TAKs, Thylakoid Membrane Protein Kinases Associated with Energy Transduction*

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The phosphorylation of proteins within the eukaryotic photosynthetic membrane is thought to regulate a number of photosynthetic processes in land plants and algae. Both light quality and intensity influence protein kinase activity via the levels of reductants produced by the thylakoid electron transport chain. We have isolated a family of proteins called TAKs, Arabidopsis thylakoid membrane threonine kinases that phosphorylate the light harvesting complex proteins. TAK activity is enhanced by reductant and is associated with the photosynthetic reaction center II and the cytochrome b$_6$f complex. TAKs are encoded by a gene family that has striking similarity to transforming growth factor β receptors of metazoans. Thus thylakoid protein phosphorylation may be regulated by a cascade of reductant-controlled membrane-bound protein kinases.

Protein phosphorylation in the photosynthetic membrane was first observed over 20 years ago (1), and since then threonine phosphorylation has been linked to a number of regulatory cascades that modulate photosynthetic rates (2). The phosphorylation of the light harvesting complex has been implicated in the modulation of light energy that is transmitted to the photosynthetic reaction center (3), either by separation of the macromolecular complexes (4) or controlled protein degradation (5). The phosphorylation is coupled to the association of the reduced plastoquinone and the cytochrome b$_6$f complex (6) and is influenced in different ways by both the quality and the amount of light (7). The D1 protein of reaction center II is also phosphorylated in a regulated fashion such that its turnover is linked to light quality and intensity via reductants produced from the photosynthetic electron transport chain (7, 8). Thus phosphorylation of thylakoid membrane proteins is an important regulative process in land plants and algae. Protein kinase activities that can phosphorylate thylakoid proteins have been described (9, 10), yet these proteins have not been purified or cloned. We report here on the isolation of a thylakoid protein kinase that is associated with the cytochrome b$_6$f complex and photosystem II (PS II) and that can phosphorylate the LHCP complex.

**EXPERIMENTAL PROCEDURES**

Construction of pGem-Tak1 and in Vitro Translation—Tak1 was PCR amplified using BAC clone T10P11 (Arabidopsis Ohio stock center) as a template using the following primers: 5′-CGG GAA TTC CAC ACA AGA AAA AAC-3′ and 5′-CGG GAA TTC CAC ACA AAA GCA-3′ and 5′-CGG GAA TTC CAC ACA AGA AAA AAG-3′; introducing EcoRI sites in both the 5′ and 3′ regions. This PCR product was digested, gel purified, and ligated into an EcoRI linearized, calf alkaline phosphatase-treated pGEM4 (Amersham Pharmacia Biotech) vector. The correct orientation of the insert was determined by restriction analysis. pGem-Tak1 DNA was linearized with Kpn1 (Promega) and used as a template for in vitro transcription (Sp6 RNA Polymerase, Amersham Pharmacia Biotech) and in vitro translation in a wheat germ extract (Amersham Pharmacia Biotech) using [35S]methionine (ICN Biochemicals) as described earlier (11).

**TAK Purification and Kinase Assay**—5 μg of purified thylakoids (11) were resuspended in 10 ml of IB (50 mM HEPES, pH 7.5 (KOH) and 330 mM sorbitol) and incubated with 5 μM of DBMIB, 125 mM NaCl, 10 mM MgOAc, 5 mM MnCl$_2$, 50 μM ATP, pH 7 (KOH), and 0.05 mM EDTA, pH 8, 1 mM MnCl$_2$, and 1 mM phenylmethylsulfonyl fluoride, and then applied to a Sephacryl G200 column at 4 °C (40 ml of packed volume, 27 × 1.5 cm) equilibrated and swollen in 50 mM HEPES, pH 7.5 (KOH), 10 mM EDTA, pH 8, and 1 mM MnCl$_2$.

**In Vitro Light Harvesting Complex Phosphorylation and TAK Auto-phosphorylation—Thylakoid membranes, equivalent to 2 μg of chlorophyll, were resuspended in 50 mM HEPES, pH 7.5 (KOH), 10 mM EDTA, pH 8, and 1 mM MnCl$_2$, with or without 25 μM 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-benzoquinone (DBMIB), respectively, were preincubated in the light (120 μmol photons·cm$^{-2}$·s$^{-1}$) dark for 2 h at 25 °C, 10 μCi [γ-32P]ATP and kinase buffer components to the amounts indicated above (see “TAK Purification and Kinase Assay” were added along with 0.5 μg/ml purified TAK (in 50 mM HEPES, pH 7.5 (KOH), 10 mM EDTA, pH 8, 1 mM MnCl$_2$, and 1 mM phenylmethylsulfonyl fluoride). The reactions were incubated for 1 h with agitation every 15 min at 25 °C in the dark.

**Peptide Sequencing—Arabidopsis purified TAK, resuspended in 50 mM HEPES, pH 7.5 (KOH), 10 mM EDTA, pH 8, 1 mM MnCl$_2$, was subjected to electrophoresis on a 10% SDS-PAGE gel, followed by transfer to polyvinylidene difluoride (Millipore, Schleicher & Schuell) in 10 mM CAPS, pH 11, for 2 h at 400 mA. The membrane was rinsed with filtered double distilled H$_2$O and stained with 0.1% Amido Black (Sigma) in 40% methanol and 1% acetic acid followed by destaining in filtered double distilled H$_2$O. The appropriate bands were excised, subjected to CNBr treatment, and sequenced.

We expected the cleaved peptides to have amino-terminal methionines, but this was not observed. The abbreviations used are: PS II, photosystem II; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DTT, dithiothreitol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; CAPS, 3-cyclohexylamino)propanesulfonic acid; LHCP, light harvesting protein; TGF, transforming growth factor.
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RESULTS AND DISCUSSION

To identify proteins that interact with the major light harvesting protein (LHCP), we developed a yeast assay that allowed selection of cDNAs encoding activities that bind a known target (12). We used as target the LHCP amino terminus, which contains the site of threonine phosphorylation, and we reported on the isolation of an Arabidopsis cDNA that encoded a 68-kDa protein kinase, WAK1, that could interact with LHCP in yeast (13). However, WAK1 in plants is responsible for interaction between the plant cell wall and plasma membrane (14), and we reasoned that its isolation was due to reduced substrate specificity when expressed in yeast. We expressed the kinase domain of WAK1 in Escherichia coli (14) and used this purified protein as denatured antigen in rabbits or reconstituted antigen in mice. The rabbit serum recognized WAK1 protein in Arabidopsis (14), but the mouse serum specifically identified a 55-kDa protein in leaf extracts (Fig. 1A, lane Th). Leaves were separated into soluble, microsomal, nuclear, and chloroplast fractions, and the 55-kDa protein was found exclusively in the chloroplast. Further fractionation (11) revealed that the 55-kDa protein was on the thylakoid membrane (Fig. 1A, lane Th) and not in the envelope (Fig. 1A, lane E) or stroma (Fig. 1A, lane S) of the chloroplast. We tentatively named the 55-kDa protein TAK for thylakoid-associated kinase. TAK can be extracted from a low-speed supernatant (Fig. 1A, lanes S versus lanes P) but not by NaHCO₃ (lanes P versus lanes S), indicating that it is integral or internal to the thylakoid membrane (11). TAK is sensitive to protease K treatment of intact thylakoid membranes (Fig. 1A, lane PrTK). Because these levels of protease K do not disrupt thylakoid membranes (11), we conclude that TAK is integral to the membrane and has a large portion exposed to the chloroplast stroma. This stromal portion reacts with antiserum raised to the kinase, and so we predict that TAK contains a stromal kinase domain, a necessary location for a LHCP-specific kinase.

TAK was first identified by an antiserum to a kinase domain, and we were interested to see whether TAK was indeed a protein kinase. We purified TAK from Arabidopsis leaves by first isolating chloroplasts and then fractionating the thylakoid complexes by sucrose gradient centrifugation (11, 15). TAK was enriched in a PS II particle containing the cytochrome b₆f complex (6). The PS II-enriched membrane preparation was incubated with urea and centrifuged, and the dialyzed supernatant was separated on a Sephadex sizing column. The eluted fractions were tested for kinase activity, and a sample containing only a 55-kDa protein band that also reacted with the TAK antiserum was able to phosphorylate BSA in vitro (see “Experimental Procedures”). Silver staining of this active fraction reveals no other proteins in addition to the 55-kDa band.

We next determined whether the purified TAK preparation would phosphorylate thylakoid membrane proteins. When thylakoid membranes are incubated in the light with [³²P]ATP and analyzed by denaturing gel electrophoresis and autoradiography, the 28-kDa LHCP, 31-kDa D1, 43-kDa cp43, and a 9-kDa protein are the most abundant phosphorylated species (Fig. 1B, lane 6) (16). The thylakoid phosphorylation activity can be greatly diminished if thylakoids are first incubated in the dark in the presence of both a PS II (DCMU) and b₆f electron acceptor (DBMIB), which leads to the oxidation of the PQ pool and thus inhibits kinase activity (as shown in Fig. 1B, lane 1) (16). Purified TAK was incubated with the inhibitor treated thylakoids along with [³²P]ATP in the dark, and the reaction was separated on denaturing polyacrylamide gels followed by autoradiography. The results are shown in Fig. 1B (lanes 4 and 5). TAK phosphorylated the 28-kDa LHCP in the presence (lane 5) but not the absence (lane 4) of the reductant DTT. Phosphorylation of other thylakoid proteins is also detected under these conditions, but these levels are significantly lower than the LHCP phosphorylation. This lower level of phosphorylation is also detected using BSA as a substrate (data not shown), indicating that it might represent nonspecific activity. Incubation of the TAK preparation with only [³²P]ATP in the presence or absence of DTT did not lead to phosphorylation (Fig. 1B, lanes 2 and 3), indicating that TAK is not capable of phosphorylating itself under these conditions. Incubation of TAK with stromal proteins also does not lead to phosphorylation (data not shown), and thus we conclude that TAK preferentially phosphorylates LHCP.

To obtain cDNAs for TAK we attempted to screen an Arabidopsis cDNA expression library with TAK antiserum, but surprisingly we isolated only WAK1 clones. We therefore obtained peptide sequence from the TAK preparation. Four distinct sequences were obtained from a cyanogen bromide-degraded preparation, and these were each used in four separate searches of the Arabidopsis data base. One gene sequence was common to each of the four independent searches, and this was named Tak1. The predicted amino acid sequence of the encoded 55-kDa protein is shown in Fig. 2A where the four peptides are lightly underlined. The amino-terminal 26 residues are predicted to serve as a chloroplast targeting sequence (17), followed by a 20-amino acid hydrophobic region that could serve as a transmembrane domain (bold dashed line above sequence). No signal cleavage site is predicted (18). Amino acids 167–190 may form a distinct ATP binding P-loop (19), and the remaining sequence includes the 11 conserved domains (amino acids 290–450) characteristic of protein serine/threonine kinases (20). Amino acid analysis for membrane topology predicts that the amino-terminal regions would lie in the thylakoid
lumen and the kinase would be stromal (21). A search for related proteins identifies numerous protein kinases, including members of the metazoan TGFβ receptor family, and their identities are shown in Fig. 2A where identical amino acids are in bold and similar sequences in gray. The sequence shown is ALK5, a subclass of human TGFβ-I receptors, but identities are also found with C. elegans and Drosophila receptors (22). These similarities lie both within and outside of the kinase domains, but the reason this receptor class is chosen above the other kinase families is the presence of the SGSGSG box (bold underline) in both TAK1 and the TGFβ-I receptor. This region is the TGFβ-I receptor family signature and acts as the recognition sequence for the partner receptor TGFβ-II, which phosphorylates the TGFβ-I receptor upon ligand binding (23).

The similarity of TAK1 to the TGFβ receptor suggested that there might be a kinase cascade in the thylakoid. We also predicted that there could be multiple TAK proteins and that TAK1 would be phosphorylated by another TAK. A search of the Arabidopsis DNA data base indeed identifies two additional Tak sequences, Tak2 and Tak3, and predictions of both encoded proteins show that they contain the four sequenced peptides. These genes were not identified in the initial search because they both contain introns. The TAKs are 90% identical except Tak3 lacks the carboxyl-terminal 41 amino acids of Tak1 and Tak2 (Fig. 2B), but the significance of this is unknown. Tak1, but not Tak2 and Tak3, contains the SGSGSG motif (Fig. 2C), and because the TGFβ-II receptor phosphorylates the TGFβ-I receptor by identifying the SGSGSG box, we draw a parallel between TGFβ-I receptor and Tak1 and the TGFβ-II receptor and Tak2 or Tak3. To explore this similarity we determined whether isolated Tak was indeed phosphorylated and whether the 55-kDa protein band represented multiple Tak proteins. Fig. 3A shows that the 55-kDa Coomassie Brilliant Blue-stained purified Tak fraction can indeed be resolved into two bands of 55 and 56 kDa upon higher resolution gel electrophoresis (lane Coom), and both proteins react with antiserum against Tak (aTAK). The 56-kDa and to a much lesser degree the 55-kDa Tak react with antiphosphothreonine and antiphosphoserine antiserum (Fig. 3A), indicating that the larger Tak is preferentially phosphorylated on threonine and serine...
residues. Anti-phosphotyrosine serum does not react with TAK (data not shown). These observations strengthen but do not confirm the parallel between TAKs and the TGFβ receptors, and additional analysis is required to explore these similarities. Although the TAK preparation does not phosphorylate itself (Fig. 1B), TAK phosphorylation may be context-specific and require the b,f complex.

To confirm that the TAK genes indeed encode thylakoid proteins, we imported an in vitro translated TAK1 into isolated chloroplasts. The TAK1 gene does not contain any introns, and the transcribed region was amplified by PCR, cloned, and expressed in vitro as a 35S-labeled protein (Fig. 3B, lane Trl) that migrates as a 55.5-kDa protein, between the TAK doublet isolated from tissue (Fig. 3B, lane Coom). The in vivo phosphorylation of the Arabidopsis TAK may alter the protein mobility relative to the in vitro translated protein. Southern analysis indicates that only three TAK genes are present in Arabidopsis (data not shown). Thus at present we can not conclude which gene encodes which TAK band. The in vitro synthesized TAK1 (Fig. 3B, lane Trl) was incubated with isolated Arabidopsis thylakoids (11), and the intact organelles were treated with protease to remove material not imported. TAK1 appears as a protease-resistant 55.5-kDa protein (Fig. 3B, lane Chl) and thus is imported and as predicted is not cleaved by the stromal protease. The 55.5-kDa labeled protein is located in the thylakoid but not stromal fraction (Fig. 3B, lane Thy versus Str). Because TAK1 is imported into the thylakoid and the predicted amino acid sequence contains the four sequenced peptides, we conclude that TAK1 and probably the 90% identical TAK2 and 3 are indeed thylakoid membrane proteins and encode at least one of the proteins identified as TAK in plant tissue.

The biochemical analysis of native TAKs, recombinant TAK1, and the predicted amino acid sequence all indicate that TAKs are anchored in the thylakoid membrane such that the protein kinase domain is stromal and can phosphorylate the amino terminus of LHCP. The data are consistent with a membrane topology where the 26 amino-terminal residues lie in the thylakoid lumen, and this raises the possibility that this domain not only serves as an uncleaved signal sequence but also as a sensor of the lumen. It has been demonstrated that kinase activation depends upon the binding of reduced plastoquinone to the b,f complex (16), and it was predicted that the kinase is intimately associated with the b,f complex. TAK indeed copurifies with both the b,f complex and PS II. Although we do observe reduc tant dependence of TAK activity in vitro, it is neither known how this is effected nor how the transmembrane domain or the luminal tail may sense the reduced form of the b,f complex as models predict. Our experiments assayed the activity of an exogenously added TAK, and the analysis of a kinase activity that is correctly associated with the b,f complex and PS II awaits the generation of TAK null alleles. The confirmation of a TAK cascade requires the analysis of recombinant protein and mutant plant analysis, and the similarities with the TGFβ receptor cascade remain suggestive but predictive. TAK antiserum detects a 55/56-kDa protein band in the thylakoids of pea and Chlamydomonas (data not shown), indicating that TAK is of general significance to land plants and algae. Future analysis can now employ these novel organelle kinases to probe the mechanism of redox-controlled phosphorylation of membrane proteins.

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