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Receptor-like kinase activity in membranes of *Arabidopsis thaliana*

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A class of protein kinases in the plant *Arabidopsis thaliana* has been identified which has biochemical characteristics similar to those of the animal receptor protein kinases. These plant protein kinases are membrane-associated, glycosylated, exhibit a preference for Mn^{2+} relative to Mg^{2+}, and range from 115 to 135 kDa when identified by renaturation after protein blotting. Evidence is presented that the TMK1 protein, a receptor-like protein kinase identified in *Arabidopsis* from its cloned gene, is also glycosylated in its native form, and shows a preference for Mn^{2+} over Mg^{2+} when assayed for kinase activity. All of the plant receptor-like protein kinases identified phosphorylate only serine and threonine residues.

Receptor protein kinase; Membrane phosphorylation; Signal transduction; *Arabidopsis thaliana*

1. INTRODUCTION

Receptor protein kinases play an integral role in the signal perception of animal systems, and mediate the responses to various growth factors and hormones. These receptor protein kinases have a number of defining structural and biochemical characteristics. They contain a glycosylated amino-terminal domain which binds the ligand, a single transmembrane domain, and a carboxyterminal domain with kinase activity [1,2]. Most known animal receptor kinases phosphorylate tyrosine residues, however a number of animal receptor kinases have now been identified that phosphorylate serine and threonine residues [3–5]. Unlike most other characterized kinases, the animal receptor kinases show a preference for Mn^{2+} over Mg^{2+} as the divalent cation required for activity [1]. Because the animal receptor kinases contain large ligand-binding and kinase domains, they typically have a molecular mass greater than 80 kDa [1].

Recently, plant genes have been cloned that code for polypeptides with the structural characteristics of receptor protein kinases. These genes code for proteins with single transmembrane domains and carboxyterminal kinase domains. The amino-terminal domains vary, one group having homology to the S-locus glycoproteins of Brassica [6,7], a second group containing leucine-rich repeats [8,9], and a third group containing epidermal growth factor repeats [10]. All the plant receptor-like protein kinases identified to date contain the consensus amino acids indicative of serine/threonine substrate specificity. Although structural data exists, little is known about the nature and function of the receptor-like protein kinases in plants.

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We have used a biochemical approach to identify protein kinases in the higher plant *Arabidopsis thaliana* which share features with the animal receptor protein kinases. A class of protein kinases has been identified which are membrane-associated, glycosylated, and have a similar cation requirement to that of the animal receptor protein kinases. These results indicate that plants may utilize a signal transduction pathway that is biochemically similar to that of animals.

2. MATERIALS AND METHODS

2.1. *Partial purification of receptor-like kinase activity from* Arabidopsis

Arabidopsis thaliana (ecotype Columbia) was grown in liquid culture for 2–4 weeks [8]. Leaves (2 g) were homogenized with 2 ml extraction buffer (30 mM Tris, pH 8.5, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 20% glycerol, and 1 mM phenylmethylsulfonylfluoride, 1 μg/ml pepstatin, 10 μg/ml aprotinin, and 10 μg/ml leupeptin as protease inhibitors) at 4°C, and the homogenate strained through Miracloth (Calbiochem). For isolation of microsomes, the homogenate was centrifuged at 8,000 × *g* for 15 min to remove intact organelles, and microsomes then pelleted from the supernatant by centrifugation at 100,000 × *g* for 30 min. For rapid isolation of a membrane-enriched fraction, the low-speed centrifugation step was eliminated. The pelleted membrane fraction was resuspended in 500 μl solubilization buffer (10 mM Tris, pH 7.3, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100) with the above protease inhibitors, and centrifuged at 100,000 × *g* for 30 min. This supernatant fraction is referred to as the Triton X-100 solubilized membranes.

Glycosylated proteins were isolated [11] by incubating 100 μl Triton X-100 solubilized membranes with either 100 μl concanavalin-A agarose or wheat-germ-lectin agarose (Sigma) for 30 min at 4°C. The lectin agarose was then washed three times with 1 ml solubilization buffer, once with 1 ml solubilization buffer containing 1 M NaCl, and three times with 1 ml solubilization buffer. The material bound to lectin agarose was either then assayed for kinase activity or eluted in loading buffer and subjected to SDS-PAGE for blot renaturation.

2.2. *Glycosylation analysis of the TMK1 protein*

Binding of glycosylated proteins to wheat-germ-lectin agarose and

wash steps were as above, however wash buffers contained 0.1% Triton X-100. Sugar elution was with 0.5 M *N*-acetylglucosamine in wash buffer. Binding to concanavalin-A agarose was at room temperature with a 0.5 ml column of the lectin agarose. Sugar elution was with wash buffer containing 0.2 M methylglucoside and 0.2 M methylmannoside. Samples were subjected to SDS-PAGE and immunoblotted as described [8], using a polyclonal antibody (Ab1930) directed against the amino-terminal domain of the TMK1 protein.

For enzymatic deglycosylation [12], Triton X-100 solubilized membranes were brought to 0.5% SDS, 1% β-mercaptoethanol and heated at 75°C for 10 min. The sample was then brought to 5% Triton X-100, an equal sample volume of 100 mM sodium acetate pH 5.0 added, and the sample brought to 10 mM EDTA. The sample (25 μl) was incubated with endoglycosidase F/*N*-glycosidase F (Boehringer Mannheim Biochemicals; 0.05 units) for 18 h at 37°C. A control was incubated without endoglycosidase F/*N*-glycosidase F.

2.3. *Kinase activity and phosphoamino acid analysis*

Material bound to lectin agarose (25 μl agarose) was assayed in 50 μl of 50 mM Tris, pH 7, 1 mM dithiothreitol, 10 mM $MgCl_2$ or $MnCl_2$ as indicated, 0.1% Triton X-100, 20 μM ATP and 20 μCi [γ-^{32}P]ATP for 20 min at room temperature. The reaction was stopped and the lectin agarose washed with 10 mM Tris, pH 7.3, 150 mM NaCl, containing 100 mM EDTA. Proteins were eluted from the lectin agarose with SDS-containing buffer, subjected to SDS-PAGE [13], and analyzed by autoradiography.

For blot renaturation, proteins bound to lectin agarose were eluted with SDS-containing buffer, subjected to SDS-PAGE, electroblotted to Immobilon-P membrane (Millipore), denatured, and renatured as described [14]. The blot was incubated in 8 ml of 30 mM Tris, pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 250 μCi [γ-^{32}P]ATP for 30 min at room temperature, washed as described [14], and subjected to autoradiography.

The maltose binding protein/TMK1 kinase fusion protein was expressed in *E. coli*, purified, and assayed for kinase activity as previously described [8].

For phosphoamino acid analysis, radioactively labeled protein was electroblotted to Immobilon-P membrane and hydrolyzed for 1.5 h in 200 μl 6 N HCl at 110°C under nitrogen [15]. Two-dimensional thin-layer electrophoresis of phosphoamino acids was then performed on 20-cm-square cellulose sheets (EM Separations), pH 1.9 in the first dimension and pH 3.5 in the second dimension [16]. Position of samples was determined ninhydrin visualization of phosphoamino acid standards.

3. RESULTS

Animal receptor protein kinases traverse the membrane and, because they contain only a single transmembrane domain, can be solubilized with the nonionic detergent Triton X-100 [1,17–19]. Animal receptor protein kinases are also glycosylated, and can be partially purified by taking advantage of their capacity to bind to lectin columns [1,17–19]. Using a similar strategy to that employed for the animal receptor protein kinases, glycosylated membrane proteins were purified from the higher plant *Arabidopsis thaliana*. A 1% Triton X-100 extract was prepared from a membrane fraction, and the solubilized glycosylated proteins then isolated by binding to lectin agarose.

In trial experiments it was determined that glycosylated kinase activity was associated with the microsomal fraction, and was substantially enriched for when compared to the soluble fraction, with the relative specific activity in the soluble fraction only 13% of that in the microsomal fraction. In addition, whereas 1% Triton X-100 could solubilize glycosylated kinase activity, 1% Tween-20 was relatively ineffective. In order to minimize proteolysis, a rapid extraction procedure was subsequently used for isolation of a membrane-enriched fraction from *Arabidopsis*.

A 1% Triton X-100 extract of the membrane-enriched fraction was incubated with lectin agarose, and a kinase assay using [γ-^{32}P]ATP then performed directly on material that bound to the lectin agarose. Phosphorylated proteins were identified by SDS-PAGE and autoradiography (Fig. 1A). Both concanavalin-A agarose and wheat-germ-lectin agarose bound proteins with kinase activity, although greater activity was found associated with the concanavalin-A agarose purified fraction. This result is consistent with the findings that many plant glycans are of the high-mannose variety and would be expected to show a preference for concanavalin-A [20]. The cation-specificity's of the partially purified kinase activities were tested. With both lectin purified fractions, the majority of the kinase activity exhibited a preference for Mn^{2+} over Mg^{2+} (Fig. 1A). This specificity was observed at either 5 or 10 mM concentrations of the cation. With the concanavalin-A purified frac-

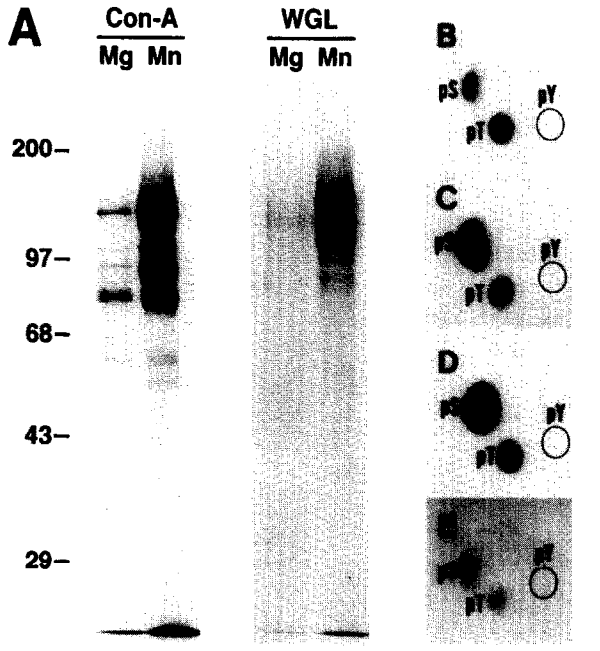

Fig. 1. Kinase activity of glycosylated proteins isolated from a membrane-enriched fraction of *Arabidopsis*. (A) Proteins bound to concanavalin-A agarose (Con-A) or wheat-germ-lectin agarose (WGL) were assayed for kinase activity in the presence of 10 mM $MgCl_2$ or $MnCl_2$, and subjected to SDS-PAGE. An autoradiogram of phosphorylated polypeptides is shown. Migration positions of molecular mass markers are indicated in kilodaltons. Tick marks indicate positions of polypeptides which show greater phosphorylation in the presence of Mn^{2+} than Mg^{2+}. Phosphoamino acid analysis was performed on the regions marked B, C, D, and E in panel (A), and the results are shown in panels (B), (C), (D), and (E), respectively. Positions of nonradioactive phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) references are indicated.

tion, greater phosphorylation of polypeptides at 80, 95, 100, 115, 125, and 135 kDa was observed in the presence of Mn^{2+} than in the presence of Mg^{2+}. With the wheat-germ-lectin purified fraction, greater phosphorylation of polypeptides at 90, 98, 105, 115, 125, 135 kDa was observed in the presence of Mn^{2+}. Several polypeptides of the same size class were phosphorylated with both the concanavalin-A and the wheat-germ-lectin purified fractions, although the relative degree of phosphorylation varied. Of particular interest is the observation that the most strongly phosphorylated polypeptides are in a size-range typical for receptor protein kinases [1]. This phosphorylation may represent autophosphorylation since the kinase assays were performed on proteins bound to lectin agarose and in the presence of detergent, both of which would minimize the ability of a kinase to interact with other substrate proteins.

To determine the amino acid specificity of the kinase activity, phosphorylated polypeptides were subjected to acid hydrolysis and analyzed by two-dimensional thin-layer electrophoresis (Fig. 1B–E) Only phosphoserine and phosphothreonine were detected. No phosphotyrosine was detected for any of the phosphorylated polypeptides.

In order to specifically identify proteins with kinase activity, the technique of renaturation on protein blots was used [14]. In this technique, proteins are subjected to SDS-PAGE, electroblotted to membrane, renatured,

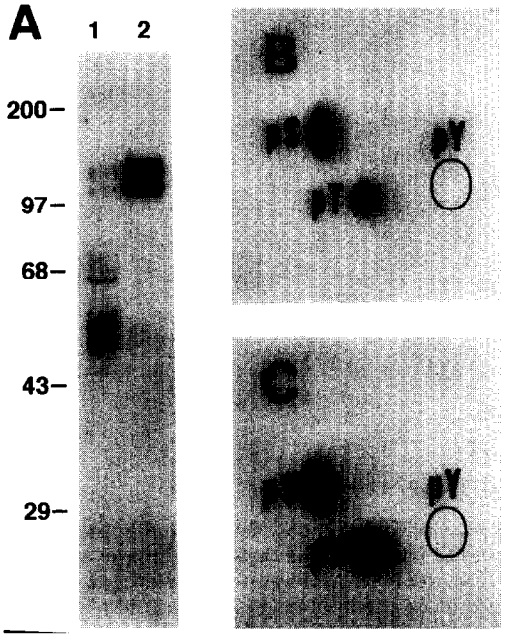

Fig. 2. Blot renaturation of protein kinases. (A) 10 μl Triton X-100 solubilized membranes (lane 1) or concanavalin-A purified proteins from 100 μl solubilized membranes (lane 2) were analyzed for kinase activity following blot renaturation. An autoradiogram is shown. Tick marks indicate positions of autophosphorylating polypeptides enriched for by binding to concanavalin-A. Phosphoamino acid analysis was performed on the regions marked B and C in panel (A) and the results are shown in panels (B) and (C), respectively.

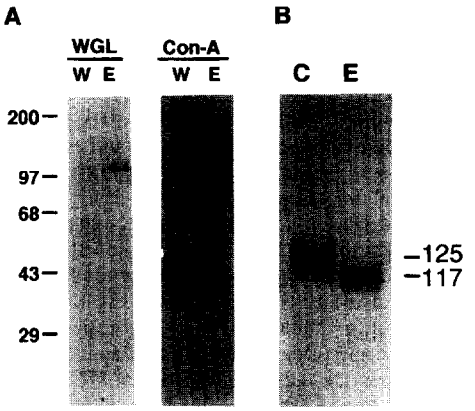

Fig. 3. Glycosylation of the native TMK1 polypeptide. (A) Binding and elution from lectin agarose. Triton X-100 solubilized membranes were incubated with either wheat-germ-lectin agarose (WGL) or concanavalin-A agarose (Con-A). Lanes show the final wash fraction (W) or the first sugar-elution fraction (E), with the TMK1 protein identified by immunoblotting. (B) Enzymatic deglycosylation. Triton X-100 solubilized membranes were either left untreated as a control (C) or incubated with endoglycosidase-F/*N*-glycosidase-F (E), and the TMK1 protein identified by immunoblotting. Molecular masses of untreated and treated TMK1 protein are at right.

and then incubated in the presence of [γ-^{32}P]ATP. Autophosphorylating kinases are identified by autoradiography. We observed four polypeptides capable of autophosphorylation that were specifically enriched for after partial purification on concanavalin-A agarose (Fig. 2A). The molecular masses of these protein kinases were 115, 123, 125, and 135 kDa, similar to what was previously observed when proteins bound to concanavalin-A were directly assayed for kinase activity (Fig. 1A). These polypeptides represent glycosylated high-molecular-weight kinases, characteristics in common with the animal receptor protein kinases. Phosphoamino-acid analysis of the phosphorylated polypeptides revealed phosphoserine and phosphothreonine, but no phosphotyrosine (Fig. 2B,C).

To complement these general studies, we have also examined the characteristics of the product of the TMK1 gene of *Arabidopsis*. The TMK1 gene codes for a polypeptide having the structural characteristics one would expect of a receptor protein kinase. It contains a hydrophobic signal sequence for membrane targeting, an amino-terminal domain with 6 potential glycosylation sites and 11 leucine-rich repeats, a single transmembrane domain, and a kinase domain [8]. The native TMK1 gene product in *Arabidopsis* was examined for glycosylation. A membrane-enriched fraction of *Arabidopsis* was solubilized with 1% Triton X-100 and this extract incubated with concanavalin-A or wheat-germ-lectin agarose. Western blotting was then performed using polyclonal antibodies directed against the amino-terminal domain of the TMK1 protein. The immunodecorated polypeptide bound to the immobilized lectins and was found capable of sugar-specific elution (Fig. 3A), indicative of glycosylation. Furthermore,

treatment of the Triton X-100 extract from *Arabidopsis* with endoglycosidase-F reduced the apparent molecular mass of the immunodecorated polypeptide from 125 to 117 kDa (Fig. 3B).

We also examined the divalent cation requirement for kinase activity of the TMK1 gene product. The carboxyterminal half of the TMK1 gene was expressed as a fusion protein with the maltose-binding protein in *E. coli*, using the expression vector pMAL-c [8]. The fusion protein was purified and examined for kinase activity. The kinase exhibited greater autophosphorylating activity with Mn^{2+} than with Mg^{2+} (Fig. 4).

4. DISCUSSION

Two approaches have been used to characterize receptor-like kinase activity in *Arabidopsis*, a general approach aimed at identifying proteins in *Arabidopsis* with the biochemical characteristics of receptor protein kinases, and a specific approach in which the TMK1 gene product, a polypeptide having the structural features of a receptor protein kinase [8], is biochemically characterized. We have identified a class of protein kinases in *Arabidopsis* which are membrane-associated, soluble in 1% Triton X-100, glycosylated, and exhibit a preference for Mn^{2+} over Mg^{2+} when assayed for kinase activity. Using the technique of renaturation on protein blots, four polypeptides of high molecular weight have been identified that belong to this class of protein kinases. These characteristics have been previously observed with the animal receptor protein kinases [1]. The class of protein kinases described here in *Arabidopsis* may also represent receptor protein kinases and play a similar role in plant signal transduction.

In order to minimize proteolysis in the characterization of *Arabidopsis* receptor-like protein kinases, we used a rapid extraction procedure with a membrane-enriched fraction. Evidence that the glycosylated kinases characterized are indeed membrane-associated is as follows. First, a similar pattern of glycosylated kinase activity was found in more highly purified microsomal preparations. Second, glycosylated kinase activity was enriched for in membrane preparations relative to soluble plant extracts. Third, the kinase activity was not removed from membranes by 150 mM NaCl, which was included in all isolation buffers, indicating that association is not mediated by electrostatic interactions. Finally, solubilization of glycosylated kinase activity required detergent extraction with 1% Triton X-100; detergent extraction with 1% Tween-20 was not effective. It will be of interest to determine if receptor-like kinase activity is present in plasma membranes or perhaps in other membrane systems as well. In this regard, there is evidence that a cloned receptor-like protein kinase from *Arabidopsis* may be present in chloroplasts [10].

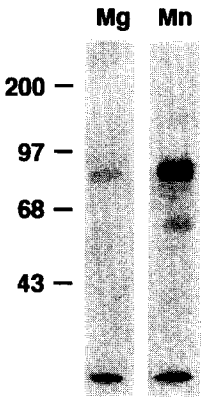

Fig. 4. Cation dependence of the TMK1 kinase. A fusion protein of maltose binding protein with the kinase domain of TMK1 (88 kDa) was expressed in *E. coli*, affinity purified, and assayed for kinase activity in the presence of 10 mM $MgCl_2$ or $MnCl_2$. An autoradiogram is shown.

Although we present evidence for a class of receptor-like protein kinases in *Arabidopsis* membranes, the exact number of independent protein kinases cannot be accurately determined. We observe four discrete polypeptides with kinase activity following renaturation, however the lower molecular weight polypeptides could be derived from proteolysis of the higher molecular weight polypeptides. For these studies, we have attempted to minimize proteolysis by using a rapid extraction procedure coupled with the use of protease inhibitors. A western blot analysis of proteins carried through the same procedure indicates that at least the TMK1 receptor-like protein kinase is isolated as a single size class on SDS-PAGE. The biochemical evidence presented here, along with sequence analysis of genes cloned from *Arabidopsis* [6–10], supports the concept that plants, like animals, may contain a battery of receptor protein kinases involved in diverse signal transduction processes.

The native product of the TMK1 gene, a receptor-like protein kinase in *Arabidopsis* [8], can also be solubilized from membranes with 1% Triton X-100, and based on several criteria is a glycoprotein. In addition, the kinase activity of the TMK1 polypeptide shows a preference for Mn^{2+} as a cation. As such, it also has biochemical characteristics in common with the animal receptor protein kinases. It should be noted that the native TMK1 polypeptide, as visualized by immunodecoration, is not one of the four autophosphorylating kinase polypeptides seen on renaturation (results not shown). This may be the result of insufficient protein for renaturation or simply an inability to renature with activity.

The receptor-like protein kinases biochemically identified in *Arabidopsis* were all found to be serine/threonine specific for phosphorylation. This is also true for the cloned receptor-like protein kinases of higher plants, including the TMK1 gene of *Arabidopsis*, that have been examined for amino-acid specificity after expression as fusion proteins in *E. coli* [8,21]. To date, no tyrosine protein kinase has been definitively identified

in higher plants. This contrasts with the animal receptor protein kinases of which most members are tyrosine-specific [1,2], although serine/threonine-specific examples are known [3–5]. Like the tyrosine receptor kinases [1], the plant receptor-like kinases exhibited a preference for Mn^{2+} over Mg^{2+} when assayed for kinase activity. The specific cation requirements for the recently identified serine/threonine receptor kinases of animals have not been reported. It is perhaps not surprising that the plant receptor-like kinases show a preference for Mn^{2+} since sequence of those cloned shows strong homology to tyrosine receptor kinases, except for the subdomains involved in amino acid specificity. The approaches described here should provide a foundation for further characterization of the plant receptor-like protein kinases and the animal serine/threonine receptor kinases, both of which are little understood biochemically.

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