Volume 296, number 2, 222–224 FEBS 10601 January 1992

A plant homologue to mammalian brain 14-3-3 protein and protein kinase C inhibitor*

Stephan Hirsch[1], Alastair Aitken[2], Uwe Bertsch[3] and Jürgen Soll[1]

[1]*Botanisches Institut, Universität Kiel, Olshausenstraße 40, 2300 Kiel, Germany,* [2]*National Institute for Medical Research, Mill Hill, London NW7 1AA, England* and [3]*Fachrichtung Botanik, Universität Saarbrücken, 6600 Saarbrücken, Germany*

Received 13 November 1991

We have isolated cDNA clones of *Spinacea oleracea* L. and *Oenothera hookeri* of 930 and 1017 base pairs, respectively. The open reading frame deduced from the *Oenothera* sequence codes for a protein of a calculated molecular mass of 29 200. The primary amino acid sequence exhibits a very high degree (88%) of homology to the 14-3-3 protein from bovine brain, and protein kinase C inhibitor from sheep brain. Subsequently the plant protein was partially purified from leaf extract. The partially purified plant protein inhibited protein kinase C from sheep brain in a heterologous assay system. The active fraction consisted of 5–6 different polypeptides of similar molecular size. One of these proteins crossreacted with a peptide-specific antibody against protein kinase C inhibitor protein from sheep brain.

Protein phosphorylation; 14-3-3 protein; Protein kinase C inhibitor

1. INTRODUCTION

A new family of regulatory proteins has emerged from studies in mammalian brain tissue [1,2]. One member is a group of acidic proteins within a mol. wt. range of 29–33 kDa which had been isolated from sheep brain and were found to function as a novel type of potent inhibitors of protein kinase C (PKC) [3]. Another member is the 14-3-3 protein, a set of at least 7 polypeptides of mol. wt. between 29–32 kDa [1,4]. These proteins are localized preferentially in neurons, and function as protein kinase-dependent activators of tyrosine and tryptophan hydroxylases, which are the rate-limiting enzymes in the pathway of monoamine biosynthesis [1,5]. The PKC inhibitor proteins (KCIP) and the 14-3-3 protein share a very high degree of amino acid sequence homology (>90%) [2]. Whether they can also substitute for each other in their biological function is not known.

In this communication we report on the existence of a member of this protein family in higher plants, e.g. spinach, *Oenothera* and pea. This finding strongly indicates an ubiquitous distribution and central regulatory functions of these polypeptides in eukaryotes.

Abbreviations: PKC, protein kinase C; KCIP, inhibitor protein of PKC.

*The cDNA sequences reported in this paper have been deposited in the EMBL-database, accession number X62837 for PHP-S and X62838 for PHP-O.

Correspondence address: J. Soll, Botanisches Institut, Universität Kiel, Olshausenstraße 40, 2300 Kiel, Germany. Fax: (49) (431) 880 1527.

2. MATERIALS AND METHODS

Immunoscreening of a λgt 11 cDNA expression library was done as in [6]. Positive clones were subcloned into Bluescript (Genofit, Geneva), and both strands sequenced as in [7]. A 250-bp fragment was isolated by *BglI*/*PstI* digestion and used to produce a digoxigenin-labelled DNA probe according to the manufacturer's instruction. A cDNA library derived from *Oenothera* mRNA was screened using this probe (Boehringer, Mannheim, Germany). Positive clones were subcloned and analysed as above. The protein was synthesized by an in vitro transcription-translation system as in [8].

The plant KCIP/14-3-3 homologue was purified from pea leaf extract essentially as described for sheep brain [3]. In brief, soluble proteins were first applied to anion exchange chromatography on DEAE-cellulose. The column was developed with a linear NaCl gradient (0–500 mM) in 20 mM Tris-HCl. Fractions which showed PKC inhibitory activity were pooled and further purified by phenyl-sepharose. Proteins were eluted from the phenyl-sepharose with a descending NaCl gradient (2.5–0 M NaCl). Active fractions were further purified by anion exchange chromatography on Mono-Q (Pharmacia, Uppsala, Sweden). Proteins were analysed by SDS-PAGE [9], silver staining [10] and immunoblot [11].

PKC-inhibitory activity was assayed in a heterologous assay system using PKC purified from sheep brain. The assay is described in [3,12]. PKC from sheep brain was purified by chromatography on DEAE cellulose [3].

Polyclonal antibodies were raised in a rabbit against a synthetic peptide identical to amino acid positions 52–71 in the KCIP protein of sheep brain (compare also position 56–75 of the plant homologue) (H_2N-KNVVGARRASWRVISSIEQK-CO_2H). The peptide was coupled to CNBr-activated sepharose according to the manufacturer's conditions (Pharmacia, Uppsala, Sweden) prior to immunisation.

3. RESULTS

During the course of screening of λgt 11 expression library derived from spinach mRNA using an antibody against a mixture of chloroplast envelope proteins [8] a cDNA clone, PHP-S, was isolated and sequenced (Fig.

Published by Elsevier Science Publishers B.V.

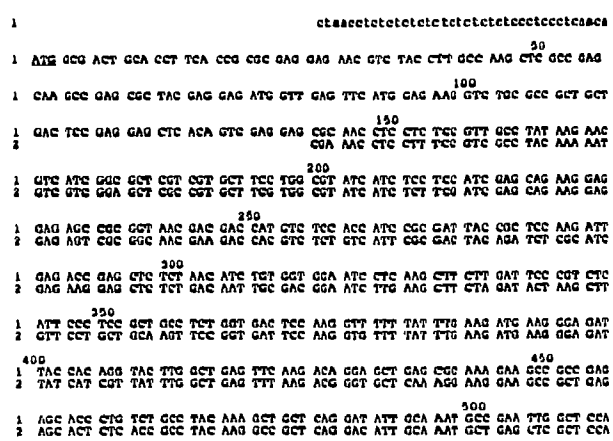

Fig. 1. Sequences of cDNA clones PHP-O (1) and PHP-S (2) from *Oenothera* and spinach, respectively. The start codon of the PHP-O clone is underlined.

1). A search of the EMBL database revealed that the PHP-S clone was highly homologous to the 14-3-3 protein from bovine brain [1], but it represented only the C-terminal part of the polypeptide. A full-length cDNA clone (PHP-O) was subsequently isolated from an *Oenothera* cDNA library (Fig. 1). The alignment of the deduced amino acid sequence of the *Oenothera* cDNA clone with the bovine brain 14-3-3 protein and the KCIP from sheep brain showed an identity of 58 and 62%, respectively, or 88% homology for both proteins, if conservative amino acid exchanges are also considered (Fig. 2). Such a pronounced preservation on the amino acid level not only between plant and animal but also in very different organs, e.g. leaf mesophyll cells (Table I) and mammalian brain, might indicate pivotal roles in regulatory processes.

Methods developed to purify KCIP from sheep brain [3] were applied here to purify the homologous protein from soluble protein of pea leaf mesophyll cells (Table I). The final 2 steps resulted in a 150-fold enrichment of an activity which inhibited PKC from sheep brain. The inhibitory activity in the leaf extract could not be determined due to interfering protein kinase activity at this stage. Thus the partially purified plant protein was able

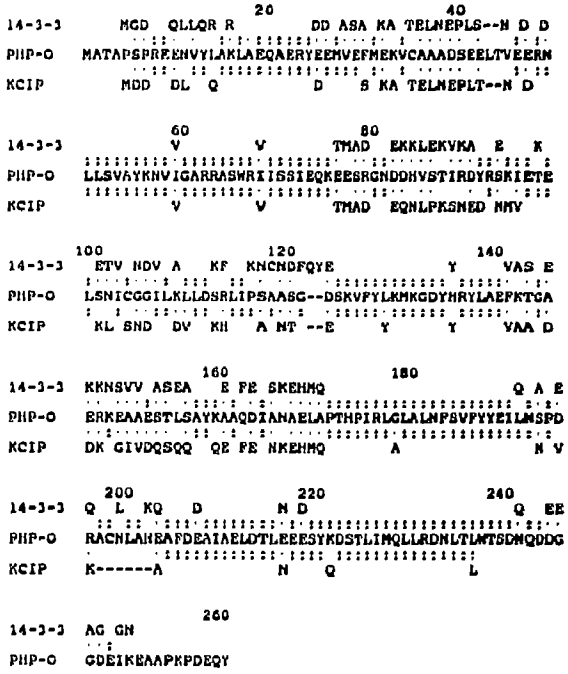

Fig. 2. Comparison of the deduced amino acid sequences of clone PHP-O with the amino acid sequences of the 14-3-3 protein [1] and KCIP [2] from mammalian brain. Identical amino acids are marked by (:); homologue exchanges by (.).

to inhibit in vitro PKC from sheep brain, demonstrating also the close functional relationship between the animal and the plant polypeptide.

The protein fraction which exhibits the highest KCIP activity after the Mono-Q purification step is composed of about 6 polypeptides in the mol. wt. range of 22–32 kDa (Fig. 3, lane 1). Whether these proteins are all members of a closely related family, as is the case for the bovine brain 14-3-3 and the sheep brain KCIP protein family, seems possible but remains to be proven. A polyclonal antibody raised against a synthetic peptide of KCIP (see Fig. 2) recognized 1 of the proteins present

Table I

The plant homologue of mammalian 14-3-3 and KCIP was purified from pea leaf extract

Sample	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Chromatographic step
Leaf extract	30	600	–	–	
Pool I	80	36	4800	133	DEAE-cellulose
Pool II	10	1.6	400	250	phenyl-sepharose
Pool III	3	0.03	342	11400	Mono-Q

The purification protocol used was essentially as in [3]. One unit is defined as 50% inhibition of Ca^{2+}- and phospholipid-activated PKC from sheep brain [3].

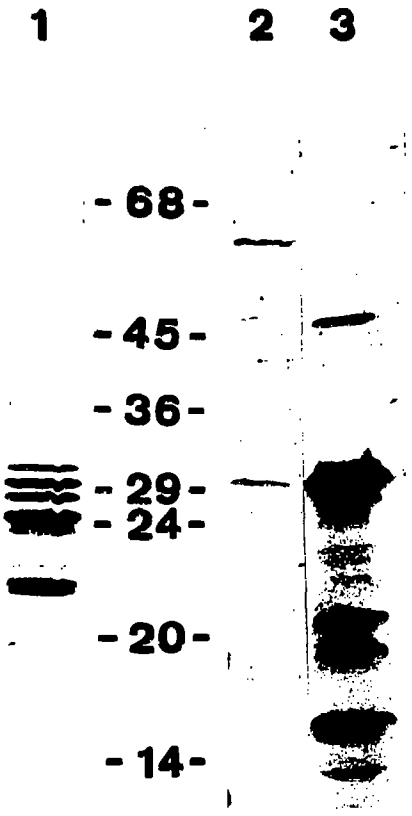

Fig. 3. Purification and identification of a protein homologue to mammalian brain KCIP and 14-3-3 purified from pea leaves. (Lane 1) Silver-stained SDS-PAGE of proteins present in fractions with PKC inhibitory activity from the Mono-Q column (compare Table I); (lane 2) Immuno-blot analysis of an active fraction obtained from the DEAE purification using a peptide-specific antibody against KCIP from sheep brain; (lane 3) in vitro transcription-translation of clone PHP-O in the presence of [^{35}S]methionine. A fluorogram of the SDS-PAGE analysis is shown.

in the active fraction. The apparent mol. wt. on SDS-PAGE of about 30 kDa corresponds well with the calculated mol. wt. of 29.2 kDa. The protein obtained by in vitro transcription-translation of the cDNA clone (PHP-O) also runs at a mol. wt. of 30 kDa. Together these data strongly indicate that the purified polypeptide band at 30 kDa is identical to the isolated cDNA clone and should represent the PKC inhibitory activity. We have not studied the subcellular localization of the protein in detail, but the radiolabelled translation product failed to be imported into intact chloroplasts (not shown).

4. CONCLUSIONS

The present study establishes the existence of a family of closely related proteins in higher plants which has been described so far only for brain tissue of mammals. Future work will probably show that this class of proteins is ubiquitous in eukaryotes.

The biochemical functions ascribed to the 14-3-3 protein and KCIP in mammals are vital for the animal. The 14-3-3 protein influences the synthesis of catecholamine and serotonin, a prerequisite for the biosynthesis of dopamine and other neurotransmitters, by activating tryptophan- and tyrosine hydroxylases. In contrast its close relative, KCIP from sheep brain, inhibits PKC in its Ca^{2+} and phospholipid activated form, and seems to play an important role in the down-regulation of PKC. A plant protein with all properties of PKC from animals i.e. Ca^{2+}-, phospholipid- and diacylglycerol-dependent, has not yet been described [13,14]. Biochemical and genetic studies reported so far demonstrate only a limited relationship between plant PKC homologues and their animal counterpart. The exact role of the plant 14-3-3/KCIP homologues remains to be elucidated. However, we would like to propose similar important functions for the plant proteins, e.g. the regulation of PKC-like protein kinases in plants.

Acknowledgements: We would like to thank Yasmina Patel for her excellent technical assistance and Norbert Wedel for supplying the Oenothera cDNA library. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 246). The work at Mill Hill was funded by the Medical Research Council, UK.

REFERENCES

[1] Ichimura, T., Isobe, T., Ohuyama, T., Takahashi, T., Araki, K., Muwano, R. and Takahashi, Y. (1988) Proc. Natl. Acad. Sci. USA 85, 7084–7088.
[2] Aitken, A., Ellis, C.A., Harris, A., Sellers, L.A. and Toker, A. (1990) Nature 344, 594.
[3] Toker, A., Ellis, C.A., Sellers, L.A. and Aitken, A. (1990) Eur. J. Biochem. 191, 421–429.
[4] Yamauchi, T., Nakata, H. and Fujisawa, H. (1981) J. Biol. Chem. 256, 5404–5409.
[5] Boston, P.F., Jackson, P., Kynoch, P.A.M. and Thompson, R.J. (1982) J. Neurochem. 38, 1466–1474.
[6] Young, R.A. and Davis, R.W. (1983) Science 222, 778–782.
[7] Sanger, F., Nichlen, S. and Coulsen, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[8] Salomon, M., Fischer, K., Flügge, U.-J. and Soll, J., Proc. Natl. Acad. Sci. USA 87, 5778–5782.
[9] Laemmli, U.K. (1970) Nature 227, 680–685.
[10] Ansorge, W. (1982) Electrophoresis 82, 235–242.
[11] Towbin, A., Staehlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[12] Parker, P.J., Stabel, S. and Waterfield, M.D. (1984) EMBO J. 3, 953–959.
[13] Lawton, M.A. (1990) Curr. Top. Plant Biochem. Physiol. 9, 373–382.
[14] Elliot, D.C. and Brennan, P.A. (1990) Curr. Top. Plant Biochem. Physiol. 9, 383–398.