Development of the aleurone layer of maize grains requires the activity of the Defective kernel 1 (Dek1) gene, encoding a predicted 240-kDa membrane-anchored protein with a C terminus similar to animal calpain domain II&III. Three-dimensional modeling shows that DEK1 domain II contains a conserved calpain catalytic triad and that domain II&III has a predicted structure similar to m-calpain. Recombinant DEK1 domain II&III exhibits activity in the caseinolytic assay in the absence of calcium, although the activity is enhanced by calcium. This is in sharp contrast to animal calpains, which require Ca\(^{2+}\) to be active. Bacterially expressed DEK1 domain II does not display caseinolytic activity, suggesting an important role for DEK1 domain III. Mutation of the catalytic Cys residue to Ser leads to a loss of caseinolytic activity of DEK1 domain II&III. Two features of DEK1 calpain may contribute to maintaining the active site triad in an "active" configuration in the absence of Ca\(^{2+}\): both of which are predicted to keep m-calpain domains IIa and IIb apart. First, DEK1 lacks key charged residues in the basic loop of domain II, and secondly, the absence of an acidic loop in domain III, both of which are predicted to be neutralized upon Ca\(^{2+}\) binding. The Dek1 transcript is present in all cell types in developing maize endosperm, suggesting that the activity of the DEK1 calpain is regulated at the post-transcription level. The role of DEK1 in aleurone signaling is discussed.

In cereal grains, the aleurone layer consists of densely cytoplasmic cells covering the surface of the endosperm, the grain storage tissue that is used for feed, food and industrial raw material (1). Aleurone cells contain a large numbers of protein and oil bodies and are cytologically and biochemically distinct from the storage cells of the underlying starchy endosperm. Upon imbibition of the grain, aleurone cells secrete enzymes and oil bodies and are cytologically and biochemically distinct material (1). Aleurone cells contain a large numbers of proteins that are involved in cell proliferation, development of the aleurone layer of maize grains (3). From the double fertilization through a cellularization process that results in a peripheral layer of aleurone cell initials (3). From the Pioneer Hi-Bred International, A DuPont Company, Johnston, Iowa 50131

Cunxi Wang, Jennifer K. Barry, Zhao Min, Gabrielle Tordsen, A. Gururaj Rao, and Odd-Arne Olsen‡

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† To whom correspondence should be addressed. Tel.: 515-253-2417; Fax: 515-254-2619; E-mail: odd-arne.olsen@pioneer.com.

‡ To whom correspondence should be addressed. Tel.: 515-253-2417; Fax: 515-254-2619; E-mail: odd-arne.olsen@pioneer.com.

The abbreviations used are: dek1, defective kernel 1 gene; GST, glutathione S-transferase; DAP, days after pollination; IPTG, isopropyl-1-thio-β-D-galactopyranoside.
apoptosis, differentiation, and signal transduction. In addition, calpains have been implicated in endocytosis, exocytosis, and intracellular membrane fusion (30).

Although an understanding of calpain at the molecular, biochemical, and cellular levels has advanced greatly in animals since calpain was first described in 1964 (31), the functional significance of the DEK1 calpain in plants was not identified until 2001 (9). In maize, the phenotype of dek1 mutations suggests a role for Dek1 in global developmental regulation. In addition to the lack of aleurone cells, knockout of the Dek1 gene results in improper embryo axis formation and a missing shoot apical meristem (9). Also, as shown by mutant sector analysis, leaf differentiation, and in particular epidermis cell formation, requires Dek1 gene function (11). The predicted structure of DEK1 calpain domains (9) was amplified by PCR. The flanking primers used for domain II and III were 5'-CTGTTGGAGACCTCCTGTTCTAAGTG-3' and 5'-CAGTTAGAAGACAGATTCTCCAAACCGA-3' (altered codon is underlined). The mutation was verified by sequencing.

**EXPERIMENTAL PROCEDURES**

**Molecular Modeling of DEK1 Calpain Domains**—The crystal structure of rat (Rattus norvegicus) m-calpain catalytic subunit (PDB ID-0_A, 2.4 Å resolution) was used as a template to model the calpain domain of DEK1. Heterogeneous calpain catalytic subunit (PDB ID-0_A, 2.6 Å resolution) was used as a template to model the calpain domain of DEK1. Following a manual sequence alignment, the two proteins shared >30% sequence identity and >45% sequence similarity. Modeling studies were done on a SGI workstation with Modeler, an automated homology modeling program, using the Insight II software from Accelrys (San Diego, CA). Ten energy-minimized models were generated and three models with the lowest value of the objective function were further evaluated for appropriate stereochemistry using the Verify3D program (32). The model with the highest self-compatibility score in the Verify program was selected for the final study.

**Cloning of cDNA Domains Encoding DEK1 Domain Proteins in Escherichia coli**—Expression Vectors—cDNA fragments corresponding to DEK1 calpain domains (9) were amplified by PCR. The flanking primers used for domain II and III were 5'-GTGCTGATCCTACTGATCAAGAGTTCCCTC-3' with SalI site (forward) and 5'-GGCGCGCCCTAAACCGCCCTGTTAGGTTG-3' with NotI site (reverse) and 5'-GTGCTGATCCTACTGATCAAGAGTTCCCTC-3' with SalI site (forward) and 5'-GGCGCGCCCTAAACCGCCCTGTTAGGTTG-3' with NotI site (reverse), respectively. The cDNAs were ligated into pCR2.1 TOPO (Invitrogen) and then into pGEX-4T vector (Amersham Biosciences), which produced a glutathione S-transferase (GST) fusion at the N terminus. The recombinant plasmids were transformed into TOP10 E. coli (Invitrogen). After insert sequences were verified by sequencing, the constructs were transformed into BL21 (DE3) E. coli (Invitrogen) for protein expression.

**The Expression of DEK1 Domain II&III using Fermentation**—The Dek1 domain II and III pGEX 4T-3 construct was transformed into BL21 Codon + RP cells (Strategene). A 5-liter fermentor containing 5 liters of Terrific Broth (Yeast Extract 24 g/liter, pancreatic digest of casein 12 g/liter, dipotassium phosphate 9.4 g/liter, monopotassium phosphate 2.75 g/liter, magnesium sulfate 0.1 g/liter, 100 μg/ml ampicillin, and 5 ml of antifoam (Mazda DF 204 Defoamer) was inoculated with 50 ml of overnight culture grown at 37 °C. The fermentation culture was grown at 37 °C until an OD600 of 1.0 was reached. The temperature was then lowered to 30 °C. The dissolved oxygen was maintained at greater than 30% throughout the run by controlling both stirring and airflow. The pH was maintained at 7.2 with NH4OH. The pH was maintained at 7.2 with NH4OH. The culture was incubated overnight at 18 °C. The cells were then harvested for the GST-DEK1 domain II purification as mentioned above.

**Site-directed Mutagenesis of DEK1**—Site-directed mutagenesis of DEK1 domain protein was generated by a PCR-based overlapping method described by Ho et al. (33). The flanking primers were the same as those used for domain II and III. The internal primers used for generating mutation were 5'-CTGTTGGAGACCTCCTGTTCTAAGTG-3' and 5'-CAGTTAGAAGACAGATTCTCCAAACCGA-3' (altered codon is underlined). The mutation was verified by sequencing.

**DEK1 Activity Assay**—The method used to determine the proteolytic activity of DEK1 domains is based on an electrophoretic casein degragation assay used in previous studies of animal m-calpain (34). In our assays, a typical reaction contained 2 μg of DEK1 domain protein, 3 μg of purified β-casein (Sigma-Aldrich C6905, more than 90% purity) in 20 μl of reaction buffer (50 mM imidazole-HCl, pH 7.5, 10 mM β-mercaptoethanol). Reaction mixtures were incubated at 30 °C for a variable time interval. The reaction was stopped by adding NuPAGE LDS sample buffer (Invitrogen). After incubation at 70 °C for 10 min, the mixture was separated by a NuPAGE 10% bis-tris gel and then stained with Colloidal Blue kit (Invitrogen). Change in the intensity of the β-casein band was used to quantify DEK1 proteolytic activity. Three independent assays were carried out to calculate standard deviation (S.D.) in these assays. Intensity was measured by using the ChemiDoc system (Bio-Rad).

**CD Studies**—CD spectra were measured using a Jasco J-715 model spectropolarimeter. Far-UV spectra were recorded from 190 to 260 nm in a 0.1 mm pathlength quartz cuvette. Protein was dialyzed into 35 mM potassium phosphate buffer (pH 7.8) and diluted to a concentration of 0.3 mg/ml. Data are reported as mean residue ellipticity.

**In Situ Hybridization Analysis**—Digoxigenin-11-UTP-labeled RNA probes were used to localize Dek1 mRNA in cells according to the protocol described by Jackson (35). RNA probes were made using Digoxigenin-11-UTP-labeled NTP mixture with SP6 and T7 RNA polymerases (Roche Applied Science). The 721-bp fragment of 3'-region Dek1 cDNA was subcloned into pSPORT I vector (Invitrogen). The clone was linearized by XbaI (sense) and PstI (antisense) and transcribed into RNA in vitro. Unincorporated ribonucleotides were removed using QiaGen RNeasy purification kit and probes were subjected to carbonate hydrolysis in order to reduce probe length to ~150 nucleotides. Microscopic analysis was carried out in dark field using a Nikon Eclipse E800 microscope.

**RESULTS**

**Molecular and Structural Features of DEK1**—Searching plant databases using the maize Dek1 sequence (9) identified several Dek1 orthologues in other plant species, including rice, Arabidopsis, sugarcane, sorghum, and soybean. Sequence analysis indicated that DEK1 proteins from different plant species are highly conserved, sharing 70~98% sequence identity (Table 1). All plant species investigated were found to have a single copy of Dek1. Structurally, the deduced DEK1 sequence can be divided into several domains, including two transmembrane domains (B1 and B2), an extracellular loop region (C), a hydrophilic and charged domain (D), domain II and domain III as described previously (9). In addition, there is a predicted membrane targeting signal sequence located at the N terminus. Domain B1, B2, C, and D show no significant amino acid sequence similarity to any other sequences in databases, and their functions remain unknown. In the C terminus, the amino acid sequence of maize DEK1 domain II and III shares about 40~50% similarity and 30 to 40% identity to that of the same domains of animal calpains. To establish the evolutionary relationship among animal calpains and plant DEK1 proteins, a phylogenetic tree was constructed using domain II and III (Fig. 4).
The comparison was generated by the Gap program in GCG (Wisconsin package).

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Accession No.</th>
<th>Corresponding region to maize DEK1</th>
<th>Similarity</th>
<th>Identities</th>
</tr>
</thead>
<tbody>
<tr>
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<td>NP_175932</td>
<td>1–2159</td>
<td>82</td>
<td>70</td>
</tr>
<tr>
<td>Sugarcane</td>
<td>AAN10107</td>
<td>1000–2159</td>
<td>98</td>
<td>97</td>
</tr>
<tr>
<td>Rice</td>
<td>AAL38190</td>
<td>1–2159</td>
<td>94</td>
<td>91</td>
</tr>
<tr>
<td>Sorghum</td>
<td>BG464268</td>
<td>1358–1538</td>
<td>98</td>
<td>97</td>
</tr>
<tr>
<td>Soybean</td>
<td>BE298863</td>
<td>1807–2002</td>
<td>90</td>
<td>84</td>
</tr>
<tr>
<td>BM567824</td>
<td></td>
<td>1751–1942</td>
<td>94</td>
<td>94</td>
</tr>
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</table>

This analysis suggests that DEK1 domain II&III is evolutionarily most closely related to the animal SOL (small optic lobes) and SOLH (SOL homologue from humans) calpains.

Amino acid sequence alignment of DEK1 calpain and m-calpain demonstrates a conserved domain organization for domain II and domain III (Fig. 2). Indeed, this architecture appears to be remarkably well conserved even in the modeled three-dimensional structure of the protein (Fig. 3A). In addition to the overall fold, the conservation and arrangement of residues important for catalysis and substrate binding in domain II are particularly significant. As in m-calpain, the catalytic Cys-71 (Cys-105 in m-calpain) is located in the α-helix of domain IIα, on the opposite side of the interface with domain IIβ which harbors the two other residues of the catalytic triad, His-229 and Asn-249 (corresponding to His-262 and Asn-286 in m-calpain, Fig. 2). Importantly, the distance of His-229 and Asn-249 (corresponding to His-262 and Asn-286 in m-calpain, Fig. 2). Importantly, the distance of −10.5 Å between the catalytic Cys Sγ and the His Nδ1 in the model compares favorably with the −10.5 Å measurement for the same atoms in the calcium-free conformation of m-calpain (14, 36) and validates the modeled structure. Additional residues conserved within the context of the active-site cleft comprising the subdomains of the catalytic domain include Gln-65, Trp-72, Gly-160–161, Pro-250, and Trp-251 (Fig. 3B). Despite this strong conservation in domain II, however, there are two significant differences to be noted in domain III between m-calpain and DEK1. First, while the basic loop comprising residues His-415-His-427 of m-calpain contains 4 Arg residues and 1 Lys, the corresponding loop in DEK1 (residues 371–383 in Fig. 2) contains just one Arg residue. Even more importantly, in contrast to the very acidic loop of m-calpain (residues 392–402) consisting of 10 negatively charged residues, there is just one conserved Asp-347 in the DEK1 loop (residues 344–354 in Fig. 2; asterisk in Fig. 3A).

Expression and Purification of Recombinant DEK1 Wild-type and Mutated Domain Proteins—The partial cDNAs encoding DEK1 domain II&III and domain II alone were cloned into pGEX-4T-3 vector to produce the corresponding GST fusion proteins in E. coli. Under conditions normally used to express fusion proteins in E. coli, the majority of the expressed DEK1 domain II&III protein appears in inclusion bodies. Production of the wild-type and mutant (see below) versions of DEK1 domain II&III was therefore carried out by fermentation, yielding a significant amount of soluble domain II&III protein. The expressed DEK1 domain II&III protein was purified to near homogeneity with glutathione-Sepharose 4B beads (Fig. 4A). In contrast to DEK1 domain II&III protein, DEK1 domain II appears both in soluble and insoluble forms under standard expression conditions (Fig. 4A). The identity of the purified protein was confirmed by immunoblotting using an antibody raised against GST and by measuring GST activity (data not shown).

The far-UV CD spectra demonstrate that DEK1 domain II&III, domain II and a mutant, C71S, of the DEK1 domain II&III protein all have a similar secondary structure (see below for details on the mutant protein) (Fig. 4B). These results indicated that no gross structural rearrangements had occurred in the mutated protein.

Characterization of DEK1 Domain II&III Proteinase Activity—The proteolytic activity of DEK1 domain II&III was tested in an assay in which purified β-casein (Fig. 5A, lane 2) was incubated with the DEK1 domain II&III protein under various conditions (Fig. 5A, lane 3). In the presence of 5 mM Ca²⁺, casein degradation leveled off after approximately 9 h of incubation (Fig. 5A, lanes 4–9). This result suggests that DEK1 domain II&III possesses an activity that is similar to that of...
m-calpain. Surprisingly, however, DEK1 domain II&III also displays a strong proteolytic activity in the absence of Ca\(^{2+}\).

The same proportion of \(\beta\)-casein was degraded after 9 h in the absence of Ca\(^{2+}\) (Fig. 5A, lanes 10–15). Notably, in the presence of Ca\(^{2+}\), DEK1 proteolysis of casein produced two bands after 5 h of incubation (Fig. 5A, lane 6). In the absence of Ca\(^{2+}\), the second breakdown product (lower molecular weight) appeared as a weak band only after 12 h of incubation (Fig. 5A, lane 15). From these observations we conclude that Ca\(^{2+}\) acts as an activator of DEK1 in this in vitro assay. Incubating \(\beta\)-casein with different concentrations of purified DEK1 domain II&III demonstrates that the casein degradation is concentration dependent (Fig. 5C and D).

**Role of Cys-71 in DEK1 Domain II&III Proteolytic Activity**—

The sequence alignment of DEK1 and m-calpain predicts that Cys-71 is an active site residue of the catalytic triad. To verify whether or not DEK1 is a true cysteine proteinase, we created a mutant in which the Cys-71 was replaced with Ser as was previously done for animal calpains (37, 38). The mutant protein was expressed and purified in the same manner as wild-type DEK1 domain II&III protein. This mutation did not cause secondary structural rearrangements as evidenced by the CD spectrum (Fig. 4B). Activity measurements show that the mutant protein is inactive compared with the wild-type protein (Fig. 6A, lanes 4 and 5). This loss of activity clearly identifies the mutated cysteine as part of the active triad of the DEK1 domain II protein, and is in agreement with previous reports showing that the same mutation in m-calpain abolishes this activity.

To evaluate the effect of DEK1 domain III on the proteinase activity of DEK1 domain II&III, DEK1 domain II alone was incubated with \(\beta\)-casein. This experiment showed that in vitro DEK1 domain II alone was inactive (Fig. 6B, lane...
demonstrating that domain III is necessary for DEK1 proteolytic activity.

Expression of Dek1 in Maize Grains—As shown previously, the Dek1 transcript is present at a low level in most plant tissues (9). To determine if Dek1 mRNA shows an aleurone preferred pattern of expression in endosperm, we carried out in situ hybridization experiments using sections from various grain developmental stages. These experiments show that the Dek1 transcript is detectable in all grain cell types, including the maternal pericarp, aleurone cells, and the starchy endosperm (Fig. 7, A and B). This result corresponds well with the previously reported LYNX MPSS data (9), suggesting that the activity of DEK1 in the aleurone layer is post-transcriptionally regulated.

DISCUSSION

Two sets of data presented in this paper support the conclusion that Dek1 encodes a functional member of the calpain super-family of proteins. First, sequence alignment and three-dimensional modeling show a significant sequence and structure similarity between DEK1 and animal calpain (Figs. 2 and 3). Secondly, recombinant DEK1 calpain is active in the caseinolytic assay previously used for animal calpains, and this activity is abolished by changing the cysteine of the active site triad to serine (Figs. 4–6).

Regulation in vivo of conventional animal calpains such as m-calpain is complex, and operates at several levels, including signal transduction, Ca$^{2+}$ activation, subcellular relocation from cytosol to, or near, the plasma membrane, interaction with phospholipids, autocatalytic cleavage, and interaction with a regulatory small subunit. Extensive studies of the three-dimensional structure of m-calpain and other members of this cysteine proteinase family have revealed that the inactive ground state of the enzyme is one in which the amino acids of the catalytic triad are sterically separated. Calcium binding

Fig. 3. Three-dimensional modeling of DEK1 calpain. A, modeled structure of DEK1 calpain showing the active site residues Cys-71, His-229, Asn-249. The individual domains are color-coded. The acidic loop region of domain III is indicated by an asterisk. B, other conserved residues in the vicinity of the active site: Gln-65, Trp-72, Gly-160–161, Pro-250, and Trp-251. Figure prepared with the program MOLMOL (45).
induces a conformational change that assembles the catalytic triad of residues into its active conformation (14, 36). In m-calpain, activation involves a series of events including a relief of the conformational restraint imposed by the interaction between domain I of the large subunit and the penta-EF-hand domain VI of the regulatory subunit. Concomitantly, Ca\(^{2+}\)-induced conformational changes in domain IV are transduced via domain III to the protease domain, domain II (39, 40). These structural features include an acidic loop consisting of 10 negatively charged residues in domain III that makes direct contact with domain II (13, 40, 41). The negative electrostatic potential in the loop is somewhat counterbalanced by interdomain salt bridges between some of the negatively charged residues and the basic residues in domain II, including the Lys residues at positions 226, 230, 234, 354, 355, and 357 (Fig. 2). Furthermore, it has been hypothesized that domain III itself can bind calcium at this negative cluster, thereby further lowering the strongly negative potential. The role of calcium binding in this model is to drive the conformational change that simultaneously overcomes the considerable steric hindrance from the Pro-287–Trp-288 loop in the active site interface and permits movement of domains IIA and domains IIB toward each other to “fuse” into the catalytically active domain. In this state, where the competent catalytic triad is reassembled, the Cys S–His N pair is reduced to ~3.7 Å. This model explains why recombinant conventional animal m-calpain is inactive in the absence of calcium, as well as the underlying mechanism for the calcium-dependent activation of the enzyme.

The lack of measurable activity of DEK1 domain II presented here is in accordance with the result reported for m-calpain domain II, showing less than 1% of full-length m-calpain activity (34). The similar characteristics of domain II from m-calpain and DEK1 appear reasonable considering the high similarity in their predicted structures (Fig. 3). In contrast, recombinant DEK1 domain II&III displayed significant activity in the absence of calcium, a characteristic that differs dramatically from that of m-calpain. In our interpretation of the DEK1 calpain 3D model (Fig. 3), the lack of an absolute calcium requirement for DEK1 calpain activity may be attributed to a ground state for the enzyme in which the catalytic triad is assembled close to its optimal configuration for activity. As mentioned above, several features of DEK1 calpain contribute to our conjecture. First, it has been suggested that a cluster of 4 negatively charged residues in m-calpain, Asp-96, Glu-172, Glu-320, and Glu-321 provide a strong repulsive force that prevents domains IIA and IIB from coming together and that charge compensation through calcium binding relieves this repulsive force and facilitates the fusion of the catalytic domain (41). In DEK1 calpain, only Asp-62 (corresponding to Asp-96 in m-calpain) is conserved, suggesting that this repulsive force is not operating in DEK1 calpain, thus making charge-compensation by calcium binding less needed. Secondly, DEK1 domain III is missing 9 of the 10 negatively charged residues in the loop corresponding to the acidic loop of m-calpain. Furthermore, key Lys residues at positions 226, 230, 234, 354, 355, and 357 of m-calpain are also absent in DEK1 domain III, making it unlikely that this domain can bind calcium and thereby regulate calpain activity (Fig. 2). We suggest that the increase in DEK1 calpain activity when calcium is added is probably caused by a slight repositioning of domain IIA and IIB, leading to an optimization of the catalytic triad configuration. The mechanism, as well as the biological role of Ca\(^{2+}\)-activation in DEK1 calpain functioning remains to be determined.

In addition to domain II&III discussed above, the conventional Ca\(^{2+}\)-dependent calpain holoenzymes consist of a domain I and an EF-hand domain IV, as well as a regulatory small subunit. Animal calpains, however, are a diverse group of proteins with domain II as the main conserved feature. In contrast to the diversified structure of animal calpains, plants appear to possess only one member of the calpain super family, namely DEK1, showing a high degree of conservation among plant species (9). Similar to animal calpains, the catalytic domain II of plant DEK1 homologues is the most highly conserved domain. For example, domain II, which is 302 amino acids long, is 100% identical between maize and sugarcane. A comparison of domain II sequences between maize and loblolly pine, a gymnosperm, reveals 79% similarity (9). The high con-

![Fig. 4. Purification of DEK1 domain proteins. A, colloidal blue staining of partially purified DEK1 domain proteins separated on a 10% NuPAGE gel. DEK1 domain proteins were expressed in E. coli as GST-fusions. II&III: DEK1 domain II&III, II: DEK1 domain II. 1 μg of purified protein was loaded per lane. B, CD spectra of recombinant wild-type and mutant DEK1 domain proteins. CD spectra of DEK1 domain II&III (closed square), domain II (open circle), and domain II&III mutant protein (closed triangle).](http://www.jbc.org/content/34472/1/13.full)

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Characterization of DEK1 Calpain

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domain is unusual for animal calpains, which mostly are cytosolic enzymes that are translocated to the plasma membrane upon activation (42, 43). The only known example of an animal calpain with a predicted membrane anchor is the Drosophila calpain CG3692 that has a transmembrane domain structure similar to DEK1 calpain. The calpain domains are linked to the membrane part of the DEK1 protein by a 600 amino acid segment with few recognizable features.

The Dek1 gene plays an important role in plant development, being essential for the proper development of the aleurone layer in maize grains, embryo shoot apical meristem function as well as leaf epidermis formation (14, 36). A second gene known to function in signal transduction in the same tissues is the TNFR-like receptor like kinase CR4 (4, 5). Recently, we cloned a third gene implicated in the same developmental pathways, Superal1 (supernumerary aleurone layer 1), encoding a plant homologue of CHMP1, a member of the E-vacuolar protein sorting family (44). This finding may suggest that regulation of aleurone cell fate involves CR4 receptor internalization through endosome trafficking and targeted proteolysis in vacuoles. Interestingly, animal calpains have been implicated in vesicle trafficking, including the formation of coated vesicles.

**Fig. 6.** Dependence of DEK1 proteolytic activity on Cys-71 active site residue and domain III. A, purified mutant protein (C71S, 2 μg) was incubated with β-casein (3 μg) in the presence of 5 mM Ca2+ at 30 °C for 7 h (lane 5). The mixture was separated by 10% NuPAGE and stained by Colloidal Blue. Lane 1, molecular marker; lane 2, β-casein; lane 3, mutated domain II&III alone (mutant C71S); lane 4, wild-type domain II&III was incubated with β-casein (3 μg) in the presence of 5 mM Ca2+ at 30 °C for 7 h. B, DEK1 domain II protein (2 μg) was incubated with β-casein (3 μg) in the presence of 5 mM Ca2+ at 30 °C for 9 h (lane 6). The reaction mixture was separated by 10% NuPAGE and stained by Colloidal Blue. Lane 1, molecular marker; lane 2, β-casein; lane 3, GST; lane 4, GST was incubated with β-casein; lane 5, DEK1 domain II only. Arrowhead and arrow indicate DEK1 domain II and β-casein, respectively.

**Fig. 7.** In situ hybridization using Dek1 mRNA as a probe. A, antisense probe; B, sense (control) probe. AL, aleurone layer; P, pericarp; S.E., starchy endosperm.

**Fig. 5.** Activity of bacterially expressed DEK1 domain II&III. A, DEK1 domain II&III (2 μg) was incubated with β-casein (3 μg) in the presence of 5 mM Ca2+ (lanes 4–9) or 2 mM EDTA (lanes 10–15) at 30 °C for the time indicated in the figure. The reaction mixture was separated by 10% NuPAGE and stained by Colloidal Blue. Lane 1, molecular marker; lane 2, β-casein; lane 3, domain II&III alone. Arrowhead and arrow indicate the DEK1 domain II&III and β-casein, respectively. B, the time course of β-casein degradation (band marked by arrow) in the presence of 5 mM Ca2+ (solid line) or 2 mM EDTA (broken line). Error bars (S.D.) were calculated based on quantitation of three independent assays. C, dependence of β-casein degradation (band marked by arrow) on DEK1 domain II&III concentration. Reaction mixture was incubated at 30 °C for 7 h. Lanes 1, 2, and 3, 0.5, 1, 2 μg of DEK1 domain II&III, respectively. Arrowhead and arrow indicate the DEK1 domain II&III and β-casein, respectively. D, degradation of β-casein (band marked by arrow) as a function of increasing concentration of DEK1 domain II&III protein. Error bars (S.D.) were calculated as described above.

Preservation between all these sequences suggests an important function for the DEK1 protein in all plants. Most notably, DEK1 calpain represents the C-terminal domain of a 240-kDa protein that is predicted to be anchored in the plasma membrane by 21 transmembrane segments interrupted by a putative extra cytosolic loop domain (9). A membrane-anchoring
and vesicle fusion to endosomes (30). One possibility, therefore, is that DEK1 functions in the endosome trafficking pathway by modifying membrane proteins participating in the formation and targeted transport of membrane vesicles. The in situ hybridization results presented here suggest that Dek1 is transcribed in all cell types, despite the fact that in the endosperm, Dek1 function is essential only in the aleurone layer (9). We are currently exploring the possibility that the DEK1 calpain is activated only in epidermal cell layers mediated through interaction(s) between the predicted extracellular loop region of DEK1 and extracellular factors.

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The Calpain Domain of the Maize DEK1 Protein Contains the Conserved Catalytic Triad and Functions as a Cysteine Proteinase
Cunxi Wang, Jennifer K. Barry, Zhao Min, Gabrielle Tordsen, A. Gururaj Rao and Odd-Arne Olsen

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