Vol. 137, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1986 Pages 397-403

PHOSPHORYLATION OF P36 IN VITRO BY PROTEIN KINASE C

Navin C.Khannna+, Masaki Tokuda+, Sarah M. Chong, and David M. Waisman*

Department of Medical Biochemistry University of Calgary
Calgary, Alberta T2N 4N1 CANADA

Received April 11, 1986

The 36kDa subunit of protein I (p36) is a major substrate of several tyrosine protein kinases. Here we demonstrate that protein kinase C catalyzes the incorporation of 1.7 moles of phosphate per mole of protein I. Phosphorylation is absolutely dependent on the presence of both calcium and phospholipid, and is specific for serine and threonine residues. Phosphorylation of protein I by the c-AMP dependent protein kinase, phosphorylase kinase, casein kinase I, and casein kinase II was not observed. The in vivo significance of protein kinase C dependent phosphorylation of p36 is discussed.

A 36,000kDa protein, called p36 has been identified as the major phosphoprotein in the Rous sarcoma virus transformed cells (1,2) and in EGF stimulated A431 cells (3). Gerke and Weber (4) isolated the 36kDa protein from porcine intestinal epithelium and showed that the 36kDa protein was a subunit of a larger protein which they named protein I. Protein I was shown to be a tetramer consisting of two copies of both of a 36kDa and 10kDa subunit (Mr 85,000). Protein I was shown to be immunologically related to the 36kDa phosphoprotein of Rous sarcoma virus-encoded tyrosine protein kinase and the EGF stimulated tyrosine protein kinase of the A431 cells (5).

*To whom all correspondence should be addressed.

+Postdoctoral fellows of the Alberta Heritage Foundation For Medical Research. Supported by a grant from the Medical Research Council of Canada.

Abbreviations: EGF, Epidermal Growth Factor; SDS-PAGE, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis; DTT, Dithioerythritol; CM, Carboxy-methyl; DEAE, Diethylaminoethyl; EGTA, Ethylene glycol bis-(-aminoethyl ether)-N,N,N',N-Tetraacetic acid.

0006-291X/86 $1.50

P36 has been shown to exist in A431 cells not only as a component of protein I but also as a monomer (5).

Recently Sawyer and Cohen (3) have immunoprecipitated p36 from EGF stimulated, intact, Pi-labelled, A431 cells and demonstrated the presence of both phosphotyrosine and phosphoserine residues in the protein. Since the previous data (6) suggested that p36 was in vitro substrate of the purified EGF receptor/kinase these authors concluded that the EGF receptor/kinase catalyzed the phosphorylation of p36 on tyrosine residues. The protein kinase responsible for EGF stimulated serine phosphorylation of p36 has never been identified. In the present communication, the 36kDa subunit of protein I is identified as an in vitro substrate of protein kinase C. This data presents the possibility that the EGF dependent activation of protein kinase C may result in the serine phosphorylation of p36 in vivo.

Materials and Methods

Calcium and phospholipid-dependent protein kinase C was purified from bovine brain as described (7). Protein I (36K and 10K) was purified from bovine lung according to the procedure of Shaddle et al (5). [γ-^{32}P] ATP (10 Ci/mmol) was purchased from New England Nuclear. DEAE-cellulose (DE-52) and CM-cellulose was obtained from Whatman. Phenyl-sepharose-4B was from Pharmacia. 1,2-diolein, L-α-phosphotidyl-L-serine, diisopropylflurophosphate (DIFP), soyabean trypsin inhibitor, phosphoserine, phospho- threonine, and phosphotyrosine were obtained from Sigma Chemical Company. Reagents for protein determination and gel- electrophoresis were obtained from Bio-Rad . Calcineurin was a generous gift from Dr. J.H.Wang (University of Calgary). Casein kinase I, casein kinase II and phosphorylase kinase were generous gifts from Dr. T.S. Singh (University of Calgary).

Calcium-activated phospholipid-dependent protein kinase was assayed by measuring P_i incorporation from [γ-^{32}P]ATP into protein I as described by (7). The assay was carried out in a total volume of 200ul containing 25mM Tris-HCl, pH7.5, 10mM magnesium chloride, 0.5mM DTT, 100ug L-α-phosphatidyl-L-serine, 10ug diolein, 12.5uM [γ-^{32}P]ATP (1400cpm/pmol), 600uM calcium chloride, and enzyme protein. Aliquots of phosphotidyl serine (5mg/ml) and diolein (5mg/ml) were mixed in chloroform and evaporated under nitrogen gas. The residues were resuspended in 20mM Tris-HCl, pH7.5, and sonicated for a total time of 5 minutes on ice with a Branson sonicator. Phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP. Incubations were carried out at 30°C for various time intervals up to two hours. Reaction was terminated by the addition of 1ml of 25% trichloroacetic acid containing 2% sodium pyrophosphate. The acid precipitated material was collected

on Whatman 3mm filterpaper funnel and washed five times with 4mls of 5% TCA containing 1% sodium pyrophosphate. 10 mls of scinti-verse was added to the filters and the radioactivity determined. Control experiments with enzyme protein alone in the absence of either/or calcium and phospholipid were also carried out by including 1mM EGTA (in the absence of calcium chloride) or replacing the phospholipid mixture with 20mM Tris-HCl, pH7.5, containing 10mM magnesium chloride. Phosphorylase kinase activity was assayed at 20mM Tris, pH7.5 containing 10mM magnesium chloride and 0.1mM calcium chloride. Endogenous phosphorylating activity of all kinases was determined in the absence of substrate and served as blanks.

To evaluate the effect of calcineurin or alkaline phosphatase on the calcium and phospholipid-dependent phosphorylation of p36, the reaction was terminated by addition of 2mM EGTA to remove the calcium. Calcineurin (5µg) or alkaline phosphatase (5µg) was added to the reaction mixture and incubation continued for another 30 minutes. Aliquots were removed at 5 and 30 minutes for SDS-PAGE.

SDS-PAGE was carried out according to the procedure of Laemmli (8). Coomasie Blue was used to visualize the protein bands on the gel. For autoradiography the stained gel was dried on a filter paper with a gel dryer and then exposed to X-Omatic AR film in a Kodak X-Omatic cassette at -70°C.

Phosphoamino acid analysis was performed according to the procedure of Wong et al (9). Gel bands containing the phosphorylated 36kDa subunit were cut from the stained gel. The p36 was electroeluted and dialyzed against water to remove the electrode buffer. To the dialysate, 50µg of bovine serum albumin was added and the protein was precipitated with 20% TCA. After centrifugation the protein pellet was washed first with ice cold ethanol and then with ethanol, ether mixture (1:1, v/v). The washed pellet was hydrolyzed with 6N HCl for 3 hours at 110 C. The solvent was evaporated using a Savant speed vaccum. The hydrolysate was resuspended in 60µl of water and an aliquot was applied to 20 x 20 cm precoated cellulose thin-layer plates. A solution containing phosphoserine, phosphotyrosine and phosphothreonine (50mM each, 2ul) was spotted with the sample. Electrophoresis was carried out at pH3.5 at 1000V using pyridine/acetic acid/water, 1:10:189 (v/v). The plate was then dried and exposed for autoradiography. After autoradiography the plate was sprayed with ninhydrin reagent and baked at 100 C for 5 minutes to allow visualization of the phosphoamino acid standards.

Results

Protein I was purified from bovine lung according to Shaddle et al (5). The purified protein gave two bands of 36kDa (p36) and 10kDa on SDS-PAGE (figure 1). Consistent with the observations of Gerke and Weber (4) gel permeation chromatography of the purified protein yields a single protein peak of Mr 85,000 (data not shown), therefore suggesting the absence of the monomeric form of the 36kDa subunit.

Incubation of protein I (36k and 10k) with protein kinase C and analysis by SDS-PAGE autoradiography suggest that Pi was

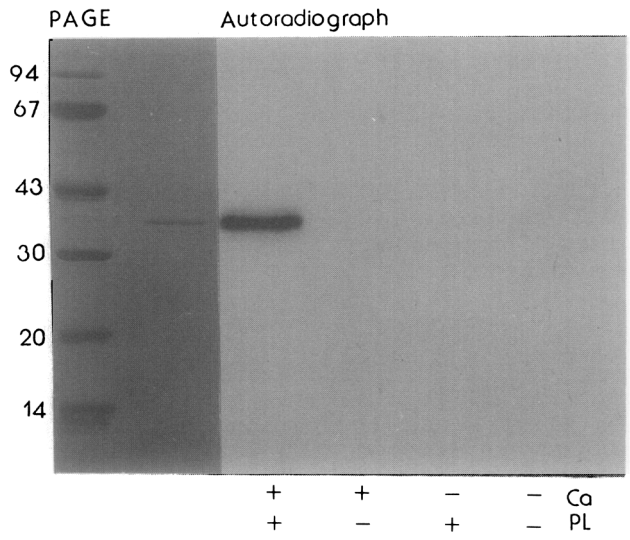
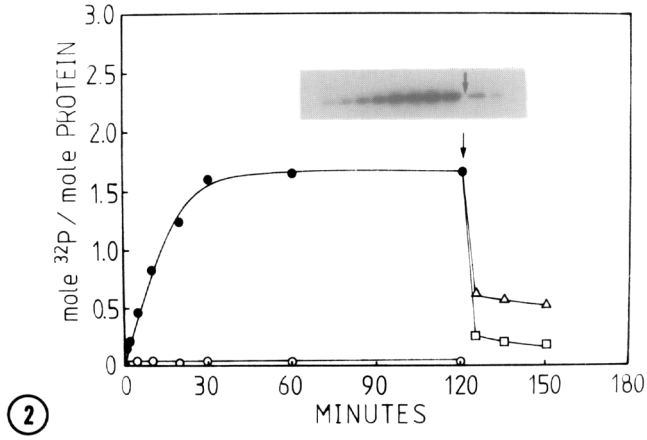


Figure 1. One-dimensional SDS polyacrylamide gel (left) and autoradiography (right) of the p36 preparation (20 µg/ml) after in vitro phosphorylation by protein kinase. The numbers indicate the mobilities of molecular mass standards in kilodaltons (kDa): phosphorylase b (94); bovine serum albumin (67); ovalbumin (43); carbonic anhydrase (30); soyabean trypsin inhibitor (20.1); α-lactalbumin (14.4).

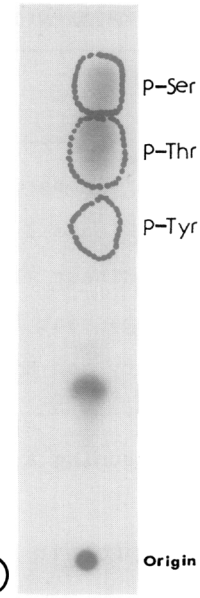
incorporated directly into the 36kDa subunit of the protein I (figure 1 inset). The phosphorylation of p36 was totally dependent on the presence of both calcium and phospholipid (figure 1). Figure 2 presents a time course of the protein kinase C catalyzed phosphorylation of p36. Assuming Mr 85,000 for the tetramer (5), 1.7 moles of phosphate/mol protein I were incorporated after 60 minutes. The addition of calcineurin or alkaline phosphatase resulted in the rapid dephosphorylation of p36 (figure 2, inset).

In order to identify the phosphoamino acid residues of p36 the protein was phosphorylated by protein kinase C and partially hydrolyzed with 6 N HCl and analyzed by chromatography on thin cellulose sheet. The autoradiograph is presented in figure 3. Radioactivity was detected on phosphoserine and phosphothreonine.

In addition to protein kinase C, protein I was tested as a substrate for several other protein kinases. As shown in table 1 the c-AMP



(2)



(3)

Figure 2. Time course of phosphorylation and dephosphorylation of p36. Purified p36 preparation was phosphorylated by protein kinase C under various conditions. Aliquots were taken at different time intervals for liquid scintilation spectrophotometry and for autoradiography. Calcineurin (20µg/ml) or alkaline phosphatase (20µg/ml) was added at the arrow and reaction continued. Inset: Shows the autoradiograph of SDS-PAGE of the time course reaction of phosphorylation and dephosphorylation. Time course is as follows: 1', 2', 5', 10', 20', 30', 60', 120', 125', 150'. The arrow indicates the addition of Calcineurin or Alkaline Phosphatase.

● in the presence of Calcium (0.6mM) and Phospholipid (see text)
○ in the absence of Calcium and Phospholipid
△ Calcineurin
□ Alkaline Phosphatase

Figure 3. Phosphoamino acid analysis of the phosphorylated p36. Portions containing p36 were cut from the SDS-PAGE and electroeluted with 2% Agarose gel. Partial acid hydrolysis was performed and phosphoamino acids were seperated on a cellulose plate by electrophoresis (1000V, 60min) at pH3.5 (pyridine/acetic acid/water, 1:10:189) as described under "Materials and Methods". Marker phosphoamino acids are phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr), identified by ninhydrin staining. Autoradiography with an intensifying screen was carried out on a Kodak X-omatic AR film.

dependent protein kinase, phosphorylase kinase, casein kinase I (10) or casein kinase II (11) did not catalyze significant incorporation of labelled phosphate into either the 36kDa subunit or the 10kDa subunit of protein I.

Discussion

The results presented in this communication suggest that protein kinase C catalyzes the stoichometric phosphorylation of the 36kDa

Table 1 Phosphorylation of Protein I by Various Protein Kinases

Kinase	mole P_i incorporation/mole protein[a]
Protein Kinase C	1.7
Casein Kinase I	0.006
Casein Kinase II	0.002
cAMP-dependent Protein Kinase	0.003
Phosphorylase Kinase	0.01

a. Assuming a molecular weight of 85kDa

subunit of protein I, *in vitro*. Phosphorylation is dependent on the presence of both calcium and phospholipid and is localized to both serine and threonine residues.

An impressive body of information has accumulated suggesting that many extracellular signals, such as EGF, elicit cytosolic calcium mobilization and diacylglycerol formation in their target cells. Diacylglycerol derived from receptor-linked phosphoinositide turnover serves as a second messenger for the activation of protein kinase C (12,13). Considering the report (5) that the major substrate of the EGF receptor/kinase, p36, is phosphorylated on both serine and tyrosine residues in vivo, and the demonstration that p36 is an *in vitro* substrate of protein kinase C (figure 1), it is reasonable to postulate that EGF activation of the EGF receptor/kinase and protein kinase C responsible for the phosphorylation of p36 on tyrosine and serine residues. The significance of threonine phosphorylation of p36 by protein kinase C is unclear but might reflect *in vitro* vs *in vivo* differences. For example, the EGF receptor/kinase has 3 *in vitro* autophosphorylation sites but only 1 *in vivo* autophosphorylation site (14). Further studies comparing the p36 *in vitro* serine site with the *in vivo* serine site will be neccessary

to unequivocally establish the *in vivo* significance of protein kinase C phosphorylation of p36.

References

1. Padke, K., and Martin, G.S. (1979) Proc. Natl. Acad. Sci USA 76, 5212-5216
2. Erikson, R.L., Colett, M.S., Erikson, E., Purchino, A.F., and Brugge, J.S.(1979) Cold Spring Harbor Sym. Quant. Biol. 44, 907-917.
3. Sawyer, S.T., and Cohen, S. (1985) J. Biol. Chem. 260, 8233-8236.
4. Gerke, V., and Weber, K. (1984) Embo. J. 3, 227-233.
5. Shaddle, P.J., Gerke, V., and Weber, K. (1985) J. Biol. Chem. 260, 16364-16360.
6. Fava, R.A., and Cohen, S. (1984) J. Biol. Chem. 259, 2636-2645.
7. Kikikawa, U., Takai, Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982). J. Biol. Chem. 257, 133431-13348.
8. Laemmli, U.K. (1970) Nature 227, 680-685.
9. Wong, T.W., and Goldberg, A.R. (1983) J. Biol. Chem. 258, 1022-1025.
10. Singh, T.J., and Huang, K.P. (1985) FEBS Lett. 190, 84-88.
11. Huang, K.P., Itarte, E., Singh, T.J., and Akatsuka, A. (1982) J. Biol. Chem. 257, 3236-3234.
12. Takai, Y., Kaibuchi, K., Tsuda, T., and Hoshijima, M. (1985) J. Biol. Biochem. 29, 143-155.
13. Beridge, M.J. (1984) Biochem. J. 220, 345-360.
14. Dawnward, J., Waterfield, M.D., and Parker, P.J. (1985) J. Biol. Chem. 260, 14538-14546