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Characterization of Phosphotyrosyl-Protein Phosphatase Activity Associated with Calcineurin*

Jonathan Chernoff**, Mary Ann Sells, and Heng-Chun Li***

Department of Biochemistry
Mount Sinai School of Medicine
of the City University of New York
New York, New York 10029

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Calcineurin purified from bovine brain is shown to possess phosphotyrosyl-protein phosphatase activity towards proteins phosphorylated by the epidermal growth factor receptor/kinase. The phosphatase activity is augmented by Ca^{2+}/calmodulin or divalent cation ($Ni^{2+} > Mn^{2+} > Mg^{2+} > Co^{2+}$). In the simultaneous presence of all three effectors, the enzymatic activity is synergistically increased. Ca^{2+}/calmodulin activates the Mg^{2+}-supported activity by decreasing the K_m value for phosphotyrosyl-casein from 2.2 to 0.6 μM, and increasing the V_{max} from 0.4 to 4.6 nmol/min/mg. These results represent the first demonstration that calcineurin can dephosphorylate phosphotyrosyl-proteins and suggest a novel mechanism of activation of this enzyme.

The phosphorylation of proteins at tyrosine (Tyr)[1] has been hypothesized to be an important step in the chain of events leading to neoplastic transformation in cells infected by certain retroviruses (1). In addition, the receptors for insulin, epidermal growth factor (EGF), and platelet-derived growth factor have been shown to possess Tyr-protein kinase activity, which may

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*** To whom correspondence should be addressed.

[1] The abbreviations used are: Tyr, tyrosine; EGF, epidermal growth factor; Tyr(P)-proteins, phosphotyrosyl-proteins; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; NP40, Nonidet P-40; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

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act as a mediator in the cellular responses to these polypeptides (2-4). As with Ser-protein kinases, the phosphorylation reaction catalyzed by tyrosine-specific kinases is readily reversible and several phosphatases capable of acting upon phosphotyrosyl-proteins (Tyr(P)-proteins) have been described (5-14). It is not known which of these enzymatic species have important physiologic function.

Calcineurin, a major calmodulin binding protein in the brain, has recently been found to possess phosphatase activity towards Ser(P)- and Thr(P)-proteins (15,16) and nonprotein phosphoesters such as p-nitrophenyl phosphate (PNPP) (17). However, whether calcineurin can function as a Tyr(P)-protein phosphatase has not been determined. In this communication, we demonstrate that calcineurin possesses activity towards Tyr(P)-proteins and that full expression of this activity is observed in the simultaneous presence of Ca^{2+}, calmodulin, and an additional divalent cation.

Experimental Procedures

The membrane fraction enriched in EGF-receptor was prepared from A-431 cells (5,18). The preparation is free of detectable Ser(Thr)-protein kinase activity. Calcineurin and calmodulin were purified from bovine brain (19). ^{32}P-Ser-casein was prepared by phosphorylating α-casein (Sigma) with [γ-^{32}P]ATP (Amersham) and cAMP-dependent protein kinase as previously described (20). ^{32}P-Tyr-casein and -histone were prepared by incubating α-casein or histone H2b (Worthington) (2 mg/ml) with A-431 membrane (0.15 mg/ml), EGF (2 μg/ml), [γ-^{32}P]ATP (0.3 mM, 3050 cpm/pmol), $MnCl_2$ (2 mM), ammonium vanadate (0.1 mM) Nonidet P-40 (NP40) (0.2%) and Hepes (20 mM, pH 7.4), in a volume of 0.3 ml for 6 hrs at 30°C. The reaction was terminated by the addition of an equal volume of 25% (w/v) trichloroacetic acid and the precipitated protein was washed as described previously (12). Phosphoamino acid analysis (20) indicated that the $^{32}P_{32}$ was incorporated into the specified amino acid residue in each ^{32}P-protein prepared.

Phosphatase activity was assayed at 30°C by determining the release of $^{32}P_i$ from ^{32}P-protein (21). The standard assay (25 μl) contained 50 mM Tris-HCl, pH 8.6, 0.5 mM dithiothreitol, 0.2 mg/ml bovine serum albumin (BSA), 0.25 μM ^{32}P-protein, 0.1 mM $CaCl_2$, 0.3 μM calmodulin and 20 mM $MgCl_2$. When other divalent cations (1 mM) were substituted for $MgCl_2$, the reaction was performed at pH 7.4 in the absence of dithiothreitol. Enzyme concentrations were adjusted so that no more than 20% of substrate was dephosphorylated. One unit of phosphatase activity: 1 nmol P_i/min at 30°C. Protein concentration was determined according to Lowry *et al* (22).

Results and Discussion

Synergistic Activation of the Calcineurin Phosphatase Activity Towards ^{32}P-Tyr-casein by Ca^{2+}, Calmodulin and Mg^{2+}: Calcineurin shows little activity in the absence of added activators (Table I). The addition of Ca^{2+} or calmodulin alone has little effect, but Ca^{2+} plus calmodulin stimulates the activity. Mg^{2+} alone is slightly more effective than Ca^{2+} plus calmodulin. The Mg^{2+} activating effect is not significantly affected by calmodulin alone, but is stimulated by Ca^{2+} alone more than 5-fold. Full expression of the phosphatase activity requires the simultaneous presence of Ca^{2+}, calmodulin and Mg^{2+}. The level of activity achieved with these three effectors is far in excess of the sum of the activity observed when these agents are added alone. Similar results are obtained when ^{32}P-Tyr-histone or PNPP is used as substrate (not shown). The data indicate (a) the synergistic interaction of Ca^{2+} and Mg^{2+} leads to partial activation (about 15% of the full activity) and (b) the synergistic interaction of Ca^{2+}, calmodulin and Mg^{2+} leads to full activation of the phosphatase.

Divalent Cation Specificity: Besides Mg^{2+}, all transition metal ions examined (Ni^{2+}, Mn^{2+}, and Co^{2+}) serve to activate calcineurin (Table II). Zn^{2+}, Cu^{2+}, Fe^{2+} are ineffective.

Table I. Synergistic activation of the calcineurin phosphatase activity by Mg^{2+}, Ca^{2+}, and calmodulin. The enzymatic activity was measured in the presence of 50 mM Tris-HCl, pH 8.6, 0.5 mM dithiothreitol, 0.2 mg/ml BSA, 0.5 μM ^{32}P-Tyr-casein, and where present, 50 μM EGTA, 0.1 mM $CaCl_2$, 0.3 μM calmodulin, or 20 mM $MgCl_2$, alone, and in the indicated combinations, for 20 min.

Addition	Phosphatase Activity (U/mg)
EGTA	0.01
Ca	0.01
calmodulin + EGTA	0
Ca + calmodulin	0.13
Mg + EGTA	0.17
Mg + Ca	0.88
Mg + Ca + calmodulin	5.89
Mg + EGTA + calmodulin	0.18

Table II. Divalent-cation specificity of the calcineurin phosphatase. The enzymatic activity was assayed with the indicated divalent cations in the presence of 50 μM EGTA or 0.1 mM Ca^{2+}, 0.3 μM calmodulin, for 20 min as described in "Experimental Procedures".

Addition	Phosphatase Activity (U/mg)	
	EGTA	Ca/calmodulin
none	0	0.26
Mg^{a} (20 mM)	0.1	2.6
Mg^{b} (20 mM)	0.1	3.9
Ni (1 mM)	4.5	17.6
Mn (1 mM)	1.9	8.2
Zn (1 mM)	0.0	0
Cu (1 mM)	0.04	0.04
Co (1 mM)	0.4	3.3
Fe (1 mM)	0.03	0
Ca (1 mM)	0.01	0.19

[a] Assayed at pH 7.4
[b] Assayed at pH 8.6

The activating effects of these transition metal ions differ from that of Mg^{2+} in several respects: (a) In the absence of Ca^{2+} and calmodulin, the transition metal ions activate the enzyme to a much higher level than observed with Mg^{2+}. However, Ca^{2+}/calmodulin stimulates the Mg^{2+}-supported activity to a much greater extent than the transition metal ion-supported activities. (b) The Mg^{2+}-supported activity is higher at pH 8.6 than 7.4, while the opposite is true for the transition metal ion-supported activity (not shown). When measured at their respective optimal conditions in the presence of Ca^{2+}/calmodulin, the order of effectiveness of these divalent cations is Ni^{2+} > Mn^{2+} > Mg^{2+} > Co^{2+} (Table II).

It has been reported that Mn^{2+} (15-17) and Ni^{2+} (23) can substitute for Ca^{2+} in the activation of the calcineurin phosphatase activity towards phosphoproteins (15,16,23) and PNPP (17). Mg^{2+} was found to be ineffective (15-17,23). In contrast, our study shows that calcineurin requires the synergistic interaction of Ca^{2+}, calmodulin, and another divalent cation, such as Mg^{2+}, Ni^{2+}, Mn^{2+} or Co^{2+} for full expression of

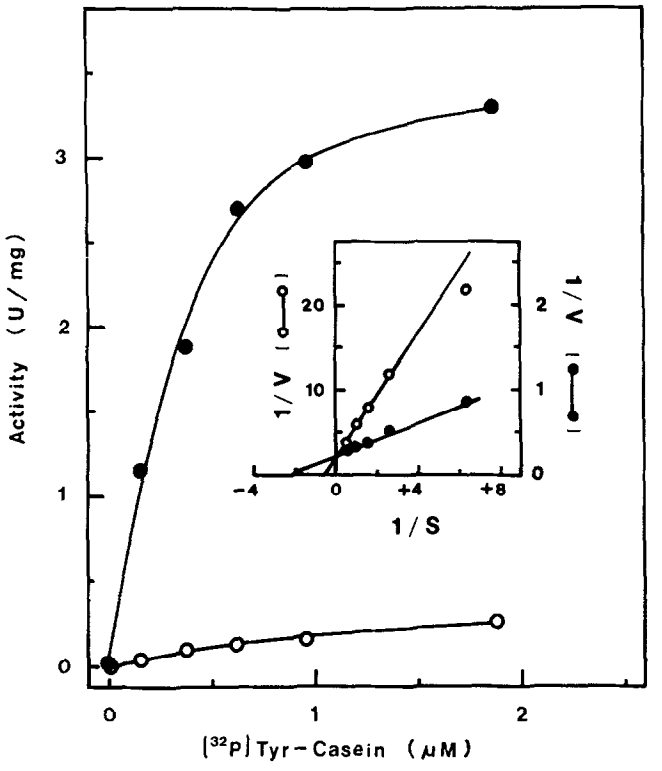

Fig. 1. Effects of Ca^{2+}/calmodulin on the kinetics of calcineurin phosphatase activity towards ^{32}P-Tyr-casein. The initial reaction rate was measured at 30° C in an incubation volume of 50 μl containing 50 mM Tris-HCl, pH 8.6, 0.2 mg/ml BSA, 0.5 mM dithiothreitol, 20 mM $MgCl_2$, and various ^{32}P-Tyr-casein concentrations in the presence of 50 μM EGTA (o-o) or 0.1 mM Ca^{2+} and 0.3 μM calmodulin (●-●). The insert shows a double reciprocal plot of the same data.

its activity. Furthermore, Mg^{2+} does not act as a Ca^{2+} analog in interacting with the B-subunit of calcineurin or with calmodulin, since it cannot substitute for Ca^{2+} in activating the phosphatase either in the absence or presence of calmodulin (Table I). These results are consistent with a model wherein Ca^{2+} and Ca^{2+}/calmodulin act as allosteric activators while Mg^{2+} acts as an essential cofactor for the catalytic action.

Kinetic Parameters: As shown in Fig. 1, the Mg^{2+}-supported activity towards ^{32}P-Tyr-casein follows Michaelis-Menten kinetics, either in the absence or presence of Ca^{2+}/calmodulin. With Mg^{2+} alone the K_m and V_{max} values are 2.2 μM and 0.4 nmol/min/mg, respectively. The addition of Ca^{2+}/calmodulin

decreases the K_m to 0.6 μM, while increasing the V_{max} to 4.6 nmol/min/mg. Thus, the major effect of Ca^{2+}/calmodulin on the Mg^{2+}-supported activity is to increase the V_{max} for this substrate. Similar experiments performed with ^{32}P-Ser-casein indicate that, in the presence of Mg^{2+} and Ca^{2+}/calmodulin, the K_m for these this substrate are slightly higher than that measured for ^{32}P-Tyr-casein (1.0 μM) but that this substrate is dephosphorylated at a much faster rate than ^{32}P-Tyr-casein (V_{max} = 121 nmol/mg/min).

Comigration of Ca^{2+}/Calmodulin- and Mg^{2+}-Stimulated ^{32}P-Tyr-protein Phosphatase Activity on Polyacrylamide Gel Electrophoresis: As shown in Fig. 2, polyacrylamide gel

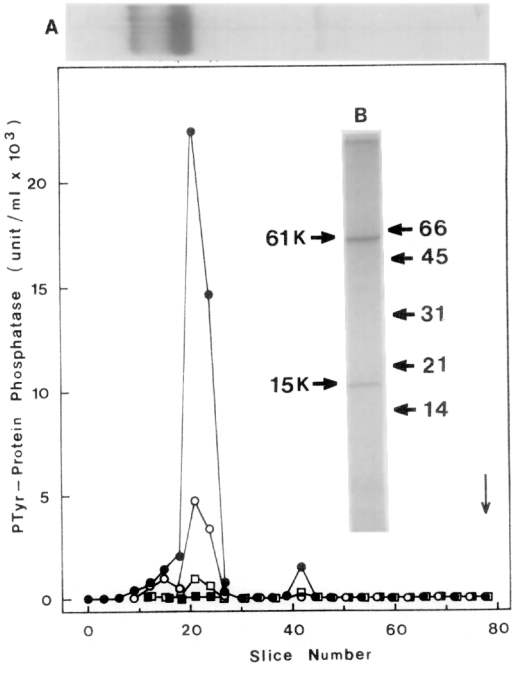

Fig. 2. Polyacrylamide gel electrophoresis of calcineurin. 25 μg of enzyme was loaded onto two lanes of a 7% polyacrylamide slab gel (25). Following electrophoresis, one lane (gel A) was stained for protein and the second was sliced and assayed for ^{32}P-Tyr-protein phosphatase activity in the presence of no effector (■—■); Ca^{2+}/calmodulin (□—□); Mg^{2+} (○—○); and Mg^{2+}, Ca^{2+}/calmodulin (●—●), as described in "Experimental Procedures". The arrow indicates the dye front. Gel B shows the protein staining pattern of the enzyme following electrophoresis on a 10% polacrylamide gel in the presence of SDS (26). The standards are phosphorylase b, BSA, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme.

electrophoresis of the calcineurin preparation results in one major and two minor protein bands (Gel A). These comigrate with the major and minor peaks of phosphatase activity. The major active species, which represents more that 90% of the phosphatase activity recovered from the gel, is partially activated by Mg^{2+} alone or Ca^{2+} plus calmodulin and fully activated in the presence of all three activators. The minor active species of lower mobility than the major one is stimulated by Mg^{2+} but not by Ca^{2+}/calmodulin. The other minor species, however, remains responsive to all three activators. These minor active species may derive from calcineurin by limited proteolysis (24). Gel B shows that two major protein bands are obtained from SDS-polyacrylamide electrophoresis calcineurin. These represent the A-(61,000) and the B-(15,000) subunits of calcineurin, respectively. The data indicate that the Tyr(P)-protein phosphatase activity is an intrinsic property of calcineurin, and that the observed effects of divalent cation cannot be attributed to a contaminating enzyme.

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