the structure of the kinase domain could be hampered by inappropriate labelling of the protein by FSBA. For this reason, and in order to determine whether another affinity probe could label the kinase, we chose arylazido- β -alanyl-ATP, a photoaffinity analogue of ATP. This and a related analogue were used to label the ATP-binding sites of several ATP-utilizing enzymes (Jeng & Guillory, 1975; Russell *et al.*, 1976; Lunardi *et al.*, 1977; Cosson & Guillory, 1979) and was chosen to label the EGF kinase because the azido-bearing side chain was at a position distinct from the triphosphate region of ATP, making substrate labelling less likely. Furthermore, after labelling we identified the receptor and kinase with a monoclonal antibody to the receptor (Kawamoto *et al.*, 1983). These studies demonstrated the usefulness of arylazido- β -alanyl-ATP as a photoaffinity probe for the EGF-dependent protein kinase and contributed to the evidence that the kinase and receptor are in a single M_r 170000 molecule.

Materials and methods

Materials

Culture medium and serum were from Gibco. Mouse EGF was purified from male Swiss Webster mouse (Simonsen) submaxillary glands (Savage & Cohen, 1972). Dimethyl formamide, β -alanine and 1,1'-carbonyldi-imidazole (carbodi-imidazole) were from Aldrich; ATP, AMP-PNP, GMP-PNP and Hepes were from Sigma. [α -³²P]-ATP and [γ -³²P]ATP were from Amersham International. The mouse monoclonal antibody to the A431 EGF receptor was kindly provided and characterized (Kawamoto *et al.*, 1983) by Dr. Tomoyuki Kawamoto.

Cell cultures and plasma membrane preparations

A431 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) bovine calf serum in a CO₂ incubator on 10cm Falcon tissue culture plates. When the cells reached a density of 2×10^6 cells per plate, they were treated, using a method modified from Cohen *et al.* (1982), to cause the cells to shed membrane vesicles. Briefly, the cell monolayers were washed three times with Ca²⁺ + Mg²⁺-free Dulbecco's phosphate-buffered saline, once with hypo-osmotic Ca²⁺ + Mg²⁺-free phosphate-buffered saline (5% in water) and then incubated at 22°C in a bacterial rotary shaker at 100 rev./min for 15 min. The hypo-osmotic phosphate-buffered saline was replaced with vesiculation buffer (100mM-NaCl, 50mM-Na₂-PO₄, 5mM-KCl, 0.5mM-MgSO₄, pH8.5) and the plates were shaken for 20 min at 22°C. The temperature of the shaker was increased to 37°C and shaking was continued for a further 100min. The shed vesicles were harvested by centrifugation and stored at -75°C until use. The yield of membrane protein was determined by the Bradford (1976) method and was 20–50 μ g/plate of cells.

Synthesis of arylazido- β -alanyl-ATP

Unlabelled arylazido- β -alanyl-ATP and its parent compound arylazido- β -alanine were synthesized and purified according to Jeng & Guillory (1975). The labelled compound was synthesized by esterification of [α -³²P]ATP (Amersham International; 410Ci/mmol) with arylazido- β -alanine by using carbodi-imidazole as a catalyst.

Photodependent inactivation of EGF-dependent protein kinase with arylazido- β -alanyl-ATP

Membrane vesicles containing 20 μ g of protein were suspended in reaction buffer containing 20mM-Hepes, pH7.2, 1mM-MnCl₂, 0.1% bovine serum albumin and 0.3 μ M-EGF. After 10 min at 4°C, arylazido- β -alanyl-ATP was added to the

tubes to a final reaction volume of 50 μ l. In the standard experiments, 10 μ M of the ATP analogue was used. Photoirradiation was carried out with a tungsten-halogen projection lamp (650 W, DVY, 3400 K) with the sample at 15 cm from the lamp in an ice/water bath. The samples were irradiated for 30 s intervals interspersed with 30 s dark periods to prevent sample heating. In the standard experiments, the total light exposure time was 2 min. The tubes containing the unirradiated samples were wrapped completely in aluminium foil and exposed to light as described for the irradiated samples. After irradiation, the samples were brought to a volume of 0.5 ml with a solution containing 20 mM-Hepes, pH 7.2, and 5 mM-EDTA, and centrifuged in a Microfuge (13000 g) for 5 min. The membrane pellet was washed three times by centrifugation with reaction buffer prior to assay for EGF-dependent protein kinase activity.

Protein kinase activity in intact A431 plasma membranes was assayed as previously described (Carpenter *et al.*, 1979; Kudlow *et al.*, 1981; Buss *et al.*, 1982). Briefly, the membrane pellet was suspended in a 50 μ l volume of reaction buffer. The reaction, which was carried out in an ice bath, was initiated by the addition of [γ - 32 P]ATP (2 μ Ci, 0.5 μ M) and terminated by the addition of 6-fold concentrated Laemmli (1970) sample buffer and boiling for 5 min. In some experiments, the reaction was terminated by spotting the reaction mixture onto a filter paper disc which was dropped immediately into a solution of 10% (w/v) trichloroacetic acid containing 10 mM-sodium pyrophosphate. The extent of phosphorylation was determined either by running the reaction mixture on a 7.5% SDS/polyacrylamide gel (Laemmli, 1970) followed by autoradiography or by determining, by liquid-scintillation counting, the amount of 32 P precipitated onto the filter papers.

Photoaffinity labelling of the EGF-dependent protein kinase with arylazido- β -alanyl-[α - 32 P]ATP

Photoaffinity labelling was performed exactly as described for photodependent inactivation except that 2×10^6 c.p.m. of the labelled azido-ATP analogue was added to each sample instead of unlabelled analogue. After irradiation, Weber & Osborn (1969) sample buffer was added to the reaction mixture and the samples were boiled for 2 min prior to electrophoresis on a 4–10% SDS/polyacrylamide gel with the Weber & Osborn (1969) buffer system (sodium phosphate, pH 7.0).

In some experiments, the EGF receptor was immunoprecipitated from solubilized plasma membranes which had been subjected to photoaffinity labelling. After irradiation, the membranes were solubilized in RIPA buffer containing

EDTA (0.15 M-NaCl, 50 mM-Tris/HCl, pH 7.2, 5 mM-EDTA, 1% Triton X-100, 1% sodium deoxycholate and 0.1% SDS). After removal of non-solubilized material by centrifugation on a microcentrifuge, a monoclonal antibody to the EGF receptor was added to a final concentration of 10^{-6} M and incubation was continued for 30 min. Formaldehyde-fixed *Staphylococcus aureus*, 15 μ l of a 10% (w/v) suspension, was then added to the tubes and incubated for a further 30 min. The entire mixture was layered onto a 0.75 ml cushion of 1 M-sucrose buffered with 20 mM-Hepes, pH 7.2, and centrifuged for 2.5 min. The bacterial pellet was washed twice by centrifugation with RIPA buffer, once with 0.5 M-NaCl in 20 mM-Hepes, pH 7.2, and once with water. The pellet was then boiled for 2 min in Weber & Osborn (1969) sample buffer. The bacteria were removed by centrifugation and the supernatant was subjected to SDS/polyacrylamide-gel electrophoresis on a 4–10% gel with the Weber & Osborn (1969) buffer system. The gel was fixed and stained in 10% (v/v) propan-2-ol prior to drying. Autoradiographic exposure was carried out at -75°C with an intensifying screen for 1–2 weeks.

In some experiments, the immunoprecipitate bound to the bacteria was treated with 10 μ l of 0.01 M-NaOH for 1 h at 37°C . The alkali was neutralized with HCl prior to processing for gel electrophoresis.

Results and discussion

To determine whether arylazido- β -alanyl-ATP was capable of interacting with the ATP-binding site of the EGF-dependent kinase in a photo-dependent manner, unlabelled analogue was incubated with intact A431 plasma membranes with and without exposure to white light. The concentration of the analogue was varied from 1 to 30 μ M and the time of exposure was fixed at 2 min. After washing the membrane vesicles free of analogue, the activity of the protein kinase was determined by measuring its ability to phosphorylate the EGF receptor. No photodependent inactivation of the enzyme was observed in the absence of analogue (Fig. 1). Some inactivation was observed on exposure of the kinase to 1 μ M-analogue and maximal inactivation of the kinase occurred at an analogue concentration of 10 μ M (Fig. 1). Therefore, the EGF-dependent protein kinase bound arylazido- β -alanyl-ATP with an apparent affinity between 1 and 10 μ M which is comparable with its affinity for ATP (Erneux *et al.*, 1983). If membrane vesicles were exposed to 100 μ M-arylazido- β -alanine, which had not been covalently bound to ATP, exposure to light resulted in no detectable alteration in kinase activity (Fig. 1, lanes A). Con-

Fig. 1. Photodependent inactivation of EGF-dependent protein kinase by arylazido- β -alanyl-ATP
A431 plasma membrane vesicles were incubated with various concentrations of azido-ATP with (L) or without (D) 2 min of photoirradiation. Remaining EGF-dependent protein kinase activity was assayed by determining the extent of the EGF receptor phosphorylation seen on autoradiography of a 7.5% polyacrylamide gel used to separate the phosphorylation products of the protein kinase reaction. The effect of 100 μ M-arylazido- β -alanine (lanes A) and 100 μ M-ATP (lanes B) on EGF-receptor kinase activity in the presence (L) and absence (D) of light was also determined.

Fig. 2. Effect of the duration of light exposure on the photodependent inactivation of EGF-dependent protein kinase by arylazido- β -alanyl-ATP

A431 plasma membrane vesicles were incubated with 10 μ M-azido-ATP with (O) and without (●) exposure to light for various lengths of time. Remaining EGF-receptor kinase activity was assayed by determining the amount of 32 P transferred from [γ - 32 P]ATP to acid-precipitable radioactivity.

versely, exposure of the kinase to 100 μ M-ATP and light also did not result in decreased protein kinase activity (Fig. 1, lanes B). Therefore, inactivation of the kinase by the analogue depended on the presence both of the ATP moiety for targeting to

the ATP-binding site and of the photoreactive group for covalent modification and inactivation of the enzyme.

The optimal light exposure time was determined by exposing membrane vesicles in the presence of 10 μ M-arylazido- β -alanyl-ATP to light for various lengths of time and measuring the remaining protein kinase activity as the rate of incorporation of 32 P into acid-precipitable protein. An exposure of 2 min gave near-maximal enzyme inactivation (Fig. 2).

To confirm that the analogue was a specific label for the ATP-binding site, various nucleotides were assessed for the ability to block the photodependent inactivation of the protein kinase by arylazido- β -alanyl-ATP (Fig. 3). ATP itself at concentrations of up to 20 mM was only partially effective at preventing photodependent inactivation. However, 20 mM-AMP-PNP could nearly prevent photodependent inactivation. AMP-PNP may have been more effective than ATP because it was not hydrolysed during the incubation with the vesicles (Yount *et al.*, 1971). GMP-PNP (20 mM) was as effective as AMP-PNP at preventing the inactivation, confirming that guanosine nucleotides are also substrates for the EGF-dependent protein kinase (Carpenter *et al.*, 1978). Even when the blocking nucleotide concentration exceeded the azido-ATP concentration by 4000-fold, complete protection of the enzyme from photodependent in-

Fig. 3. Effect of nucleotides on the photodependent inactivation of the EGF-receptor kinase by arylazido- β -alanyl-ATP

A431 membrane vesicles were incubated with 5 μ M-azido-ATP and 20mM of one of AMP-PNP, GMP-PNP or ATP with (L) or without (D) exposure to light. Remaining kinase activity was determined as described for Fig. 1.

activation was not observed. This incomplete protection may have resulted from competition between reversible binding by the nucleotides and covalent binding by the azido-ATP. It was unlikely to be due to indiscriminate inactivation by the analogue, since arylazido- β -alanine at 10-fold the concentration of analogue had no effect on the kinase activity. The requirement that arylazido- β -alanine be linked to ATP to achieve photo-dependent inactivation, the high apparent affinity of the analogue for the kinase and the ability of AMP-PNP and GMP-PNP to protect against inactivation indicate that arylazido- β -alanyl-ATP is ATP-binding-site-specific and that the inactivation is not simply a result of light exposure or incubation with ATP.

To determine the M_r of the EGF-receptor-associated peptide to which arylazido- β -alanyl-ATP binds, A431 membrane vesicles were photoaffinity labelled with arylazido- β -alanyl-[α - 32 P]ATP. Plasma membrane vesicles were prepared in such a way that proteolysis of the native receptor would be minimized (Cohen *et al.*, 1982). The pattern of labelled proteins obtained using the azido-ATP differed from that obtained using [γ - 32 P]ATP (Fig. 4, lanes 1 and 3), suggesting that the labelled proteins were ATP-binding proteins and not substrates. While several peptides were labelled, the most intensely labelled peptide had an M_r of

Fig. 4. Photoaffinity labelling of the EGF-dependent protein kinase with arylazido- β -alanyl-[α - 32 P]ATP

A431 plasma membranes were incubated with [γ - 32 P]ATP to label endogenous substrates of the EGF-receptor kinase (lane 1). Membranes were incubated with azido-[α - 32 P]ATP in the absence (lane 2) and presence (lane 3) of light. In lanes 4–11, immunoprecipitates with an anti-(EGF receptor) monoclonal antibody were prepared from solubilized membranes after labelling. Membranes were labelled with azido-ATP in the absence (lane 4) and presence (lane 5) of light prior to immunoprecipitation. Also shown: immunoprecipitation of membranes labelled with azido-ATP in the presence of light using 1 μ l of normal mouse serum (lane 6); membranes labelled with azido-ATP in the presence of light and AMP-PNP (lane 7) or GMP-PNP (lane 8) prior to immunoprecipitation with monoclonal antibody; immunoprecipitate of azido-ATP-labelled membranes treated with 0.01M-NaOH prior to running on the polyacrylamide gel (lane 9); immunoprecipitate of [γ - 32 P]ATP-labelled membranes treated with 0.01M-NaOH (lane 11) or without NaOH (lane 10) prior to polyacrylamide-gel electrophoresis. Autoradiography of lanes 1–3 and 10 and 11 was for 18 h and for lanes 4–9 for 10 days.

53000. The M_r 53000 peptide has the same M_r as the β -subunit of the ($\text{Na}^+ + \text{K}^+$)ATPase (Spector *et al.*, 1980) and a glycoprotein labelled by 8-azido-ATP in sarcoplasmic reticulum membranes (Campbell & MacLennan, 1983). A very faint band was evident in the region corresponding to the EGF receptor. These results contrast with those obtained by Buhrow *et al.* (1982) using FSBA. FSBA labelled principally the M_r 170000

receptor protein, resulting in a pattern of labelling seen when substrates are phosphorylated in the presence of [γ - 32 P]ATP. The labelling pattern obtained with FSBA may have resulted from its potential ability to label substrates of the protein kinase, which in the case of the EGF-dependent protein kinase is the EGF receptor, or because FSBA is a more-specific label for the EGF-dependent protein kinase.

To determine which of the proteins photoaffinity labelled by arylazido- β -alaninyl-ATP was associated with the EGF receptor, the membrane vesicles were solubilized after labelling and the receptor was immunoprecipitated by using a monoclonal antibody to the receptor. Immunoprecipitates of the EGF receptor have been shown to contain EGF-dependent protein kinase activity (Cohen *et al.*, 1982). Immunoprecipitation of this labelled mixture of proteins effectively enriched for the receptor and associated protein kinase and revealed that only an M_r 170000 labelled protein was specifically bound by the anti-receptor antibody (Fig. 4, lane 5). This labelling of the receptor occurred at a concentration of about 40 nM arylazido- β -alaninyl-[α - 32 P]ATP, indicating a high affinity of the kinase for this analogue. The M_r 53000 protein also appeared in the immunoprecipitates, but its binding was not specific (Fig. 4, lane 6), in that it appeared in immunoprecipitates with normal mouse serum. The labelling could be decreased by AMP-PNP (Fig. 4, lane 7) and GMP-PNP (Fig. 4, lane 8) indicating nucleotide-binding-site specificity. However, as in the photoinactivation studies, these nucleotides were not capable of completely blocking the labelling by the azido-ATP analogue. Incubation of the immunoprecipitate with alkali showed that the receptor label was labile (Fig. 4, lane 9), a property of the ATP- β -alanine ester linkage but not of tyrosine phosphate (Fig. 4, lanes 10 and 11) (Hunter & Sefton, 1980). Furthermore, the labelling was strictly photodependent (Fig. 4, lanes 2 and 4). Therefore, the labelling of the receptor resulted from linkage of the [α - 32 P]ATP to the protein through the azido group and not from tyrosine phosphorylation of the receptor due to contamination of the labelled analogue with [γ - 32 P]ATP.

To determine the effect of EGF on the photoaffinity labelling of the kinase, labelling was carried out in the presence and absence of EGF. Since the monoclonal antibody used in these experiments binds to the EGF-binding site of the receptor, we first had to ensure that the presence of EGF would not interfere with the immunoprecipitation. When the receptor was labelled with [γ - 32 P]ATP and then immunoprecipitated, the appropriate increment in receptor labelling was

Fig. 5. Effect of EGF on the photoaffinity labelling of the ATP-binding site of the EGF-dependent protein kinase. Intact A431 plasma membranes were incubated with [γ - 32 P]ATP as described in Fig. 1 in the absence (lane 1) or presence (lane 2) of 0.3 μ M-EGF. The labelling was terminated by solubilization of the membranes with RIPA buffer and the labelled EGF receptor was immunoprecipitated and run on polyacrylamide gels (Weber & Osborn, 1969). The autoradiograms of the gel are shown (lanes 1 and 2). In lanes 3 and 4 are shown autoradiograms of the immunoprecipitated EGF receptor labelled with arylazido- β -alaninyl-[α - 32 P]ATP as described in Fig. 4 in the absence (lane 3) and presence (lane 4) of 0.3 μ M-EGF.

observed (Fig. 5, lanes 1 and 2). However, when the receptor was labelled with arylazido- β -alaninyl-[α - 32 P]ATP, no effect of labelling was observed with EGF (Fig. 5, lanes 3 and 4). These results confirm that the labelling of the receptor does not result from contamination of the azido-ATP with [γ - 32 P]ATP and suggest that the kinase ATP-binding site was labelled and not the substrate site on the receptor, since labelling of the substrate is stimulated by EGF. Furthermore, the failure of EGF to stimulate labelling suggests that EGF does not stimulate the kinase by increasing its affinity for ATP.

These studies demonstrate that arylazido- β -alaninyl-ATP is an ATP-binding-site-specific photo-

affinity label for the EGF-dependent protein kinase. The kinase has an apparent affinity for this analogue that is comparable with its affinity for ATP. The use of this analogue of ATP enabled us to probe the kinase at an alternative site to that probed by FSBA. These results suggest that the EGF receptor contains a domain that binds ATP, implying that the receptor and EGF-dependent protein kinase reside in the same polypeptide chain. However, because of the spacial proximity of the receptor and kinase, it is also possible that the receptor was labelled by this photoaffinity probe because of its substrate relationship to the kinase. The agreement of the results with this analogue and FSBA (Buhrow *et al.*, 1982), however, strengthen the argument that the receptor and kinase activities reside within the same molecule. Therefore, the EGF-receptor-kinase system resembles the insulin-receptor-kinase system (Roth & Cassel, 1983; Van Obberghen *et al.*, 1983), in which the insulin receptor and protein kinase also appear to reside in a single macromolecule.

Arylazido- β -alanyl-ATP is a member of a family of azido-ATP analogues in which the length of the azido-bearing side chain can be varied (Jeng & Guillory, 1975). The use of this class of analogues could therefore give important structural information about the EGF receptor kinase and may also be useful for probing other tyrosine-specific protein kinases. Such data coupled with sequence data would give further insight into the structure-function relationships in this important molecule, and help provide a data base for comparison with other tyrosine-specific protein kinases.

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