Phosphorylation of p36 in vitro with pp60^{src}

Regulation by Ca^{2+} and phospholipid

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P36 is a major substrate of the tyrosine protein kinases. P36 isolated from bovine intestine was used in phosphorylation reactions with pp60^{src}. Phosphorylation was stimulated 3–5-fold by Ca^{2+}, however the K_m was the same (2.5 μM) at high or low Ca^{2+}. Although the level of free Ca^{2+} needed for this enhanced phosphorylation was 10^{-4}–10^{-3} M, phosphatidylserine shifted the Ca^{2+} sensitivity to the 10^{-6}–10^{-5} M range. Independent evidence suggested that p36 interacts directly with liposomes containing phosphatidylserine. This raises the possibility that p36, like c-kinase, is a Ca^{2+}-activated, phospholipid-dependent protein.

Cytoskeleton *Actin* *Tyrosine kinase* *Growth factor*

1. INTRODUCTION

One of the major substrates of the tyrosine protein kinases is a protein termed p36 [1,2]. Cell fractionation studies and immunofluorescence microscopy have demonstrated that p36 is associated with the plasma membrane [3–7]. P36 is a normal cellular protein present in a variety of tissues and is especially abundant in intestine [8–10], from which it has been isolated [10]. P36 has been shown to exist as a monomer or as a complex with a 10 kDa subunit in both p36 isolated from chick fibroblasts [11] and porcine intestine [10,12]. Although intestinal p36 has been shown to bind to spectrin and actin [10], this interaction requires millimolar Ca^{2+}, levels which are orders of magnitude higher than other Ca^{2+}-binding proteins [12]. Here, it was found that Ca^{2+} enhances the phosphorylation of p36 without a change in the K_m of the reaction. Phosphatidylserine (PS) was found to shift the Ca^{2+} requirement from high Ca^{2+} (10^{-4}–10^{-3} M) to low Ca^{2+} (10^{-6}–10^{-5} M).

2. MATERIALS AND METHODS

P36 was isolated by a modification of the method in [10]. Phosphorylation was carried out using pp60^{src} immunoprecipitated from RSV-infected CEF cells with anti-C-terminal peptide rabbit antiserum (a gift from Gernot Walter [13]). The phosphorylation assays were performed in a final volume of 20 μl with 10 mM Tris, 0.05 M KCl, 5 mM $MgCl_2$, 0.5 mM DTT, pH 7.1, containing 10–20 μM unlabeled ATP and 10–20 μCi [^{32}P]ATP (Amersham) with 1 mM EGTA and the specified Ca^{2+} as described [12]. Phospholipid vesicles, prepared by sonication, were included at 100 μg phospholipid/ml, and the reaction was initiated by the addition of staph A-containing pp60^{src}. After incubation the reaction was stopped by the addition of SDS sample buffer and subjected to SDS-PAGE. Gels were stained with Coomassie blue and the p36 bands were excised and Cerenkov counted for radioactivity. For peptide maps, p36 was labeled on tyrosine with Na^{125}I using insolubilized glucose oxidase/lactoperoxidase (Enzymobeads, BioRad) or ^{32}P labeled on tyrosine with pp60^{src} as described above. Samples were run on a 15% gel, Coomassie blue stained and the band of p36 was excised and dried. The gel bands were rehydrated in 50 μg chymotrypsin/ml of 25 mM ammonium bicarbonate, incubated

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overnight at 37°C, and the soluble peptides were subjected to electrophoresis and chromatography [14].

3. RESULTS

The phosphorylation of p36 was stimulated

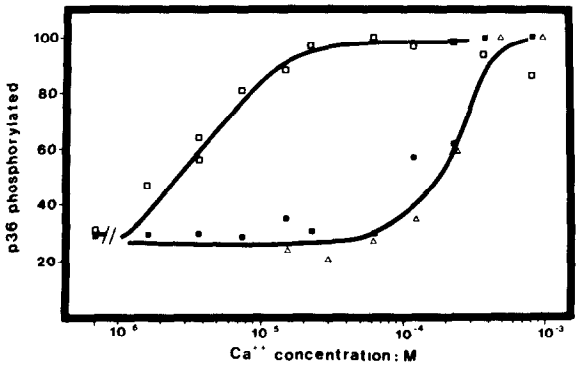

Fig.1. Effect of the free calcium level on the phosphorylation of p36. P36, in the absence of phospholipid (△) or in the presence of PC (■) or PS (□), was phosphorylated with pp60^{src} in a buffer at the specified free Ca^{2+}. Values are expressed in terms of percent of maximum level of ^{32}P incorporated into p36.

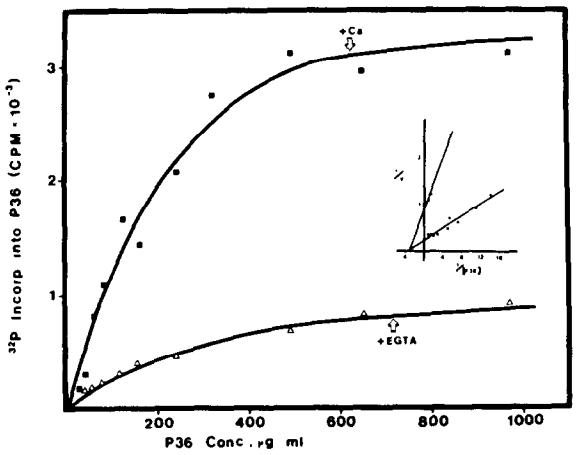

Fig.2. Effect of substrate (p36) concentration on the enzyme (pp60^{src}) activity. Increasing amounts of p36 were phosphorylated with immunoprecipitated pp60^{src} as described in the legend to fig.1 in 1 mM (final) $CaCl_2$ (■) or 5 mM EGTA (△). Inset: double-reciprocal plot of 1/rate of phosphorylation vs 1/molar p36 level. The rate is expressed in cpm × 10^{-3} incorporated per 20 min reaction (absolute rates could not be estimated since the pp60^{src} level was not known) and the units of the *x*-axis are × 10^5 M^{-1}, assuming an M_r of the p36-p10 complex of 85000 [10].

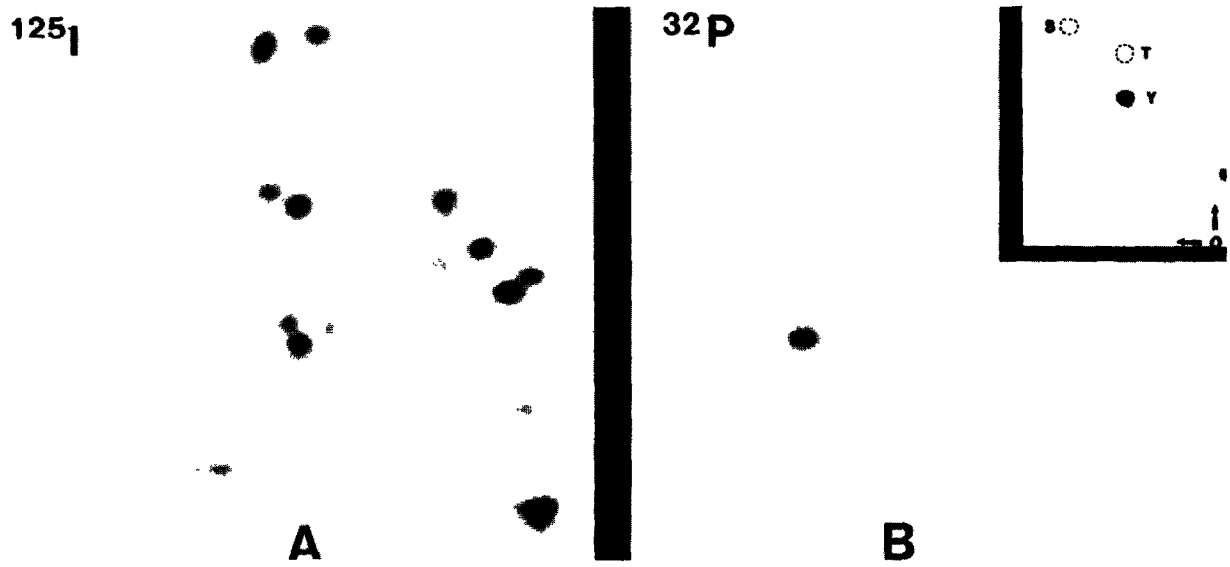

Fig.3. Resolution of tyrosine-containing peptides derived from p36. P36 was iodinated on tyrosine (A) using insolubilized lactoperoxidase/glucose oxidase and glucose or phosphorylated with immunoprecipitated pp60^{src} (B) in buffer containing 1 mM $CaCl_2$. Chymotryptic peptides were resolved by electrophoresis (left to right) and chromatography (bottom to top). Inset in B is the phosphoamino acid analysis of p36 labeled as the complex in high calcium. The positions of phosphoamino acid standards serine (s), threonine (t) and tyrosine (y) are shown.

some 3–5-fold at high Ca^{2+} (fig.1). In the absence of phospholipid the level of Ca^{2+} needed for this activation was between 10^{-4} and 10^{-3} M (fig.1), paralleling that needed for actin binding (see, e.g., fig.8 in [12]). Phosphatidylcholine (PC), PS, phosphatidylinositol (PI) and phosphatidylethanolamine (PE) were then tested for their ability to alter the Ca^{2+} sensitivity of phosphorylation. Whereas PE (not shown) and PC (fig.1) had little effect on phosphorylation, PS (fig.1) and PI (not shown) shifted the curve some 1–2 orders of magnitude such that a 3–4-fold increase in phosphorylation was observed between 10^{-6} and 5×10^{-5} M free Ca^{2+}.

Peptide maps suggest that p36 is phosphorylated by $pp60^{src}$ at a single site both at high Ca^{2+} (fig.3) or low Ca^{2+} (not shown) and only phosphotyrosine was detected (fig.3). P36 phosphorylation exhibited typical saturation by substrate (fig.2) approaching saturation at $>500\ \mu g$ p36/ml. The double-reciprocal plot revealed that the apaprent K_m for p36 was 2.5–3 μM and was unaffected by the level of Ca^{2+} (fig.2) but that the apparent V_{max} is greatly increased in the presence of Ca^{2+}.

To determine whether p36 binds directly to PS liposomes, we used a simple centrifugation assay. Whereas p36 was not pelleted with PC liposomes, it was sedimented with PS vesicles (fig.4). The co-sedimentation with PS liposomes was Ca^{2+} dependent (fig.4) and the concentration of Ca^{2+} needed to detect this interaction was in the 10^{-6}–10^{-5} M range (not shown).

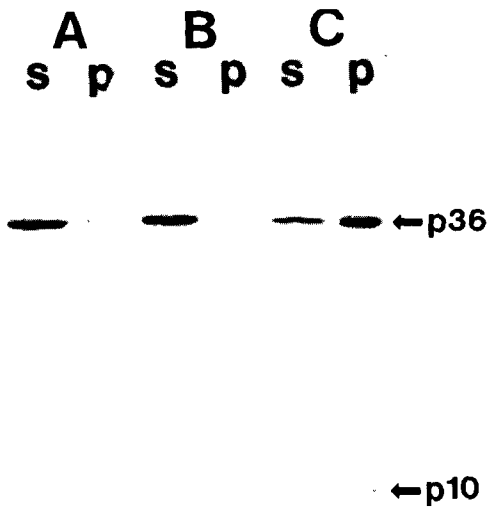

Fig.4. Co-sedimentation of p36 with PS-containing liposomes. Liposomes of PC (A) or PS (B,C) were prepared by sonication and mixed with p36 (100 mg/ml) in 10 mM Tris, 50 mM $MgCl_2$, 1 mM DTT and either 1 mM EGTA (B) or 1 mM EGTA plus 0.87 mM Ca (5×10^{-5} M free Ca^{2+}) (A,C). After incubation for 1 h, samples were centrifuged ($100000 \times g$, 20 min) and equivalent volumes of supernatant (S) and pellet (P) fractions were subjected to SDS-PAGE (15% gel) and stained with Coomassie.

4. DISCUSSION

At least 2 other proteins are known to be modulated by Ca^{2+} with the Ca^{2+} sensitivity shifted by other effectors. The Ca^{2+}-activated protease calpain is composed of 2 chains, with one subunit modulating the Ca^{2+} sensitivity of the catalytic subunit [15]. The second example is protein kinase C, in which the kinase is activated at high Ca^{2+} in the absence of lipid but is shifted to micromolar Ca^{2+} sensitivity in the presence of diacylglycerol and PS or PI [16,17]. Previous studies with p36 have shown that it binds to 2 cytoskeletal proteins, spectrin and actin, requiring millimolar Ca^{2+} for this interaction [10,12]. It seems likely that, as with Ca^{2+}-stimulated phosphorylation, the interaction between p36 and actin or spectrin will be detectable at micromolar Ca^{2+} in the presence of PS or PI.

Both in high Ca^{2+} (1 mM) and low Ca^{2+}, a typical enzyme saturation curve is observed with an apparent K_m identical in both cases at 2.5 μM. This compares favorably to the K_m of acid-treated enolase with the FPS tyrosine kinase oncogene [18] which has been reported to be in the micromolar range, with the native protein significantly higher [18]. The apparent V_{max} for p36 is quite different between high and low Ca^{2+} conditions (since the enzyme concentration was not known) which may point to a non-competitive type of inhibition under conditions of low Ca^{2+}. Here, low $pp60^{src}$ levels were used to detect changes in substrate kinetics and the resultant stoichiometry of phosphorylation was low (approx. 1%). Phosphorylation was only observed on a single tyrosine (fig.3) and other studies have shown that this is the same site used

in vivo [1]. In addition, increasing the pp60^{src} level increased the stoichiometry of phosphorylation (not shown).

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