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# Identification of a New in Vitro Substrate of Tyrosine Protein Kinase†

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ABSTRACT: Recent studies in our laboratory [Tokuda, M., Khanna, N. C., Aurora, A., & Waisman, D. M. (1986) *Biochem. Biophys. Res. Commun. 139*, 910–917] have identified in membranes of rat spleen two tyrosine protein kinases named TPK-I and TPK-II. In this paper the identification of the $Ca^{2+}$ binding protein CAB-48 as a major in vitro substrate of TPK-II is reported. TPK-II catalyzed the incorporation of 0.73 mol of phosphate/mol of CAB-48. Phosphoamino acid analysis revealed that phosphorylation of CAB-48 was specific for tyrosine residues. Phosphorylation of CAB-48 by TPK-I (rat spleen), protein kinase C, casein kinase I, casein kinase II, cAMP-dependent protein kinase, or calcium calmodulin dependent protein kinase was not observed.

That tyrosine phosphorylation may be involved in the virus-induced malignant transformation of cells and in the regulation of cellular growth and proliferation has been suggested by the observation that tyrosine protein kinase activity appears to be intrinsic to several retroviral transforming proteins (Bishop, 1983) as well as certain growth factor receptors (Ushiro & Cohen, 1980; Ek & Heldin, 1982; Kasuga et al., 1982). High levels of tyrosine protein kinase activity have been demonstrated in the membranes of rat spleen (Swarup et al., 1983). Solubilization and partial purification of the tyrosine kinase activity of the 30000*g* pellet of rat spleen have identified two tyrosine protein kinases (Brunati et al., 1985; Tokuda et al., 1986), which have been named TPK-I and TPK-II.[1] Both TPK-I and TPK-II have been characterized as oncogenic and growth factor independent tyrosine protein kinases (Tokuda et al., 1986). In contrast, bovine spleen has been shown to contain a single tyrosine protein kinase activity (Kong & Wang, 1987). Fundamental to our understanding of the cellular function of the oncogenic and growth factor independent tyrosine protein kinases is the identification of the substrates of these kinases.

Recent studies in our laboratory (Waisman et al., 1983a, 1985) have used the combined techniques of $^{45}Ca^{2+}$ autoradiography and Chelex $Ca^{2+}$ binding assay to identify the complete spectrum of $Ca^{2+}$ binding proteins of bovine brain 100000*g* supernatant. Two of these proteins, CAB-27 (Waisman et al., 1983b) and CAB-48 (Waisman et al., 1985; Tokuda et al., 1987), have been shown to be novel $Ca^{2+}$ binding proteins of unknown function. CAB-48 was shown to bind 1.0 mol of calcium/mol of protein with an apparent $K_d$ of 15 $\mu$M (in the presence of 150 mM KCl and 3.0 mM $MgCl_2$). One kilogram of bovine brain was found to contain about 100 mg of CAB-48. On the basis of the concentration of CAB-48 in bovine brain tissue we have suggested that this protein represents a major brain calcium binding protein.

In this paper, the bovine brain $M_r$ 48 000 $Ca^{2+}$ binding protein is identified as an in vitro substrate for bovine and rat spleen tyrosine protein kinases. Phosphorylation is shown to approach stoichiometry and to be specific for tyrosine residues.

## Experimental Procedures

*Materials.* [$\gamma$-$^{32}$P]ATP (10 Ci/mmol; 1 Ci = 3.7 × $10^{10}$ Bq) was purchased from Amersham. Phosphoserine, phosphothreonine, and phosphotyrosine were purchased from Sigma. Casein kinase I and casein kinase II were generous gifts from Dr. T. J. Singh (University of Waterloo). cAMP-dependent protein kinase was purchased from Sigma. Protein kinase C was purified from bovine brain as described (Kikkawa et al., 1982). $Ca^{2+}$-calmodulin-dependent protein kinase was purified from bovine brain as described (Sharma & Wang, 1986).

*Purification of CAB-48.* CAB-48 was purified from bovine brain by the procedures described by Tokuda et al. (1987).

*Purification of Tyrosine Protein Kinase.* Bovine spleen tyrosine protein kinase was purified as described (Kong & Wang, 1987). Rat spleen tyrosine protein kinases (named TPK-I and TPK-II) were purified by the procedures described previously (Tokuda et al., 1986). Enzyme solutions containing 25 mM HEPES (pH 7.5), 1 mM EDTA, 10% glycerol, 0.1% NP40, and 1 mM DTT were stored at −70 °C.

*In Vitro Phosphorylation Assay.* The phosphorylation reaction was performed as described earlier (Tokuda et al., 1986). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10 $\mu$M sodium vanadate, [$\gamma$-$^{32}$P]ATP (50 $\mu$M, 1–2 $\mu$Ci), and

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[1] Abbreviations: CAB, calcium binding protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; NP40, Nonidet P-40; DTT, dithiothreitol; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TPK-I, tyrosine protein kinase I; TPK-II, tyrosine protein kinase II; Tris, tris(hydroxymethyl)aminomethane.

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5–20 μg of CAB-48. The reaction was carried out at 30 °C for 30 min with shaking and was terminated by addition of 25% w/v trichloroacetic acid. The precipitated protein was filtered on Whatman 3MM filter paper, and the radioactivity was determined. Alternatively, an aliquot of the reaction mixture was incubated with the disruption buffer containing 0.1% SDS at 100 °C for 3 min and subjected to SDS–PAGE.

*Phosphoamino Acid Analysis.* CAB-48 (10 μg) was phosphorylated by TPK-II (0.01 μg/mL) at 30 °C for 30 min. The reaction was terminated by the addition of 25% trichloroacetic acid and 100 μg of bovine serum albumin. The precipitate was collected by centrifugation, and the pellet was then washed with ethanol and ethanol–ether (1:1) at −20 °C. Partial hydrolysis with 6 N hydrochloric acid was performed at 110 °C for 2 h, and the hydrolysates were analyzed by thin-layer chromatography on a cellulose sheet at pH 3.5 (pyridine–acetic acid–$H_2O$ = 1:89:189) as described (Wong & Goldberg, 1983). Phosphoamino acid markers were detected by ninhydrin and labeled phosphoamino acids by autoradiography with Kodak XAR-5 X-ray film.

*Alkali Treatment of Polyacrylamide Gels.* Alkali treatment was performed as described (Cheng & Chen, 1981). After being stained with Coomassie Blue, polyacrylamide gels were incubated in 10% methanol–10% acetic acid (v/v) at room temperature for 2 h. Dried gels were exposed to Kodak XAR-5 X-ray films.

*Other Procedures.* SDS–PAGE with 10% acrylamide was performed by the method of Laemmli (1970). Molecular weight standards used for calibration were phosphorylase *a* ($M_r$ 97 400), bovine serum albumin ($M_r$ 67 000), ovalbumin ($M_r$ 45 000), carbonic anhydrase ($M_r$ 30 000), trypsin inhibitor ($M_r$ 20 100), and α-lactalbumin ($M_r$ 14 400). Protein was measured by the method of Lowry et al. (1951).

## Results and Discussion

*Phosphorylation of Bovine Brain Supernatant by Tyrosine Protein Kinase.* The results of several investigators (Swarup et al., 1983; Brunati et al., 1985) have suggested that relatively high activities of tyrosine kinases exist in the membranes of normal tissues. Recently (Tokuda et al., 1986), we have reported the purification of two tyrosine protein kinases from rat spleen, named TPK-I and TPK-II. The solubilized and purified enzyme preparations phosphorylated only tyrosine residues when either endogenous proteins or extrinsic substrates such as angiotensin II and casein were used as substrates (Tokuda et al., 1986; Kong & Wang, 1987).

In previous studies (Tokuda et al., 1987) we have analyzed the complete spectrum of bovine brain $Ca^{2+}$ binding activity by a procedure involving chromatography of the brain 100000*g* supernatant on DEAE-cellulose and analysis of the resultant fractions for $Ca^{2+}$ binding activity. The three peaks of $Ca^{2+}$ binding activity were resolved, and the second peak of $Ca^{2+}$ binding activity was further purified. The major $Ca^{2+}$ binding proteins of this $Ca^{2+}$ binding activity peak were identified as CAB-48 and calcineurin. The identification of the major cellular target of the Rous sarcoma virus encoded tyrosine-specific protein kinase, p36, as a $Ca^{2+}$ binding protein (Shadle et al., 1985) has suggested that other $Ca^{2+}$ binding proteins might be substrates of tyrosine protein kinases. Therefore, $Ca^{2+}$ binding activity peaks resolved on DEAE-cellulose (peaks I–III) were incubated in the presence or absence of the rat spleen tyrosine protein kinases. Tyrosine protein kinase dependent phosphorylation was observed only in peak II by TPK-II. As shown in Figure 1, incubation of peak II with TPK-II resulted in the phosphorylation of a $M_r$ 48 000 protein, and alkali treatment of the gel clearly identified this protein

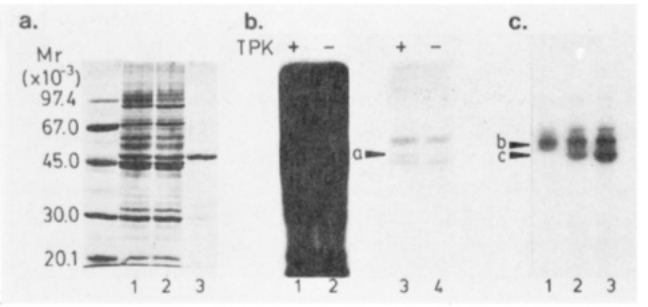


FIGURE 1: SDS–PAGE and autoradiographic analysis of CAB-48. Peak II of the DEAE-cellulose chromatography (50 μg) and purified CAB-48 were incubated in the presence or absence of rat spleen tyrosine protein kinase II (0.1 μg). Phosphorylated proteins were subjected to SDS–PAGE (a) and autoradiographic analyses (b and c). (a) SDS–PAGE of DEAE-cellulose peak II (lanes 1 and 2) and purified CAB-48 (lane 3); (b) autoradiograph of DEAE-cellulose peak II before (lanes 1 and 2) and after (lanes 3 and 4) alkali treatment; (c) TPK-II incubated in the absence (lane 1) or in the presence of 4.0 (lane 2) or 8.0 μg (lane 3) of purified CAB-48.

band as the only detectable substrate of tyrosine protein kinase (Figure 1b, lanes 3 and 4, arrow a). In the absence of added tyrosine protein kinase, phosphorylation of this band was not observed and was therefore not due to intrinsic tyrosine protein kinase activity (data not shown). In contrast, protein phosphorylation by TPK-I was not observed. The $M_r$ 48 000 phosphoprotein substrate of TPK-II was of identical molecular mass to that of CAB-48, a $M_r$ 48 000 $Ca^{2+}$ binding protein (Figure 1a, lane 3) discovered by our laboratory (Waisman et al., 1985). When purified CAB-48 was incubated with tyrosine protein kinase and analyzed by SDS–PAGE, a $M_r$ 50 000 (arrow b) phosphoprotein and a $M_r$ 48 000 (arrow c) phosphoprotein were resolved (Figure 1c). The $M_r$ 50 000 phosphoprotein (Figure 1c, lanes 1 and 2) was due to the autophosphorylation of TPK-II (Tokuda et al., 1986) and was detected when tyrosine protein kinase was incubated in the absence of CAB-48 (Figure 1c, lane 1). Phosphorylation was not observed when CAB-48 was incubated in the absence of tyrosine protein kinase (data not shown), therefore suggesting that CAB-48 was not undergoing autophosphorylation. Phosphorylation of CAB-48 by the partially purified tyrosine protein kinase of bovine spleen (Kong & Wang, 1987) was also observed. We have identified CAB-48 in the 100000*g* supernatant of a variety of bovine tissues, including spleen, by immunoblot analysis (data not shown).

*Analysis of Phosphoamino Acid.* That phosphorylation occurred on tyrosine residues could not be unequivocally established since some phosphoserine- and phosphothreonine-containing protein can be resistant to alkali treatment (Cooper et al., 1982). To identify the phosphoamino acid residues of CAB-48, the protein was incubated with rat spleen tyrosine protein kinase II, partially hydrolyzed with 6 N HCl, and analyzed by chromatography on a thin-layer cellulose sheet. The autoradiograph is shown in Figure 2. The radioactivity was detected only on phosphotyrosine. Radioactivity was not observed on either phosphoserine or phosphothreonine. This result demonstrated that CAB-48 was phosphorylated only on tyrosine residues by the tyrosine protein kinase.

*Kinetics of CAB-48 Phosphorylation.* A time course of the phosphorylation of CAB-48 by rat spleen tyrosine protein kinase II is presented in Figure 3. About 0.73 mol of phosphate/mol of CAB-48 was incorporated after 2 h. The phosphorylation pattern was analyzed by SDS–PAGE, and densitometric analysis of the autoradiograph suggested that tyrosine protein kinase autophosphorylation contributed less than 5% of the total phosphorylation (Figure 3). Similar

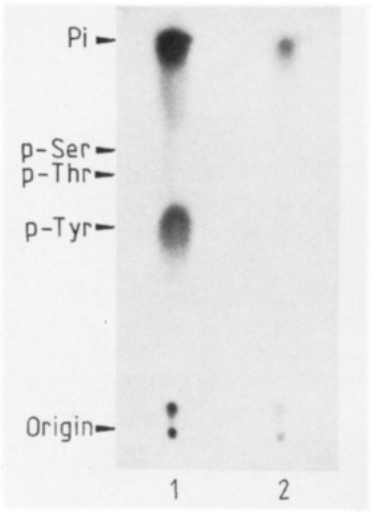


FIGURE 2: Phosphoamino acid analysis of CAB-48. Phosphoamino acid analysis was carried out as described under Experimental Procedures (1) in the presence of CAB-48 and (2) in the absence of CAB-48. p-Ser, phosphoserine; p-Thr, phosphothreonine; p-Tyr, phosphotyrosine.

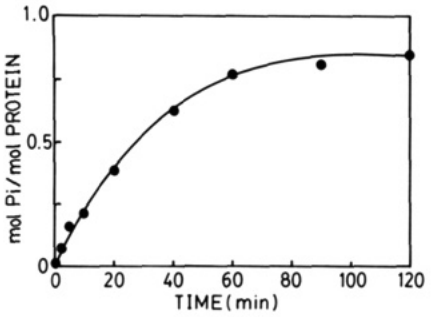


FIGURE 3: Stoichiometry of incorporation of phosphate into CAB-48. CAB-48 (50 μg) was incubated with rat spleen tyrosine protein kinase II (0.01 μg) at 30 °C, and aliquots were removed and subjected to liquid scintillation counting. Results are presented as total counts per minute minus control counts per minute, where control counts per minute refers to phosphorylation in the absence of added CAB-48.

stoichiometry (0.58 mol/mol of CAB-48) was demonstrated by bovine spleen tyrosine protein kinase (Table I).

The stoichiometry of phosphorylation of CAB-48 appears to be relatively high compared to other substrates such as vinculin (1%) (Sefton et al., 1981), p36 (10%) (Hunter & Cooper, 1984), and p46 (8%) (Cooper et al., 1983). Recently, Yoshikawa identified tubulin as a substrate of mouse liver tyrosine protein kinase and reported a stoichiometry of 2 mol of phosphate/mol of tubulin dimer after 180 min of incubation (Yoshikawa et al., 1985).

Work by many laboratories [reviewed by Hunter and Cooper (1984)] has identified several substrates of tyrosine protein kinases in the $M_r$ 40 000–50 000 range. These substrates include the p46 (p*I* 7.4) substrate of pp60$^{v\text{-}src}$ (Cooper et al., 1983), the $M_r$ 45 000 (p*I* 6.8), $M_r$ 40 000 (p*I* 6.9), $M_r$ 43 000 (p*I* 7.1), $M_r$ 43 000 (p*I* 7.3), and $M_r$ 45 000 (p*I* 7.0) substrates of EGF- and PDGF-stimulated kinase (Cooper et al., 1982; Nakamura et al., 1983; Kohno, 1985), and the $M_r$ 50 000 (p*I* 5.4) substrate of pp60$^{v\text{-}src}$ (Brugge & Darrow, 1982). Considering the p*I* 4.7 of CAB-48 (Tokuda et al., 1987), it is extremely unlikely that CAB-48 has been previously identified as a substrate of these tyrosine protein kinases.

*Phosphorylation of CAB-48 by Other Protein Kinases.* Recent studies have shown that many substrates of tyrosine protein kinases are also substrates of other protein kinases. For example, work in our laboratory has shown that one of the major in vivo substrates of the tyrosine protein kinase pp60$^{v\text{-}src}$, p36, is also an in vitro substrate of protein kinase C (Khanna et al., 1986). Similarly, we have shown that one of the major in vivo substrates of the EGF receptor tyrosine protein kinase, p35, is an in vitro substrate of protein kinase C (Khanna et al., 1986). Work in other laboratories has identified the EGF receptor tyrosine protein kinase as a substrate of protein kinase C (Fearn & King, 1985). As shown in Table I, a variety of protein kinases including protein kinase C do not catalyze the phosphorylation of CAB-48. This result presents the possibility that the phosphorylation of CAB-48 may be restricted to tyrosine residues. It is interesting to note that the major substrate of pp60$^{v\text{-}src}$, p36, and the major substrate of the EGF receptor tyrosine protein kinase, p35, are $Ca^{2+}$ binding proteins.

Table I: Phosphorylation of CAB-48 by Various Kinases

| kinases | stoichiometry of phosphorylation[a] (mol of $P_i$/mol of CAB-48) |
|---|---|
| tyrosine protein kinase (bovine spleen) | 0.58 |
| TPK-I (rat spleen) | 0.05 |
| TPK-II (rat spleen) | 0.73 |
| casein kinase I | 0.01 |
| casein kinase II | 0.01 |
| A-kinase | 0.02 |
| C-kinase | 0.04 |
| $Ca^{2+}$-calmodulin kinase | 0.02 |

[a] CAB-48 was phosphorylated by various kinases for 2 h as described under Experimental Procedures, and the radioactivity was counted for the calculation of stoichiometry. Results are presented as total counts per minute minus control counts per minute, where control counts per minute refers to phosphorylation in the absence of added CAB-48.

The results presented in this paper suggest that CAB-48 is an in vitro substrate of an oncogenic and growth factor independent tyrosine protein kinase and that CAB-48 may be an important in vivo substrate of this kinase. However, it has become apparent that tyrosine phosphorylation observed in in vitro systems may not faithfully reflect the situation in vivo (Sefton et al., 1981; Collett et al., 1980). Therefore, in vivo studies will be necessary to determine whether CAB-48 is an important in vivo substrate of tyrosine protein kinase.

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# The Eighth Component of Human Complement: Evidence That It Is an Oligomeric Serum Protein Assembled from Products of Three Different Genes†

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ABSTRACT: The eighth component of human complement (C8) consists of three nonidentical subunits arranged asymmetrically as a disulfide-linked $\alpha$–$\gamma$ dimer and a noncovalently associated $\beta$ chain. Genetic studies of C8 polymorphisms established that $\alpha$–$\gamma$ and $\beta$ are encoded at different loci. Implicit in this finding was the existence of two different genes and the likelihood that $\alpha$–$\gamma$ would be synthesized in single-chain precursor form. However, recent characterization of cDNA clones revealed separate mRNAs for human $\alpha$ and $\beta$ but no evidence of a single-chain precursor for $\alpha$–$\gamma$. A cDNA clone containing the entire coding region for human $\gamma$ has now been characterized, and its sequence supports the existence of a separate $\gamma$ mRNA. Included are a consensus translation initiation sequence, an apparent initiation methionine, and a signal peptide. By use of cDNA probes specific for human $\alpha$, $\beta$, or $\gamma$, analysis of poly(A) RNA from normal baboon liver revealed separate mRNAs of 2.5, 2.6, and 1.0 kilobases (kb), respectively. Parallel analysis of poly(A) RNA from rat liver identified mRNAs of 3.4, 2.3, and 0.9 kb. These results argue against the possibility that C8 is assembled from products of two different genes ($\alpha$–$\gamma$ and $\beta$) and suggest it is comprised of three different gene products ($\alpha$, $\beta$, and $\gamma$) that undergo both covalent and noncovalent association to yield the mature protein.

Human C8 is a glycoprotein constituent of C5b-9, the macromolecular cytolytic complex composed of complement proteins C5b, C6, C7, C8, and C9 (Müller-Eberhard, 1986). Both C8 and C9 have the exceptional ability to circulate in plasma as hydrophilic proteins but undergo hydrophilic to amphiphilic transitions leading to interaction with target membranes. This interaction is a consequence of association with other constituents and contributes directly to the cytolytic function of C5b-9. Human C8 has an atypical subunit structure consisting of $\alpha$ ($M_r$ 64 000), $\beta$ ($M_r$ 64 000), and $\gamma$ ($M_r$ 22 000) subunits arranged as a disulfide-linked $\alpha$–$\gamma$ dimer that is noncovalently associated with $\beta$ (Kolb & Müller-Eberhard, 1976; Steckel et al., 1980). Several distinct functional domains have been identified on C8. Included are those involved in interactions between subunits (Brickner & Sodetz, 1984, 1985), with other constituents of C5b-9 (Monahan & Sodetz, 1981; Stewart & Sodetz, 1985; Stewart et al., 1987), and with the target membrane bilayer (Steckel et al., 1983).

Insight into the genetic basis for the unusual subunit structure of C8 was first provided by studies of human C8 polymorphisms. Electrophoretic analysis of C8 under nonreducing conditions revealed that $\alpha$–$\gamma$ and $\beta$ polymorphic patterns segregate independently in families, thus indicating these subunits are encoded at different genetic loci (Raum et al., 1979; Alper et al., 1983; Rittner et al., 1983). Existence of separate loci was further supported by analysis of human C8 deficiencies, where, in a given individual, $\alpha$–$\gamma$ or $\beta$ is dysfunctional but not both (Tedesco et al., 1983a,b). On the basis of these observations, it has generally been assumed that $\alpha$–$\gamma$ and $\beta$ are encoded in separate genes and, by analogy with other secreted proteins containing disulfide-linked chains, that $\alpha$–$\gamma$ is synthesized in single-chain precursor form.

To determine the C8 amino acid sequence, establish the existence of an $\alpha$–$\gamma$ single-chain precursor, and directly confirm that $\alpha$–$\gamma$ and $\beta$ are products of different genes, we re-

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