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Cloning, expression, purification and characterization of the alternate splice Src variants for drug discovery

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Abstract

Protein tyrosine kinases (PTKs) are key members of intra- and extra-cellular signaling pathways. Aberrant signaling pathways are responsible for many human diseases, making these enzymes targets for drug development programs. The difficulty in PCR-amplification of Src due to the high G-C content was overcome using a commercial "G-C melt" reagent. The N06 Src was cloned along with the N12 and N23 neuronal variants. Neuronal variants of Src occur due to splicing within the N-loop of the SH3 domain. These variants have greater catalytic activity. Affinity purification methodologies were established that takes advantage of binding sites within the SH1 and SH2 domains. The purified enzyme is stable, without loss of activity for >1 year when frozen and more than 1 week at 4°C. A 96-well solution phase assay was developed and validated that overcomes many of the false positives and negatives generated by other assays. Studies of the catalytic mechanism have indicated that a second metal ion is essential for catalysis. Some transition metals can be substituted for the second metal ion and maintain activity while others act as dead-end inhibitors with binding constants in the sub-micromolar range. The precise role of this second metal ion is being studied.

Keywords: Src; Protein tyrosine kinases; Recombinant enzymes; Essential metal ions; Drug discovery

1. Introduction

Aberrant activity of protein tyrosine kinases (PTKs) through mutation, over-expression or activation via post-translational mechanisms is the basis of numerous human diseases that continue to plague man and remain elusive to manage (e.g. cancer, neuro-degenerative, immunological, etc.). The prototypical PTK is Src which represents a family of intracellular enzymes with nine members (Src, Yes, Fyn, Lyn, Lck, Hck, Blk, Fgr, and Yrk) not counting the alternate splice variants. Src was the first PTK to be described and there are two Nobel prizes associated with this enzyme (Peyton Rous in 1966 for his work on the Rous sarcoma virus; J. Michael Bishop and Harold Varmus in 1989 for their work on the proto-oncogene theory). While there is an enormous literature base describing its biological function, relatively little is known of its chemical function. The domains within the enzyme are referred to as Src Homology (SH) since it was found that numerous other signaling proteins have homologous domains [1]. Michael Eck's laboratory [2] has crystallized a

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truncated inactive form of the enzyme lacking the membrane-association SH4 domain. The SH4 domain is involved in membrane localization. The SH2 and SH3 domains are involved in protein–protein recognition while the SH1 domain contains the active site where the transfer of the gamma phosphate occurs from ATP–Mg to a tyrsoyl residue within the substrate protein.

Our laboratory has been developing an infrastructure to initiate a target-directed approach to the design of inhibitors specific for Src. There is no natural source of large amounts of this enzyme, which necessitated cloning and expression of recombinant enzyme. Suitable assays for biochemical screening and the lack of understanding regarding the role of metal ions have impeded the development of PTK inhibitors, problems we are beginning to surmount.

2. Experimental design

2.1. Protein expression and purification

Sf9 insect cells were grown to a density of 2.3×10^6 cells/ml and infected with baculovirus containing either the N06, N17, or N23 human Src construct. After 62 h of infection, cells were harvested by centrifugation and the cell pellets stored at -80°C. The frozen pellets, after thawing, were resuspended in the lysis media (10 ml/g of cells) of 20 mM MOPS (3-[*N*-morpholine] propane sulfonic acid) pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 μg/ml leupeptin, 5 μg/ml pepstatin A and 5 mM DTT. The suspension was homogenized in a Wheaton homogenizer, passed through a 23-gauge syringe needle and incubated for 30 min at 4°C. The homogenate was centrifuged at $35{,}000 \times g$ for 15 min at 4°C. The supernatant was recovered and the cell pellet discarded. The supernatant was loaded onto a P-tyrosine affinity column [3] pre-equilibrated with 10 mM MOPS pH 7.5, 0.05% Triton X-100, 10% glycerol, and 1 mM DTT. The column was washed with the above buffer and protein eluted using a step gradient of 0.2, 0.3, 0.5, 0.6, and 0.8 M NaCl in the MOPS buffer. Src activity eluted around 0.5 M NaCl. Fractions containing Src activity were pooled, concentrated and exchanged with the MOPS buffer using an Amicon ultrafiltration unit fitted with a YM 30 membrane.

2.2. PTK assay

The assays were performed at 30°C with poly E_4Y as the substrate as described [4]. The standard assay contains purified soluble recombinant enzyme, 0.2 mM ^{32}P-ATP (specific activity 600 dpm/pmol), and 6 mM $MgCl_2$. Variations from this are described in Figure legends. K_m and V_{max} determinations were performed with variable (1–20 μg/ml) poly E_4Y. To determine the effect of free magnesium, magnesium ion in excess of that needed to bind ATP was calculated based on the stability constant of 73,000 M^{-1} [5] which was determined under similar conditions and was experimentally shown to be the concentration of free magnesium ion in our assay [6].

3. Results and discussion

The human Src enzyme and its two neuronal variants were cloned from a human fetal brain cDNA library. The N06 Src is ubiquitous in its distribution and activated in many human cancers while the N12 and N23 variants are only expressed in neurons [7]. The N12 and N23 variants regulate the NMDA receptor [8]. The sequence of the N-loop in N06 Src is NNTEGD, while the sequence of the N12 Src is NNTRKVDVREGD, and the sequence of the loop in N23 Src is NNTRKVDVRQTWFTFRWLQREGD. PCR-amplification was considered impossible due to the >70% G-C content at the 5′ end. Amplification was achieved by utilizing the Clonetech (Palo Alto, CA) "GC-melt Kit". Protein was expressed using the baculovirus–insect cell system as described earlier with the avian Src protein [9]. Protein was purified in a single step to >70% purity using P-tyrosine-agarose affinity chromatography and its activity with poly E_4Y was determined (Table 1). All members of the Src family have a P-tyrosine binding site within their SH2 domain that can be used for affinity chromatography purification. The enzyme was found

Table 1
Activity of the human c-Src preparations

Src variant[a]	K_m poly E_4Y (μg/ml)	V_{max} (nmol min^{-1} mg^{-1})	Relative specificity (V_{max}/K_m)
N06	2.5	132	1.0
N17	3.7	857	4.4
N23	7.0	840	2.3

[a] N06 is the ubiquitous Src, while N17 and N23 are neuronal variants. All were purified by affinity chromotography as described in the text.

to have identical responses to pH, ionic strength, metal requirements, etc., as reported for the avian enzyme. The physical stability, judged by changes in catalytic activity, was determined. The enzyme is quite stable, with no loss of activity over a 96-h period when incubated at 4°C and less than 10% at ambient temperature. The enzyme is susceptible to irreversible loss of activity upon multiple freeze–thaw cycles. We routinely aliquot the enzyme into small volumes and store at −80°C, so the enzyme is stable for many years.

A high through-put assay was developed to allow rapid testing of combinatorial libraries for identification of inhibitors. The assay is essentially similar to the radiometric test tube assay using ^{32}P-ATP and poly E_4Y as substrates [4]. This is a solution-phase assay utilizing 0.45 microfiltration 96-well plates that traps the acid-precipitated product ^{32}P-poly E_4Y. The product is then analyzed by liquid scintillation counting. The assay has a typical signal of 40,000 dpm and a background noise of < 1000 dpm. Poly E_4Y (Sigma, St. Louis, MO) is a polydisperse heteropolymer and an ideal substrate as it has a similar mass to proteins (average mass = 40 kDa) and can be used with virtually every PTK. Thus, it is easy to add additional PTKs to the biochemical screen to assess the ability of a compound to inhibit catalytic activity, and assess the specificity against a range of PTKs.

Such first-round screens of chemical libraries for activity can be misleading if a solid-phase assay is used or if magnesium is replaced with manganese (Fig. 1). We have observed that the testing of some peptides as inhibitors gave results that were dependent upon whether a solution-phase or solid-phase assay was used. This may be due to the conformation restrictions of the enzyme when bound as a solid-phase immunoprecipitate. Relative to the choice of metal ion to use, historically, the pharmaceutical industry has replaced magnesium with manganese since most PTKs exhibit lower K_m values for ATP

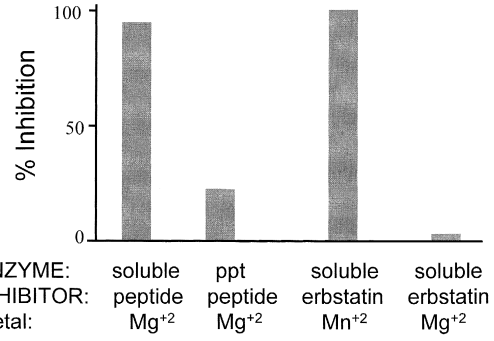

Fig. 1. Effect of assay conditions on the identification of inhibitors. The peptide is cyclo (DNEYAFXQfP) where X is *p*-carboxyphenylalanine, and erbstatin is a "known" PTK inhibitor [6]. The use of a solid-phase assay in which the enzyme is immunoprecipitated generates a "false negative" with the peptide inhibitor, while the use of manganese with erbstatin generates a "false positive".

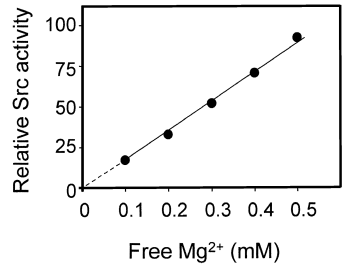

Fig. 2. The kinase activity of Src as a function of the concentration of free magnesium ion in the assay. Enzyme activity was determined with 0.2 mM ATP–Mg and the indicated free magnesium concentration calculated based on the binding of magnesium to ATP. The dashed lines are extrapolations as it is not possible to experimentally achieve a concentration of zero free magnesium.

and higher signal-to-noise ratios. However, transition metals such as manganese catalyze a destruction of compounds containing a catechol moiety [10]. Unfortunately, many natural products identified as PTK inhibitors contain a catechol moiety. The compound erbstatin [11] is an example of such a compound that inhibits in the presence of manganese and not magnesium. In effect, this generates "false positives".

While initially studying the response of the enzyme to metal ions, we noticed that the amount of magnesium required to fully activate the enzyme was much greater than that to form the ATP–Mg complex (Fig. 2). Further studies have indicated that there is an absolute requirement for a second metal ion which serves as an essential cofactor and this requirement is not mechanistically the same for all PTKs [6]. Substitution studies with different metal ions indicated that Mg^{+2} could be replaced with Co^{+2} or Ni^{+2}, albeit with reduced activity. In addition, some metal ions such as Zn^{+2} serve as dead end inhibitors and will be useful as a pre-reactive

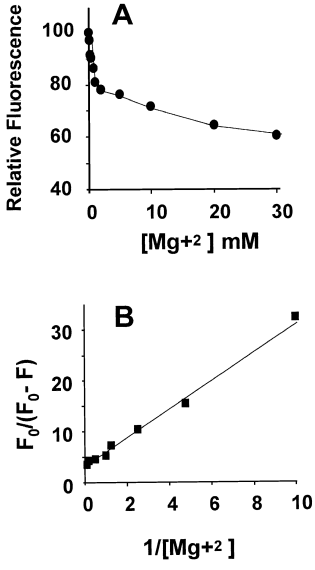

Fig. 3. Binding of free magnesium independent of ATP. The enzyme was excited with the intrinsic excitation wavelength of tyrosine at 280 nm and the fluorescence emission measured at 340 nm. (A) Ligand binding as measured by the change in intrinsic fluorescence of the apoenzyme with increasing concentration of $MgCl_2$. (B) Double reciprocal plot of $1/r$ vs. $1/[Mg^{2+}]$ where $r=(F_0-F)/F_0$, F_0 is the initial fluorescence of the enzyme and F is the fluorescence in presence of the ligand. The slope of such plot is equivalent to the K_d.

transition state model [12], which will aid in understanding catalysis. Zinc is particularly interesting since it binds with $>10{,}000$-fold higher affinity than magnesium [13]. We also demonstrated that one can take advantage of this high affinity to purify PTKs by zinc metal ion immobilized affinity chromatography (results to be described elsewhere).

Many other PTKs utilize the second metal ion in a manner which results in a reduction in the K_m for ATP–Mg [14], and can be seen as an ATP–$(Mg)_2$ complex in the crystal structure [15]. However, this is not the case with members of the Src or Csk family of PTKs as there is no change in the K_m for ATP–Mg, but a change in the K_m for the phosphate accepting substrate [6,13]. Furthermore, only a single magnesium ion is visible in the crystal structure [16]. This led us to determine if the apoenzyme can bind the free metal ion. The change in intrinsic tyrosine fluorescence was monitored as the free enzyme was titrated with $MgCl_2$ (Fig. 3). A K_d of 2.3 mM was calculated which is identical to the K_a of 2.5 mM determined from steady state kinetic analysis.

4. Conclusions

PTKs in general, and Src in particular, are exciting targets for drug development as their activation is associated with a large number of diseases and their inhibition is expected to not generate unacceptable toxicity. Molecular target directed drug discovery requires access to the target and functional assays that are predictive. The data presented here points to the successful acquisition of these Src variants and an assay to facilitate drug discovery. Combinatorial libraries have been screened and "lead compounds" identified. Future studies on the Src variants will focus on their pharmacological profile. The studies that indicate a role for a second metal ion will not only aid in enhancing our understanding of PTK catalysis, and how it differs among this large family of enzymes, but can also be exploited in the development of drugs.

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