FEBS Letters 352 (1994) 163–166

FEBS 14562

The fusicoccin receptor of plants is a member of the 14–3–3 superfamily of eukaryotic regulatory proteins

C. Oecking[a], C. Eckerskorn[b], E.W. Weiler[a,*]

[a]*Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität, D-44780 Bochum, Germany*
[b]*Abteilung Proteinanalytik, Max-Planck-Institut für Biochemie, D-82152 Martinsried, Germany*

Received 17 August 1994

Abstract The receptor for the wilt-inducing phytotoxin fusicoccin was purified to homogeneity from plasma membranes of *Commelina communis* as a complex with the radioligand [^{3}H]9′-nor-8′-hydroxyfusicoccin. The preparation consisted of two polypeptides with apparent molecular masses of 30.5 kDa and 31.5 kDa and with isoelectric points of around pH 5.2 and 5.3, respectively. The proteins were N-terminally blocked. Internal amino acid sequences were obtained for both polypeptides of the fusicoccin-binding complex. Sequence information, as well as subsequent immunological analysis, proved that both polypeptides are members of the eukaryotic 14–3–3 family, which comprises structurally conserved regulatory proteins of widespread occurrence and a wide range of functions. 14–3–3 isoform(s) constituting the fusicoccin receptor are distinguishable from other cellular 14–3–3 proteins by their tight association with the plasma membrane. Applying temperature-induced Triton X-114 phase separation experiments, they, as well as the target enzyme of fusicoccin action, the H^+-ATPase, partitioned into the phospholipid-rich fraction which contains the most hydrophobic proteins. The results discussed herein provide a basis for the elucidation of the molecular mechanism of fusicoccin action.

Key words: 14–3–3 Proteins; Fusicoccin; Fusicoccin receptor (identification)

1. Introduction

The phytotoxin fusicoccin (FC) profoundly alters plasma membrane traffic by stimulating apoplast acidification, hyperpolarizing the membrane potential and consequently increasing proton-motive force [1]. The H^+-translocating P-type ATPase is considered as target of FC action. The toxin activates the ATPase by a mechanism involving the C-terminal autoinhibitory domain of the enzyme [2] and exerts its effects by means of a high-affinity binding protein (FCBP) located at the plasma membrane of plants [3–8]. The FCBP exhibits conserved biochemical properties in all species studied so far and occurs ubiquitous from the mosses to the angiosperms [9]. Mutants of *Arabidopsis thaliana* with reduced binding affinity of the FCBP towards FC are toxin insensitive and show greatly reduced or lacking physiological responses to FC, proving that the FCBP represents the plant's FC receptor [10].

Three approaches, namely (i) photoaffinity labeling of plasma membranes [3,6,8], (ii) purification of the FCBP-radioligand complex [3] and (iii) affinity chromatography of the FCBP [4] have shown that the FCBP is composed of two subunits of approximately 31 kDa and 32 kDa apparent molecular mass in SDS-PAGE. The solubilized receptor-radioligand complex displays an apparent molecular mass of about 80 kDa, suggesting a heterooligomeric (dimeric or trimeric) structure of the FCBP in its native form [6,11].

We now have obtained amino acid sequence information for both subunits of the FCBP and show that they are members of a eukaryotic family of conserved regulatory or signal transducing proteins known as 14–3–3 proteins (for review see [12]).

2. Materials and methods

Plasma membrane vesicles were prepared from leaves of *C. communis* by partitioning between aqueous dextran and polyethylene glycol phases [13]. FC-binding assays were performed using [^{3}H]9′-nor-8′-hydroxyfusicoccin as radioligand [14], plasma membrane vesicles were photoaffinity-labeled with a biologically active azido-derivative of FC, 9′-nor-8′-[(3,5-[^{3}H]4-azidobenzoyl)diaminoethy l]-fusicoccin [6,8]. Temperature-induced phase separation of plasma membrane proteins in Triton X-114 (1% (v/v), 1 mg protein/ml) was carried out as described in [15]. Functional solubilization of the FCBP and purification of the FCBP-radioligand complex were achieved according to [3]. Proteolytic digestion of the electrophoretically separated FCBP-subunits using endoproteinase Lys-C (Boehringer, Mannheim, Germany) was performed directly in the polyacrylamide gel. Following elution, the peptides were purified by reversed-phase HPLC and subsequently sequenced [16]. Immunoblot analysis was carried out as in [17]. The monoclonal antibody raised against GF14 [18], a 14–3–3 protein from *A. thaliana* (antibody GF14–19, kindly provided by Dr. R.J. Ferl, Gainesville, USA), was used at a final dilution of 1:5,000. The antiserum against the *A. thaliana* H^+-ATPase was kindly provided by Dr. R. Serrano, Valencia, Spain, and was used at a final dilution of 1:10,000.

3. Results

The FCBP was purified from highly enriched plasma membranes of *C. communis* [3]. Tracking of the FCBP during the purification process was possible due to the slow rate of dissociation of the radioligand–FCBP complex at low temperatures [8]. From altogether 660 mg of plasma membrane protein, the receptor was obtained in 15% overall yield (based on initial total radioligand binding) and with 1,000-fold enrichment (based on specific radioligand binding). The final preparation contained less than 0.015% of the initial protein. Details of the procedure were given in [3]. SDS-PAGE of the purified receptor showed two polypeptides with relative molecular masses of 30.5 kDa and 31.5 kDa, in agreement with earlier reports [3,4,6,8]. The 90 kDa polypeptide, assumed by one group [19] to be associated with the FCBP, could not be detected.

As shown by two-dimensional gel electrophoresis (Fig. 1), both polypeptides appeared homogenous, although a sharp focus was not achieved in the first dimension (isoelectric focusing), which we attributed to the formation of aggregates. The

*Corresponding author. Fax: (49) (234) 709 4187.

SSDI 0014-5793(94)00949-X

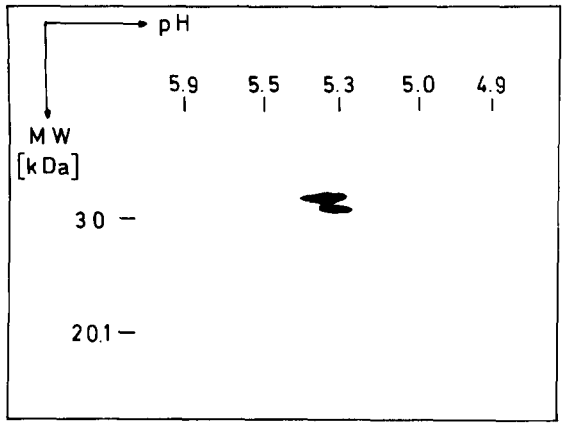

Fig. 1. Two-dimensional gel electrophoretic analysis [29] of the purified FCBP (silver stained, relevant area is shown). Protein from the appropriate fractions (500 μl) containing the FCBP was precipitated using methanol and isoelectric focusing was carried out using a 4% polyacrylamide gel which included 5% (v/v) ampholyte 4–9 and 2% (v/v) Triton X-100. The second dimension was SDS-PAGE (12.5% polyacrylamide).

isoelectric point of the 31.5 kDa polypeptide was determined around pH 5.3 and that of the 30.5 kDa subunit was around pH 5.2.

N-Terminal sequencing showed both polypeptides to be blocked. To obtain internal amino acid sequence information, ca. 20 μg of the purified FCBP were electrophoretically separated. The two FCBP subunits were excised separately and digested directly in the gel using the endoproteinase Lys-C [16] followed by separation of the peptide fragments by reversed-phase HPLC. Peptide sequences were derived from both subunits (Fig. 2). A data bank search revealed a high degree of similarity of all four sequences to 14–3–3 proteins, but no homology to any other protein was observed. An alignment of the corresponding sequence elements from various species and the plant consensus sequence are also shown in Fig. 2.

Further proof for the identity of the FC receptor came from the use of 14–3–3 specific antibodies. A monoclonal antibody raised against GF14, a 14–3–3 protein from *A. thaliana* [18], demonstrated the existence of 14–3–3 homologs in both, the soluble and the microsomal, fractions from leaves of *C. communis*. The antibody exclusively recognized a doublett of polypeptides of approximately 31 and 32 kDa in those crude fractions, confirming its specificity and applicability for *C. communis* (not shown). When partially purified FCBP–radioligand complexes were subjected to high-performance anion-exchange chromatography, all, the 14–3–3 immunoreactive polypeptides, the FCBP (marked by the reversibly bound radioligand), as well as the 30.5 and 31.5 kDa protein doublett (visualized by silver staining), co-eluted from the column, further stressing their identity (Fig. 3).

Next, we subjected highly enriched plasma membranes, after either reversible or photoaffinity labeling of the FCBP with the appropriate radioligand, to fractionation by temperature-induced phase separation in Triton X-114. This procedure is suitable to distinguish membrane proteins according to their hydrophobicity [15]. From Fig. 4A–C, it becomes obvious that 14–3–3 immunoreactive polypeptides partitioned into both, the aqueous phase containing hydrophilic proteins (Fig. 4C, lane a) and the lipid-rich, detergent-insoluble phase containing the most hydrophobic proteins (Fig. 4C, lane p), while none could be detected in the detergent phase itself. The reversibly as well as the photoaffinity labeled FCBP partitioned into the hydrophobic, detergent-insoluble phase (Fig. 4A,B, lanes p). This proves that the receptor is represented by the hydrophobic, and not by the hydrophilic, isoform(s) of the plasma membrane associated 14–3–3 proteins. The H^+-ATPase, the target enzyme of FC action and a trans-membrane protein of known structure (for review see [20]), likewise was localized in the hydrophobic, detergent-insoluble protein fraction (Fig. 5C, lane p).

In order to check, if the hydrophobic and hydrophilic fractions of the plasma membrane associated 14–3–3 proteins are interconvertible in a ligand-dependent manner, we incubated plasma membranes in the absence or presence of FC (1 μM), followed by Triton X-114 phase fractionation. In parallel experiments, plasma membranes were prepared from leaves that had been preincubated for 30 min in 1 μM FC. From earlier studies [21], it was known that such a treatment resulted in a strong and persistent activation of the H^+-ATPase, while a treatment of isolated plasma membranes with FC had no effect on enzyme activity. This also was observed for *C. communis*. The data clearly show that in-vivo treatment of leaves with FC results in activation of the H^+-ATPase (cf. Fig. 5D) without any effect on total amount of enzyme protein as determined by immunoblot analysis (cf. Fig. 5C, lanes p). In contrast to this, FC-pretreatment results in a higher abundance of FC binding sites (cf. Fig. 5B, lanes pm and p) as well as a higher abundance of the hydrophobic fraction of 14–3–3 proteins in the final membrane preparation (cf. Fig. 5A, lanes p), while the amount of the hydrophilic fraction of the membrane associated 14–3–3 polypeptides remained unaltered (cf. Fig. 5A, lanes a). The distribution of hydrophilic vs. hydrophobic plasma membrane 14–3–3 homologs was independent of the incubation of isolated plasma membranes in the presence or absence of FC (not shown).

4. Discussion

The evidence presented herein shows that both subunits of the FCBP are members of the 14–3–3 family. This conclusion rests on the facts that (i) the partial sequences obtained for the two polypeptides fall into areas of high sequence conservation

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KCIP                82  SNEDRNMVET ELKLISNDIL DVLDKHLIPA ANTGESKVFY YKM  124
14-3-3 ß            80  -KEYREKIEA ELQDICNDVL QLLDKYLIPN ATQPESKVFY LKM  121
Oenothera PHP-O     88  IRDYRSKIET ELSNICGGIL KLLDSRLIPS AASGDSKVFY LKM  130
Arabidopsis GF14    85  IREYRSKIET ELSGICDGIL KLLDSRLIPA AASGDSKVFY LKM  127
Maize GF14          75  IKDYRGKIET ELTKICDGIL KLLESHLVPS STAPESKVFY LKM  117
plant consensus     91  I..YR.KIET EL..IC.GIL KLL.S.L.P. .....SKVFY LKM  133
FCBP                    KDYRGK              KLLDSH          KVFY LK
                        -1-                 -2-             -3-

KCIP               192  ------AFDE AIAELDTLNE ESYQDSTLIM  215
14-3-3 ß           189  CSLAKTAFDE AIAELDTLNE ESYKDSTLIM  218
Oenothera PHP-O    198  CNLANEAFDE AIAELDTLEE ESYKDSTLIM  227
Arabidopsis GF14   195  CNLAKQAFDE AIAELDTLGE ESYKDSTLIM  224
Maize GF14         185  CSLAKQAFDE AISELDTLSE ESYKDSTLIM  214
plant consensus    201  C.LA..AFDE AIAELDTL.E ESYKDSTLIM  230
FCBP                    KQAFDE AIAELDTLGE ESY
                        -4-
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Fig. 2. Comparison of the partial sequences obtained for the FCBP-peptides (FCBP) with those of known 14–3–3 proteins. Amino acids are denoted in single-letter code. Peptides 1 to 3 (bold) were derived from the 31.5 kDa polypeptide while peptide 4 (bold and underlined) represents part of the 30.5 kDa polypeptide. The other 14–3–3 sequences are: protein kinase C inhibitor protein from sheep brain (KCIP) [24], 14–3–3 β isoform from bovine brain (14–3–3 β) [25], *Oenothera* PHP-O [30], *A. thaliana* GF14 [18] and maize GF14 [22].

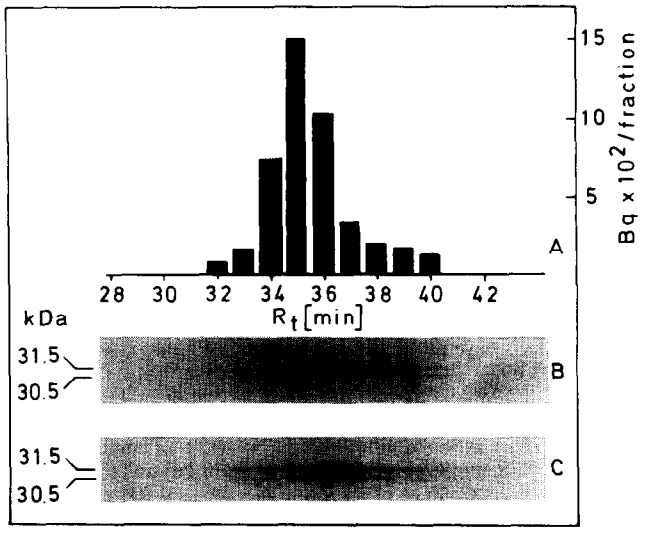

Fig. 3. Profile of protein-bound radioactivity (A), polypeptide pattern (B) (silver stained) and immunolocalization of 14–3–3 homologs (C) in fractions eluting from high-performance Mono Q anion exchange columns (last purification step of the FCBP, as detailed in [3]). Aliquots of 100 μl (B) or 300 μl (C) of the individual fractions were used to precipitate protein with methanol and the precipitates were subsequently separated on 12.5% polyacrylamide gels in the presence of SDS.

among all 14–3–3 proteins (cf. Fig. 2; for review see [12]). Peptides 1 and 3 derived from the 31.5 kDa subunit are identical in sequence to the corresponding segments of maize GF14 [22], and there is a single amino acid difference between peptide 2 of the 31.5 kDa subunit and the corresponding sequences from maize and *A. thaliana* [18]. Peptide 4 derived from the 30.5 kDa subunit is identical to the *A. thaliana* sequence, and there exists a single amino acid difference to maize. (ii) 14–3–3 homologs are acidic proteins with isoelectric points around pH 5, apparent molecular masses of 29–33 kDa in SDS-PAGE, blocked N-termini (due to acetylation) and form dimers [12], all known properties of the FCBP. (iii) Antibodies raised against plant 14–3–3 proteins [18] recognize the purified FCBP, and the immunoreactivity co-migrates with the fusicoccin radioligand, whether reversibly or covalently attached to the FCBP, in a variety of different experimental situations (cf. Figs. 3–5).

The data shown in Fig. 5 prove a functional connection between activation of the plasma membrane H^+-ATPase by FC, the concomitant increase in abundance of the hydrophobic, plasma membrane associated 14–3–3 isoform(s) and the increase in FC-binding activity. We therefore conclude that the FC receptor is an oligomer composed of 14–3–3 protein homologs. These are distinguishable from other cellular 14–3–3 isoforms by their strong apparent hydrophobicity. Similarly, distinct isoforms of animal 14–3–3 proteins (γ and ε) associate tightly with membranes by directly interacting with phospholipids [23]. The structural basis for this hydrophobicity is still unknown.

Although considerably conserved in terms of primary sequence, it is now clear that members of the family of 14–3–3 proteins serve a multitude of distinct functions, often in the regulation of specific signal transduction pathways (for review see [12]). A novel type of protein kinase inhibitor (KCIP) originally isolated from sheep brain [24] belongs to this family as well as neuronal protein kinase-dependent activators of tyrosine or tryptophan hydroxylases involved in monoamine neurotransmitter biosynthesis [25]. 14–3–3 proteins are further involved in the regulation of calcium-dependent exocytosis in animal cells [23], the timing of mitosis in *Schizosaccharomyces pombe* [26] and the development of neoplasia [27]. In plants, 14–3–3 homologs have been described in several species and various contexts. The *A. thaliana* [18] and maize [22] GF14 proteins are part of the G-box binding complex and thus are involved in transcriptional control, and the barley HV1433 mRNA increases during fungal infection, suggesting that HV1433 plays a role in pathogen defense [28].

The molecular mechanism, by which the FC–receptor complex activates the plasma membrane H^+-ATPase is currently under investigation. FC activation of the enzyme involves its C-terminal autoinhibitory domain (for review see [20]). It appears possible, therefore, that the ligand-occupied receptor associates directly with this domain of the ATPase, thereby locking the enzyme in its active state (as can be achieved alter-

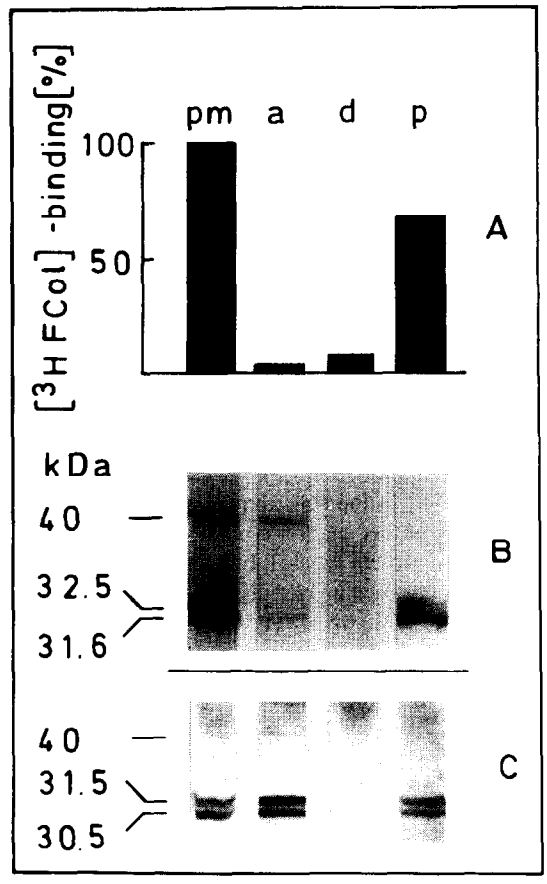

Fig. 4. Hydrophobicity of the reversibly (A) or photoaffinity (B) labeled FCBP and of 14–3–3 homologs (C) characterized by means of Triton X-114 partitioning of isolated plasma membrane fractions, pm; a, aqueous phase; d, detergent phase; p, lipid-rich detergent-insoluble phase (pellet). (A) Relative distribution of radioactivity (%); 100% = activity of the plasma membranes prior to partitioning. (B) Autoradiogram showing radiolabeled polypeptides after SDS-PAGE (11% gel); plasma membrane protein (1 mg) was subjected to Triton X-114 partitioning, and the total methanol-precipitated protein per fraction was loaded on the gel (lane pm, 700 μg protein loaded). (C) Proteins were separated by SDS-PAGE (12.5% gel) and transfered to nitrocellulose, followed by immunodetection of 14–3–3 homologs; 200 μg plasma membrane protein was subjected to Triton X-114 fractionation and the total recovered protein was loaded on the gel (lane pm, 100 μg protein loaded).

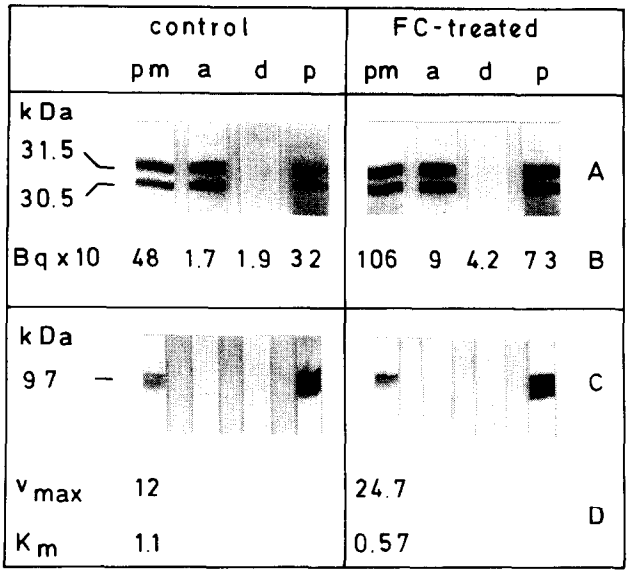

Fig. 5. Effect of in vivo treatment of leaves from *C. communis* with 1 μM FC on the immunoreactive 14–3–3 homologs (A), FC-binding (B), H^+-ATPase polypeptide (C) and kinetic properties of the enzyme (D). Control, plasma membranes from standard leaves; FC-treated, plasma membranes from leaves pretreated for 30 min in FC (1 μM). Lanes labeled as in Fig. 4. Plasma membranes (150 μg) were fractionated in Triton X-114, and the total protein recovered per fraction was applied on a gel to obtain the immunoblot data shown in A and C (pm, 50 μg protein applied to gel). B, radioactivity per fraction, corresponding to 3.03 pmol bound radioligand (mg protein)$^{-1}$, pm control; 6.67 pmol bound radioligand (mg protein)$^{-1}$, pm FC-pretreated. D, influence of FC-pretreatment on V_{max} (nkat(mg protein)$^{-1}$) and on apparent K_m for ATP (mM) of the H^+-ATPase of plasma membrane vesicles (measured at pH 7.3 [21]).

natively by proteolytic removal of the autoinhibitory domain [20]). On the other hand, P-type ATPases are subject to regulation by phosphorylation/dephosphorylation [20], and kinases have been implied in the function of several 14–3–3 proteins [12], providing another plausible model of FC action (regulation of a specific protein kinase). Further insight in the mechanism awaits the molecular cloning and functional analysis of the 14–3–3 isoform(s) comprising the FC receptor. This work is in progress.

Acknowledgements: The authors are grateful to Dr. R.J. Ferl, Gainesville, USA, for the gift of monoclonal antibody GF14–19, to Dr. R. Serrano, Valencia, Spain, for the gift of anti-H^+-ATPase antiserum and to the DFG, Bonn and Fonds der Chemischen Industrie, Frankfurt (literature provision) for financial support. We especially thank Klaus Hagemann for excellent technical assistance.

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