Requirement for an Additional Divalent Metal Cation To Activate Protein Tyrosine Kinases†

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ABSTRACT: In addition to the magnesium ion needed to form the true phosphate-donating substrate (ATP−Mg complex), we have determined that at least one additional Mg^{2+} ion is essential for the activation of protein tyrosine kinases. This activation was investigated in detail using purified Csk, Src, and the fibroblast growth factor receptor kinase, which led to the following conclusions. (1) The catalytic activity of these kinases is dependent on the Mg^{2+} concentration present in the assay, approaching saturation at 5−8 mM $MgCl_2$, while ATP was saturated at approximately 1 mM $MgCl_2$. (2) Extrapolation to zero free Mg^{2+} at a constant ATP−Mg concentration predicts zero activity, suggesting that free magnesium ion in excess of that needed to bind to ATP is essential for the activation of these enzymes. (3) The free magnesium ion activates Csk and Src kinase activity by increasing the V_{max} but does not change their apparent $K_{m(ATP-Mg)}$. In contrast, the free magnesium ion activates the fibroblast growth factor receptor kinase activity by increasing its V_{max} and decreasing its apparent $K_{m(ATP-Mg)}$. These and previous studies with the insulin receptor tyrosine kinase suggest that receptor-type protein tyrosine kinases respond to the concentration of free Mg^{2+} differently than soluble protein tyrosine kinases. (4) With the phosphate-accepting substrate as the variable ligand, increases in the concentration of free Mg^{2+} resulted in increases in the apparent V_{max} for all tyrosine kinases examined, but the apparent K_m response is dependent on the enzyme and the substrate used. While these studies do not pinpoint a single kinetic mechanism, they do suggest that additional magnesium ion(s) is(are) an essential activator for protein tyrosine kinases in addition to being a part of the ATP−Mg complex. The difference among protein tyrosine kinases in their kinetic response to the additional divalent metal cation and the potential biological significance of such are discussed.

Protein tyrosine kinases (PTKs)[1] catalyze the transfer of the γ-phosphate of ATP to a tyrosine located in an appropriate sequence within a peptide or protein (Hunter & Cooper, 1985). PTKs are divided into two general categories, receptor-type and soluble, and each category is further divided into many subfamilies (Hunter & Cooper, 1985). The insulin receptor kinase (IRK) and the fibroblast growth factor receptor kinase (FGFR) belong to the former category, while Csk and Src are soluble PTKs. Although regions outside of the catalytic domains provide the major distinction, phylogenetic studies based on sequence homology indicate that these two groups also have significant differences in their catalytic domains, representing two divergent branches in the family tree (Hanks et al., 1988). The FGFR is the receptor for a family of mitogenic polypeptide growth factors (Burgess & Maciag, 1989). Csk regulates the Src family of PTKs (Nada et al., 1991) by phosphorylation of a tyrosine residue near the carboxyl terminus which results in their inactivation (Cooper & Howell, 1993).

Mammalian cells contain 10−30 mM total magnesium (Maguire, 1990), and the concentration of free magnesium ion is in the range of 0.3−3 mM (Altura et al., 1987). Many enzymes require magnesium for their activity, and there are two general mechanisms for such an activation (Malmstrom & Rosenberg, 1959). One is through binding to a ligand, thereby making it a suitable substrate (e.g., ATP−Mg). The other mechanism is through allosteric activation in which binding to one or more forms of the enzyme in the reaction course induces a change in its conformation. Free ATP is not a substrate for phosphotransferases, and the true substrate is an ATP−divalent metal cation complex. For this reason, phosphotransferases require divalent metal cations for their activity (Knowles, 1980). All of the published data that we have found concerning the response of PTKs to increasing magnesium ion concentration have indicated that 5−10 mM divalent cations were required for maximal activity. These concentrations, however, are much higher than that needed to complex with ATP. Our initial observation of this response to magnesium with Src led us to hypothesize that there was an additional role for magnesium ion (Budde et al., 1993).

White et al. (1984) found that Mn^{2+} stimulated the insulin receptor tyrosine kinase (IRK) autophosphorylation by decreasing its apparent K_m for ATP-Mn. Consistent with this observation, Vicario et al. (1988b) found that Mn^{2+} stimulated IRK phosphorylation of angiotensin II by a similar mechanism. These results indicated that the free metal ion and the ATP−metal complex bind to the enzyme in a mutually inclusive manner. Such an activation has not been

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[1] Abbreviations: FGFR, fibroblast growth factor receptor tyrosine kinase; GST, glutathione S-transferase; IRK, insulin receptor tyrosine kinase; PTK, protein tyrosine kinase; RCM-L, carboxymethylated−maleylated, reduced lysozyme; SH domain, Src homology domain.

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examined in depth with other PTKs. A role for a divalent metal cation other than binding to ATP has been established for the cAMP-dependent protein kinase (Mildvan et al., 1985). The ATP−metal complex binds to the enzyme first and this binding induces a conformational change in the enzyme so that a binding site for a second divalent metal cation becomes available. However, the binding of the second divalent metal ion reduces the catalytic activity (Armstrong et al., 1979).

Herein we examine why PTKs seem to require more Mg^{2+} to achieve maximal reaction rates than that required for the complexation to ATP. The actual concentration of free magnesium ion in the reaction medium was determined to be the same as calculated from the ATP−Mg stability constant. Thus, the requirement for extra magnesium ion is not due to nonspecific binding to any assay component. Analysis of the free magnesium concentration versus enzymatic activity demonstrates that the additional metal ion was essential for catalytic activity. This requirement for free magnesium ions was found with Csk, Src, and FGFR, thereby indicating that this is probably the case with every PTK. Kinetic analysis of the mechanism by which these enzymes are activated indicated that there are subtle differences that may distinguish the activation of soluble versus receptor PTKs.

EXPERIMENTAL PROCEDURES

Enzymes. Csk was expressed as a fusion protein with glutathione S-transferase (GST) and strep tag in *Escherichia coli* using a dual tag expression system (Sun & Budde, 1995). The two-step affinity purification and removal of GST were carried out as described (Sun & Budde, 1995). Csk used in this study was tagged with the 10-amino acid strep tag at the C-terminus and purified to apparent homogeneity. Avian Src was expressed in the baculovirus−insect cell expression system and purified to apparent homogeneity (Budde et al., 1993). The intracellular domain of FGFR was expressed as a GST fusion protein (a gift from Dr. X. Zhan, Holland Laboratory, Rockville, MD) and purified to near homogeneity by glutathione affinity chromatography (Zhan et al., 1994).

Kinase Assay. For assaying PTK activities, we measured the phosphorylation of $polyE_4Y$ and/or carboxymethylated−maleylated, reduced lysozyme (RCM-L) using the acid precipitation onto filter paper assay. The phosphorylation reactions were performed in 50 μL at 30 °C. At the end of the reaction time (usually 30 min), 35 μL of the reaction mixture was spotted onto Whatman filter paper squares (2 × 2 cm), which were subsequently washed in 5% TCA at 65 °C 3 times for 10 min each. The radioactivity incorporated into $polyE_4Y$ or RCM-L was determined by liquid scintillation counting. The standard phosphorylation reaction contained 0.2 mM [γ-^{32}P]ATP (600 dpm pmol^{-1}), 1 mg mL^{-1} $polyE_4Y$ or RCM-L, 6 mM $MgCl_2$, 75 mM EPPS-NaOH (pH 8.0), 5% glycerol, 0.005% Triton X-100, 0.05% 2-mercaptoethanol, and the appropriate amount of enzyme. The appropriate amount of enzyme transferred approximately 200 pmol in 30 min under the assay conditions. This activity level was the result of a compromise between the needs for high signal and linearity of the reaction. When the K_m and V_{max} were determined with regard to one substrate, the concentration of that substrate was varied from 20 to 200 μM (for ATP) or from 20 to 200 μg mL^{-1} (for the polypeptide substrate) while the concentration of the other reaction components remained constant. To determine the dependence of PTK activity on total divalent metal cation, the concentration of total $MgCl_2$ or $MnCl_2$ was varied while the concentrations of the other components were the same as those in the standard reaction.

To determine the effect of free magnesium on the kinase activities, ATP and magnesium were added so that a fixed concentration of ATP−Mg complex and the desired concentrations of free magnesium ion were achieved. Magnesium ion in excess of that needed to bind ATP was assumed to be free magnesium ion and the stability constant of ATP−Mg (73 000 M^{-1}), as determined previously under similar conditions (O'Sullivan & Smithers, 1979) was used for calculating the concentrations of ATP, magnesium ion, and ATP−Mg. The $K_{0.5}$ values were determined as described (Vicario et al., 1988a).

Determination of the Concentration of Free Magnesium. Any possible nonspecific interaction between Mg^{2+} and various reaction medium components was determined by comparing the concentration of total added magnesium and experimentally determined free Mg^{2+} in the presence of the various components. The concentration of free magnesium was determined using the fluorescent magnesium indicator Magnesium Green (Molecular Probes, Inc., Eugene, OR) (van der Wolk et al., 1995). Solutions of $MgCl_2$ (1.2 mM) in the assay medium (75 mM EPPS-NaOH (pH 8.0), 5% glycerol, 0.005% Triton X-100, and 0.05% 2-mercaptoethanol), or with the addition of either 1 μg mL^{-1} Csk, 0.2 mM ATP, 1 mg mL^{-1} $polyE_4Y$, or 1 mg mL^{-1} RCM-L, were prepared. Magnesium Green (1.5 μM) was then added and the fluorescence of the dye was determined (λ_{ex} = 475 nm and λ_{em} = 532 nm). The concentrations of free magnesium in these solutions were determined from a standard curve of Magnesium Green fluorescence in the presence of 0−2 mM $MgCl_2$.

Calculations. For a model system in which magnesium ion is only required for complexation with ATP, the apparent $K_{m(ATP-Mg)}$ and V_{max} (determined with ATP as the variable substrate), as a function of changing concentration of total magnesium, were calculated using eqs 1 and 5, which are derived below. The calculation is based on the following facts or assumptions: (1) The true substrate is ATP−Mg (1:1) and not free ATP, (2) the stability constant of the ATP−Mg complex under our assay conditions is 73 000 M^{-1} (O'Sullivan & Smithers, 1979), and (3) the apparent $K_{m(ATP-Mg)}$ determined at saturating Mg^{2+} concentration is the same as $K_{m(ATP-Mg)}$. There are two binding equilibria that govern the response of the apparent V_{max} and K_m to the total concentration of Mg^{2+}. The first is the binding of ATP to Mg^{2+} to form ATP−Mg. The second is the binding of ATP−Mg to the enzyme. When Mg^{2+} is subsaturating, the apparent V_{max} is not a true V_{max} but the velocity at a sub-saturating concentration of ATP−Mg, which approaches total magnesium concentration. Therefore, the concentration of magnesium ion can be substituted for ATP−Mg in the Michaelis−Menten equation:

$$V_{maxapp} = \frac{V_{max}[Mg^{2+}]}{[Mg^{2+}] + K_{m(ATP-Mg)}} \quad (1)$$

The $K_{m(ATP-Mg)}'$ is defined as the total ATP concentration

that produces half of the apparent V_{max} (V_{maxapp}). Its calculation can be derived in two steps:

$$\frac{V_{maxapp}}{2} = \frac{V_{max}[ATP{-}Mg]}{[ATP{-}Mg] + K_{m(ATP-Mg)}} \quad (2)$$

Substituting eq 1 for V_{maxapp} in eq 2 and solving for [ATP−Mg]:

$$[ATP{-}Mg] = \frac{[Mg^{2+}]K_{m(ATP-Mg)}}{[Mg^{2+}] + 2K_{m(ATP-Mg)}} \quad (3)$$

The second step is to calculate the total ATP concentration needed to produce the required ATP−Mg concentration at any given concentration of total magnesium. The stability constant can be equated to the ratio of the ATP−Mg complex to the concentrations of free magnesium ion ([Mg^{2+}] − [ATP−Mg]) and free ATP ([ATP] − [ATP−Mg]):

$$\frac{[ATP{-}Mg]}{([Mg^{2+}] - [ATP{-}Mg])([ATP] - [ATP{-}Mg])} = K_s \quad (4)$$

Rearranging eq 4 to solve for ATP:

$$[ATP] = \frac{[ATP{-}Mg]}{K_s([Mg^{2+}] - [ATP{-}Mg])} + [ATP{-}Mg] \quad (5)$$

This ATP concentration will give half of the apparent V_{max} (eq 1) and is defined as the apparent $K_{m(ATP-Mg)}'$.

RESULTS

The physiological substrates of Csk are PTKs belonging to the Src family. Since their own kinase activities interfere with the accurate measurement of Csk activity, it is not feasible to use Src family PTKs as the substrates for the current study. Another substrate for Csk with a good reaction velocity is the copolymer $polyE_4Y$, which has been commonly used for analysis of PTK activities. With this substrate and the filter paper assay, the reaction follows Michaelis−Menten kinetics. Technically, over 100 reactions can be performed at the same time with good precision and accuracy. The background (minus polypeptide substrate) is routinely less than 3% of the signal and standard error is less than 5%. RCM-L is a less effective substrate for Csk, yet it provides an alternative substrate for the validation of the kinetic determinations. Physiological substrates of Src and FGFR other than their own autophosphorylation have not been identified, but both enzymes can phosphorylate $polyE_4Y$ and RCM-L.

The dependence of Csk and Src activity on the concentration of divalent metal cations is shown in Figure 1. There is no activity for either enzyme in the absence of divalent metal cations. Maximal Csk activity was achieved at about 8 and 4 mM $MgCl_2$ with $polyE_4Y$ and RCM-L, respectively (Figure 1A). With RCM-L as the substrate, higher concentrations of $MgCl_2$ produced a minor inhibition of Csk activity. The optimal concentration of $MnCl_2$ is about 2 mM and higher concentrations resulted in markedly lower enzyme activities with both substrates (Figure 1B). While the

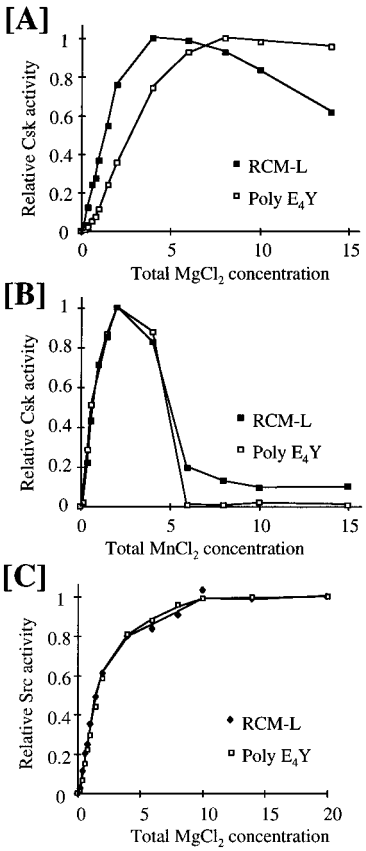

FIGURE 1: Dependence of Csk and Src activity on the presence of divalent metal cations for their activity. Protein tyrosine kinase activity was assayed in the presence of either $MgCl_2$ or $MnCl_2$ at the indicated concentrations. The phosphate accepting substrate used is indicated. (A) Csk activity was assayed with increasing concentrations of $MgCl_2$. The optimal specific activity with RCM-L = 1.9 nmol mg^{-1} min^{-1} and with $polyE_4Y$ = 110 nmol mg^{-1} min^{-1}. (B) Csk activity was assayed with increasing concentrations of $MnCl_2$. The optimal specific activity with RCM-L = 2.5 nmol mg^{-1} min^{-1} and with $polyE_4Y$ = 26 nmol mg^{-1} min^{-1}. (C) Src activity was assayed in the presence of increasing concentrations of $MgCl_2$. The optimal specific activity with RCM-L = 122 nmol mg^{-1} min^{-1} and with $polyE_4Y$ = 370 nmol mg^{-1} min^{-1}.

requirement of a divalent metal cation for Csk activity is consistent with an ATP−metal complex as the true substrate, it also raised the possibility of an additional activating role for the divalent metal cations. We calculated that ATP in the assay (0.2 mM) was 95% saturated at 0.45 mM Mg^{2+} or 0.38 mM Mn^{2+}. Yet at these concentrations, Csk has less than 15% of its maximal activity with RCM-L and less than 5% with $polyE_4Y$. Similar response curves were observed with Src (Figure 1C) and FGFR (data not shown). It was noted that the response curves of Csk activity to the changes in the concentration of $MgCl_2$ were different with $polyE_4Y$ and RCM-L. This was not caused by any difference in chelation of magnesium cation by the substrates, since such a difference was not observed with Src (Figure 1C) or FGFR (data not shown).

To be certain that the additional magnesium requirement is not a result of nonspecific chelation by the components of the reaction medium, the interaction between Mg^{2+} and each of the components was examined. This was performed by comparing the concentration of total magnesium versus the experimentally determined concentration of free Mg^{2+} in solutions containing 1.2 mM $MgCl_2$ in the assay medium alone or with the addition of each of the components (Table

Table 1: Binding of Mg^{2+} to the Components in the PTK Reaction Medium

reagent	free Mg^{2+} [a] (mM)	maximum Mg^{2+} bound[b] (mM)
control	1.2 (±0.01)	0.01
ATP (0.2 mM)	1.0 (±0.02)	0.22
Csk (1 μg mL^{-1})	1.3 (±0.2)	0.1
$polyE_4Y$ (1 mg mL^{-1})	1.2 (±0.1)	0.1
RCM-L (1 mg mL^{-1})	1.2 (±0.2)	0.2

[a] The concentrations of free Mg^{2+} in solutions containing 1.2 mM $MgCl_2$ (control) or with the addition of the indicated reagent were determined using Magnesium Green as described in the Experimental Procedures. All solutions are in the PTK assay medium given in Experimental Procedures. The standard errors are given in parentheses. [b] Maximum Mg^{2+} bound = total Mg^{2+} − (free Mg^{2+} − standard error). This represents the upper limits to the amount of Mg^{2+} that is chelated by the given reagent.

1). In the presence of 0.2 mM ATP, the concentration of free Mg^{2+} was reduced to 1 mM. This confirmed the formation of ATP−Mg complex under these conditions, as expected on the basis of the previously determined binding constant between ATP and Mg^{2+}. The presence of Csk, $polyE_4Y$, or RCM-L at the maximal concentrations used did not result in a significant loss of free magnesium ion. These results demonstrated that the components of the reaction did not nonspecifically chelate magnesium and that the additional requirement of free magnesium ion was indeed a property of these enzymes. These results, however, do not investigate the binding of magnesium to Csk in equimolar quantities, since the method is not sensitive enough to detect the loss of free magnesium from such an interaction.

Although Csk has a preference for Mn^{2+} over Mg^{2+} at low millimolar concentrations, an observation reported previously (Okada & Nakagawa, 1988, 1989) and observed by us, we decided to focus our effort on the effect of magnesium for two reasons. First, since the concentration of Mg^{2+} is over 100 times greater than that of Mn^{2+} in the cell, the observed preference by PTKs for Mn^{2+} over Mg^{2+} is likely of no physiological significance. A case in point is a mutant of a viral reverse transcriptase, which is fully active *in vitro* in the presence of Mn^{2+} yet inactive in the presence of Mg^{2+}. This mutant enzyme has no biological activity in the cell, where Mg^{2+} is the predominant divalent metal cation (Blain & Goff, 1996). This example demonstrates the misleading potential of *in vitro* results in the presence of Mn^{2+}. Second, higher concentrations of Mn^{2+} resulted in markedly reduced kinase activity in the PTK activity assays (Figure 1B). When greater than 5 mM $MnCl_2$ was present in the assay, white cottonlike precipitates become readily visible, a common observation made with transition metals in the presence of proteins (Zaworski & Gill, 1988). This nonspecific metal−protein interaction is probably responsible for the decrease in PTK activities at $MnCl_2$ concentrations greater than 2 mM (Figure 1B) and further complicates the kinetic analysis.

Classification of an activator as essential or nonessential depends on whether or not the reaction can proceed in its absence. Complicating this determination for PTKs is the fact that Mg^{2+} is also required for the formation of the ATP−Mg complex. To separate the effects of magnesium as an activator from being a part of a substrate (ATP−Mg), we determined the Csk kinase activity in the presence of 0.2 mM ATP−Mg with different concentrations of free Mg^{2+}

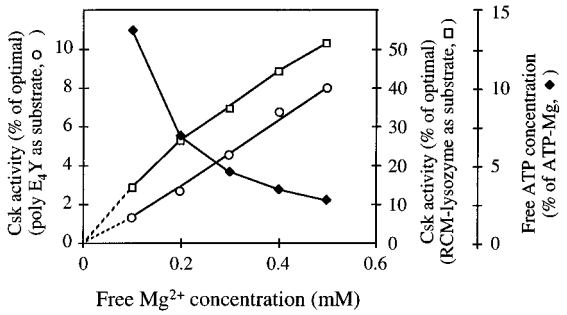

FIGURE 2: Csk activity as a function of the concentration of free Mg^{2+}. Csk activity was determined with 0.2 mM ATP−Mg and the indicated free magnesium concentration. The dashed lines are extrapolations from the determined Csk activity at different concentrations of free Mg^{2+}. The method for calculating the concentrations of free ATP, ATP−Mg complex, and free magnesium is given in the text.

(0.1−0.5 mM). The concentration of ATP−Mg and indicated concentrations of free Mg^{2+} were achieved by adding ATP and $MgCl_2$ based on the previously established stability constant (O'Sullivan & Smithers, 1979). The results with $polyE_4Y$ and RCM-L at concentrations of free Mg^{2+} below 0.5 mM are shown in Figure 2. Two points should be considered in interpreting the results. First, the combination of 0.2 mM ATP−Mg and zero free Mg^{2+} cannot be achieved experimentally; therefore, the effect of zero free Mg^{2+} can only be extrapolated from higher Mg^{2+} concentrations. Using either $polyE_4Y$ or RCM-L, Csk activity declines with the decrease in free Mg^{2+} concentration, and the trend points to zero activity when the concentration of free magnesium approaches zero (Figure 2). Second, the concentration of free ATP increases with the decrease in free Mg^{2+} concentration, and free ATP has been suggested to be a competitive inhibitor of kinases (Morrison, 1979). Therefore, it is possible that the increase in Csk activity was the result of the decrease in inhibition by free ATP. For this reason the free ATP concentration is also plotted in Figure 2. Consideration of the relative concentrations of free ATP and ATP−Mg suggests that it is unlikely that the increase in Csk activity is the sole result of the decrease in ATP inhibition. Similar analyses indicated that divalent metal cation in excess of that required for binding to ATP is also essential for the activation of Src and FGFR.

We are aware of other potential explanations for such an apparent activation by the divalent metal cation, such as a nonspecific ionic effect, stabilization of the enzymes, or chelating and removing inhibitors from the reactions. It is unlikely to be a nonspecific ionic effect since addition of NaCl to increase the ionic strength in the assay inhibits the PTK activity. Conditions used in our assay were optimized with regard to enzyme stability and other potential activity-stabilizing additives do not further affect the stability of the activity. The enzymes are stable in the absence of divalent metal cations. This excludes the possibility that the lack of activity in the absence of free divalent metal cations is due to instability. The possibility of the presence of an inhibitor that is chelated and removed by divalent metal cations is also not likely, since all three enzymes are purified to apparent or near homogeneity, and each was expressed and purified in a different manner. Furthermore, the fact that magnesium is essential for the activity of all three enzymes argues against all these alternative explanations.

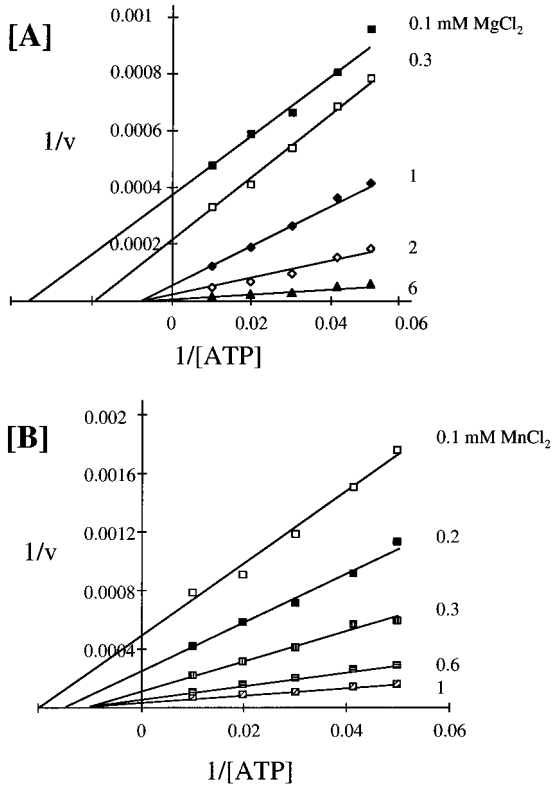

FIGURE 3: Double-reciprocal Lineweaver−Burk plots with ATP as the variable substrate at different concentrations of Mg^{2+} or Mn^{2+} within the assay. Csk activity was measured at 1 mg mL^{-1} $polyE_4Y$ and variable concentrations of ATP (micromolar) and the indicated concentrations of $MgCl_2$ (A) or $MnCl_2$ (B).

We next determined the kinetic effects of increasing concentration of Mg^{2+} and Mn^{2+} on Csk phosphorylation of $polyE_4Y$ with ATP as the variable substrate (Figure 3). The apparent $K_{m(ATP-Mg)}'$ [2] was approximately 30 μM at 0.1 mM $MgCl_2$ and increased with increasing concentration of Mg^{2+}. It reached a maximal value of 160 μM in the presence of 1 mM $MgCl_2$. The apparent V_{max} was 2.7 nmol min^{-1} mg^{-1} at 0.1 mM $MgCl_2$ and increased to 92.5 nmol min^{-1} mg^{-1} with increasing concentration of $MgCl_2$ up to 6 mM (Figure 3A). A similar activation pattern was observed with Mn^{2+} (Figure 3B). In the low millimolar range where Mn^{2+} stimulates Csk activity, the apparent $K_{m(ATP-Mg)}'$ and V_{max} of Csk have similar responses to that with increasing concentration of Mg^{2+} (Figure 3B). Initially, the apparent $K_{m(ATP-Mg)}'$ increases with increasing concentration of the divalent metal cations. This indicated that more ATP−metal complex was formed with the increasing concentration of divalent metal cations. After ATP is saturated, increasing concentration of the divalent metal cations has no effect on the apparent $K_{m(ATP-Mg)}$. In contrast to the effect on the apparent $K_{m(ATP-Mg)}$, the apparent V_{max} continued to increase with higher concentrations of $MgCl_2$.

We separated the effect of magnesium ion binding to ATP from the activation effect of free magnesium ion by calculating the expected effect based on a single ATP binding site. The V_{maxapp} and $K_{m(ATP-Mg)}'$ values were calculated using eqs 1 and 5 and compared with the observed values (Figure 4).

[2] The Michaelis constants for ATP−Mg is denoted $K_{m(ATP-Mg)}$. The apparent K_m values determined under conditions when ATP is not saturated with magnesium, such that there is free ATP present, are denoted as $K_{m(ATP-Mg)}'$.

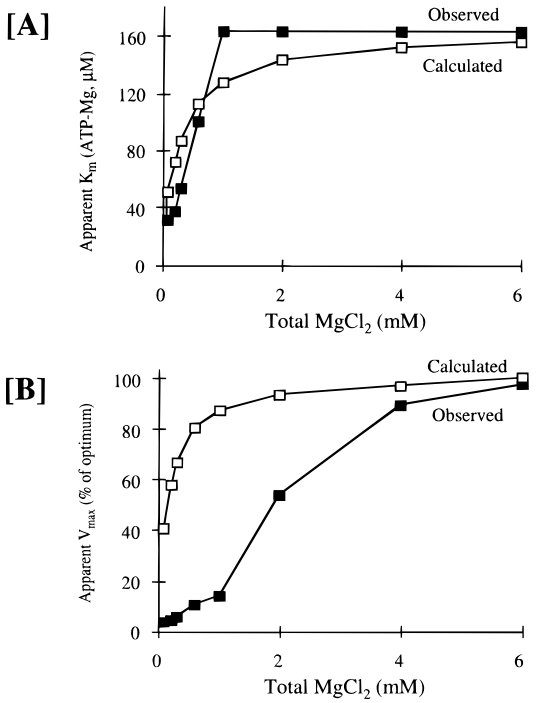

FIGURE 4: Comparison between observed and calculated apparent K_m and V_{max} values with ATP−Mg as the variable substrate. The observed apparent K_m and V_{max} values are from Figure 3 and the calculated values are obtained as described in the text.

The comparison indicated that the observed (from Figure 3A) and calculated apparent $K_{m(ATP-Mg)}'$ had similar responses to the changes in magnesium concentration (Figure 4A). Both values increased with the increase in magnesium concentration and approached a maximal value at about 1 mM $MgCl_2$. The discrepancy between the two curves is within the range of error in the determination of K_m values. This agreement indicated that the change in the observed apparent $K_{m(ATP-Mg)}'$ with the increase in the concentration of Mg^{2+} can be explained solely in terms of ATP−Mg formation. Once ATP is saturated at about 1 mM Mg^{2+}, additional Mg^{2+} does not affect the apparent $K_{m(ATP-Mg)}'$. In contrast, the observed apparent V_{max} has a response curve different from that of the calculated values (Figure 4B) that only considered a single role for Mg^{2+}, the complexation with ATP. The calculated apparent V_{max} approaches the maximum at 1−2 mM $MgCl_2$ but the observed value (from Figure 3A) continues to increase with increasing Mg^{2+} concentration up to 6 mM. This further increase is not the result of increasing concentration of ATP−Mg, since ATP is already saturated. It is the result of activation by free magnesium. Together these results further demonstrate that additional magnesium ion did not affect the affinity between Csk and ATP−Mg, but increased its V_{max}.

This conclusion does not agree with that reached with the study of the IRK. Vicario et al. (1988a) found that free Mn^{2+} stimulated IRK activity by increasing the apparent affinity between the enzyme and ATP−metal. The most obvious explanation for this disagreement is that the free divalent metal cations activate Csk and IRK through different mechanisms. Since we do not have access to IRK, we examined the effect of Mg^{2+} on the kinetics of two other PTKs, Src and FGFR. The results are shown in Figure 5. At concentrations above 1 mM $MgCl_2$ where ATP is saturated with magnesium ion, additional Mg^{2+} activates Src

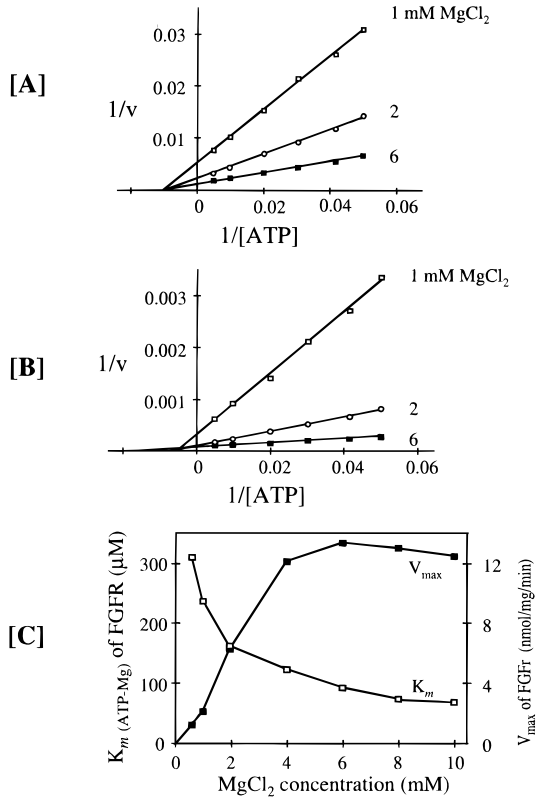

FIGURE 5: Apparent $K_{m(ATP-Mg)}'$ and V_{max} for Src and the FGFR as a function of total $MgCl_2$ concentration. Double-reciprocal Lineweaver−Burk plots of Src (A) or the FGFR (B) are determined as described in the legend for Figure 3. The velocity was in (A) nanomoles per minute per milligram or (B) and picomoles per minute per milligram. (C) Apparent $K_{m(ATP-Mg)}'$ and V_{max} for the FGFR as a function of the concentration of total $MgCl_2$ in the assay.

by increasing its V_{max} but does not affect $K_{m(ATP-Mg)}$. This pattern is similar to that with Csk activation, suggesting a similar mechanism. In contrast, increasing concentration of Mg^{2+} seems to activate FGFR by both increasing the V_{max} and decreasing its $K_{m(ATP-Mg)}$ (Figure 5B). The dual effects of increasing concentration of magnesium concentration on the FGFR are further demonstrated in a wider range of Mg^{2+} concentrations (Figure 5C). The $K_{m(ATP-Mg)}$ decreased from over 300 μM to approximately 70 μM, and the apparent V_{max} increased from 1.2 to 13.3 nmol mg^{-1} min^{-1} when the concentration of $MgCl_2$ increased from 0.6 to 10 mM. While the V_{max} effect is still different from the response of IRK, the decrease in the K_m as a result of increases in the concentration of magnesium is in agreement with the report on IRK. These results indicate that although all PTKs require magnesium ion as an essential activator, Csk and Src have a different kinetic response to the change in Mg^{2+} concentration than FGFR and IRK. Whether this is a property distinguishing soluble and receptor-type PTKs remains to be established with the study of a wider spectrum of enzymes. This difference also suggests a potential mechanism for selectively modulating the activity of different PTKs by changing the concentrations of ATP and Mg^{2+}.

We next determined the apparent K_m and V_{max} by varying the phosphate-accepting substrate, either $polyE_4Y$ or RCM-lysozyme, at one fixed ATP concentration and different concentrations of $MgCl_2$ (Figure 6). With $polyE_4Y$ as the substrate, an increase in the $MgCl_2$ concentration results in an increase in both the apparent K_m and V_{max}. The apparent K_m increased from 10 to 120 μg mL^{-1}, while V_{max} increased

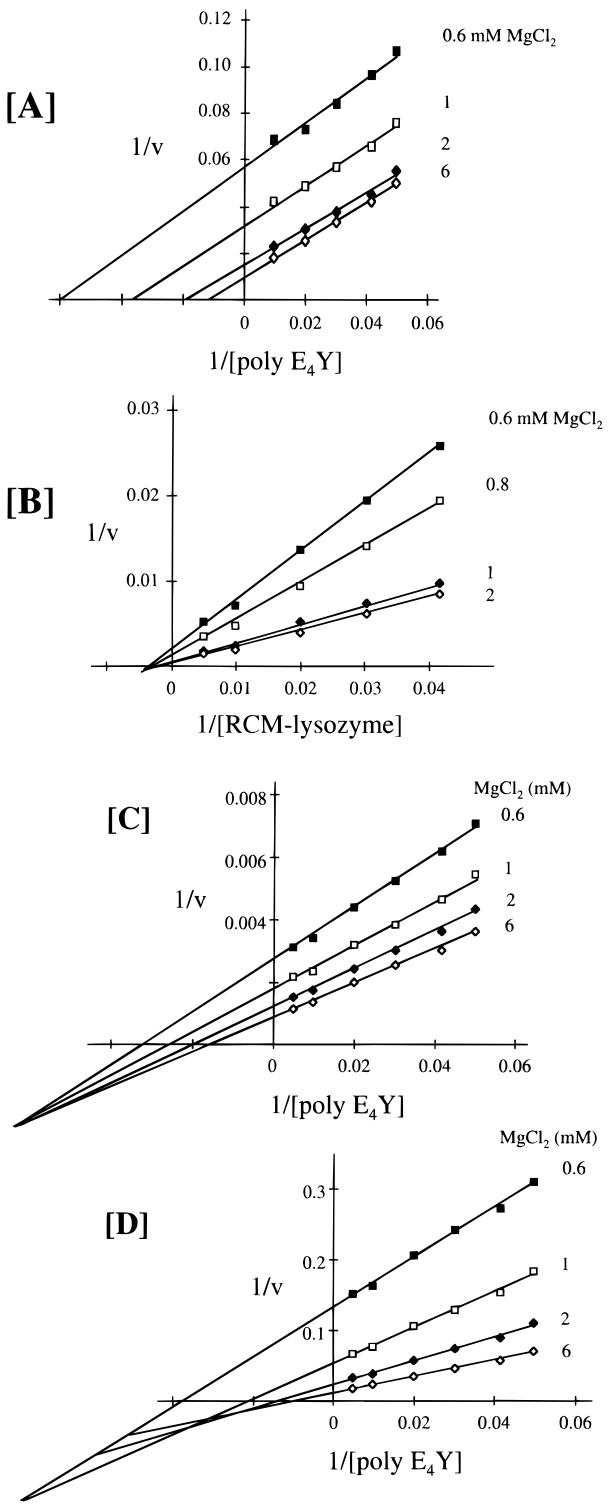

FIGURE 6: Apparent K_m and V_{max} for phosphate-accepting substrates at different concentrations of $MgCl_2$. (A) Csk activity with $polyE_4Y$ as the variable substrate; (B) Csk activity with RCM-L as the variable substrate; (C) Src activity with $polyE_4Y$ as the variable substrate; (D) FGFR activity with $polyE_4Y$ as the variable substrate. The lines in panels C and D are extended to show the different patterns of intersection. Reaction velocity units are in (A, C, D) nanomoles per minute per milligram or (B) picomoles per minute per milligram.

from 10 to 120 nmol mg^{-1} min^{-1} when the Mg^{2+} concentration increased from 0.1 to 6 mM. The efficiency of the reaction expressed as the V_{max}/K_m ratio remained essentially the same, as expected from a family of seemingly parallel lines. Surprisingly, with RCM-L as the substrate, the pattern

is markedly different (Figure 6B). Increases in the Mg^{2+} concentration had little effect on the apparent K_m for RCM-L and increased the apparent V_{max} about 4-fold, from 0.45 to 2 nmol mg^{-1} min^{-1} when the total magnesium ion concentration increased from 0.6 to 2 mM. The patterns with Src and FGFR (Figure 6C,D) appear intermediate between the two extremes, with double-reciprocal lines intersecting below the *x*-axis. It was noted that with Src the lines intersected at a common spot, but with FGFR the lines did not intersect at a common spot. A similar pattern was observed with IRK and Mn^{2+}, where increases in the Mn^{2+} concentration caused increases in both the apparent K_m for the peptide substrate, Val^5-angiotensin II, and the V_{max} value, giving lines that intersected below the *x*-axis (Vicario et al., 1988a).

Although the pattern varies with the enzyme and the substrate, this does not necessarily suggest drastically different kinetic mechanisms. In a multisubstrate reaction system, the apparent K_m and V_{max} are determined by a plethora of factors, such as the affinity between the substrate and the enzyme, the catalytic efficiency of the enzyme for a particular substrate, the kinetic mechanism, and the binding order of the substrates. Many different mechanisms can often give identical graphic patterns and one kinetic mechanism can also give different patterns depending on the combination of all these factors (Segel, 1975). Since the pattern is dependent on the substrate (polyE_4Y versus RCM-L) with Csk, the difference is more likely caused by the difference in quantitative parameters such as rate constants and/or binding constants, rather than difference in the kinetic mechanisms. In fact, polyE_4Y is about 50-fold more effective (V_{max}) than RCM-L as a substrate for Csk. We are currently investigating how the substrate specificity affects the kinetic parameters of the reaction.

Previous studies on the kinetic mechanism have concluded that the Csk-catalyzed reaction is a bi-bi sequential random mechanism (Cole et al., 1994). Our results suggest that PTKs, including Csk, should be regarded as a sequential terreactant system. Metal cations that are essential activators are generally treated as additional substrates for kinetic analysis (Segel, 1975). We eliminated some kinetic mechanisms for a Csk-catalyzed terreactant system. In an ordered mechanism, saturation by the middle reactant would create an irreversible step, and the double-reciprocal plots of the other two substrates with one variable and one changing but fixed should generate a family of parallel lines. But such patterns were not observed with any of the three substrates being saturating (data not shown), thereby excluding an ordered mechanism. This result also excluded an ordered A random BC mechanism, since in such a mechanism saturating B or C will convert it to an ordered mechanism and should produce the patterns of parallel lines of an ordered mechanism.

DISCUSSION

We demonstrated that in addition to the requirement of a divalent metal cation to complex with ATP, PTKs require an additional divalent metal cation as an essential activator. The $K_{0.5}$ values and the effect on the kinetic parameters are summarized in Table 2. The additional magnesium ion activates Csk, Src, and FGFR mainly by increasing their V_{max}. It does not affect the apparent $K_{m(ATP-Mg)}$ for Csk or Src but decreases the apparent $K_{m(ATP-Mg)}$ of the FGFR and IRK. In this aspect, Csk and Src seem to be activated by magnesium ion through a mechanism that is different from the activation of IRK and FGFR. Magnesium ion in excess of that required for ATP−Mg complex formation affected the apparent K_m (phosphate-accepting substrate) of Csk in a substrate-dependent manner. Double-reciprocal plots produced lines that seemed parallel or intersected below the *x*-axis when polyE_4Y was the variable substrate and on the *x*-axis when RCM-L was the variable substrate. With Src and FGFR, double-reciprocal plots produced lines that intersected below the *x*-axis when either polyE_4Y or RCM-L was the variable substrate and the magnesium concentration was changed. This pattern does not pinpoint any definitive reaction mechanism.

Table 2: Comparative Summary of Kinetic Parameters for the Mg^{2+} Activation of Csk, Src, FGFR, and IRK[a]

	$K_{0.5}(Mg^{2+})$ (mM)		effect of increasing free Mg^{2+} [b]			
			variable ATP−Mg		variable polyE_4Y	
enzyme	polyE_4Y	RCM-lysozyme	K_m	V_{max}	K_m	V_{max}
Csk	2.3	1.1	no effect	increase	increase	increase
Src	1.1	1.1	no effect	increase	increase	increase
FGFR	2.8	2.8	decrease	increase	increase	increase
IRK	*c*	*c*	decrease	no effect	increase	increase

[a] The data on Csk, Src and the FGFR are taken from various figures in this report and the data on IRK are taken from Vicario et al. (1988b). [b] Free Mg^{2+} is magnesium ion in excess of that bound to ATP. The effects on IRK was determined with Val^5-angiotensin II and ATP-Mn. [c] The $K_{0.5}$ value for IRK, 15 mM, was determined with Val^5-angiotensin II.

An essential activator may exert its activation by binding to the enzyme or complexing with a ligand to form a metal−substrate complex such as ATP−Mg. It does not appear that the additional divalent metal cation required for PTK activity is binding to the polypeptide to form a polypeptide−Mg complex or to ATP−Mg to form an ATP−$(Mg)_2$ complex. In such mechanisms, changes in the concentration of free magnesium would affect the apparent K_m for the affected substrate. Such a pattern was not observed with either RCM-L or ATP−Mg as the variable substrate, where changes in the concentration of magnesium ion did not affect the apparent K_m for either substrate. It cannot be excluded, however, that the peptide or ATP serves as part of the binding site for the second divalent metal cation. Crystallographic studies of magnesium coordinated in the active sites of enzymes have found that the metal is invariably coordinated by six nucleophilic groups (Villafranca & Nowak, 1992). Both ATP and the peptide substrate could provide such coordinating groups.

It is not clear how the second Mg^{2+} is coordinated in its interactions with the PTKs. It may bind to sites other than the active site of the enzymes and activate the enzymes by inducing a conformational change. Another possibility is that it is coordinated within the active site of the enzymes and directly participates in catalysis. Several kinases, such as phosphoenolpyruvate carboxykinase and pyruvate kinase, require a second metal ion for their activities (Villafranca & Nowak, 1992). Although the roles of the second Mg^{2+} vary in each case, it is always an intrinsic element of the reaction and part of the transition-state complex. We propose that the second Mg^{2+} is directly involved in the catalysis of the PTKs. Understanding this involvement could have a

significant impact on the design of PTK inhibitors.

We were unable to pinpoint the kinetic mechanism although some mechanisms were eliminated. Further elucidation of the kinetic mechanism can be expected with the help of binding studies and dead-end inhibitors competitive against ATP and the peptide substrate. It should be pointed out that after extensive studies, the definite kinetic mechanism often still cannot be identified with certainty when only two substrates were considered (Cole et al., 1994; Cook et al., 1982; Whitehouse et al., 1983).

On the basis of the results presented here and previously (Vicario & Bennun, 1990; Vicario et al., 1988a,b; White et al., 1984), it appears possible that PTK activities could be modulated by a change in the concentration of free magnesium in the cell. A magnesium transport system has been identified (Maguire, 1990). Fluxes in the intracellular concentration of free Mg^{2+} would affect the ATP−Mg concentration, the apparent K_m for their substrates (ATP−Mg and protein), and the apparent V_{max} of the enzymes. Although the total magnesium concentration is approximately 10−30 mM in the cell, the free magnesium concentration is only 0.3−2 mM (Maguire, 1990; Altura et al., 1987). Binding of insulin (Hwang et al., 1993; Sanui & Rubin, 1978) and epidermal growth factors (Grubbs, 1991; Ishijima et al., 1991) to the cell surface receptors was found to significantly increase the Mg^{2+} influx and intracellular concentration of free Mg^{2+} in a variety of cells. This increase in the concentration of free magnesium has been proposed to mediate the stimulatory effects of some hormones and growth factors. Our results suggest that PTK activities would be very sensitive to such a change and that the soluble and receptor PTKs would respond to a change in magnesium concentration in a different manner. Since magnesium activates the IRK and FGFR in part by decreasing the K_m for ATP−Mg, an increase in the concentration of free magnesium would decrease the ATP−Mg concentration required for saturation of these enzymes. This would make them more efficient at low ATP−Mg concentrations. In contrast, the ATP−Mg concentration required for saturation of Csk and Src is less affected by the cellular levels of free magnesium ion. Kinetic reaction mechanism-dependent substrate and product effects have been proposed as a general regulatory mechanism for all multisubstrate enzyme systems (Purich & Fromm, 1959). Results presented in this paper suggest that PTKs would be an ideal system for such regulation.

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