

Dictyostelium discoideum protein disulfide isomerase, an endoplasmic reticulum resident enzyme lacking a KDEL-type retrieval signal

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Abstract The primary activity of protein disulfide isomerase (PDI), a multifunctional resident of the endoplasmic reticulum (ER), is the isomerization of disulfide bridges during protein folding. We isolated a cDNA encoding *Dictyostelium discoideum* PDI (Dd-PDI). Phylogenetic analyses and basic biochemical properties indicate that it belongs to a subfamily called P5, many members of which differ from the classical PDIs in many respects. They lack an intervening inactive thioredoxin module, a C-terminal acidic domain involved in Ca²⁺ binding and a KDEL-type retrieval signal. Despite the absence of this motif, the ER is the steady-state location of Dd-PDI, suggesting the existence of an alternative retention mechanism for P5-related enzymes.

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Key words: Endoplasmic reticulum retrieval and retention; Protein disulfide isomerase; *Dictyostelium discoideum*

1. Introduction

As the founding member of the protein-folding catalyst class of enzymes, protein disulfide isomerase (PDI) is a ubiquitous protein. It has been detected in every eukaryote studied so far and has counterparts in the prokaryotic world [1]. Despite the lack of precise complete structural information, a wealth of knowledge has been accumulated about PDI function. The basic activity of PDI is to shuffle (unscramble) non-native disulfide bridges in oxidation/reduction cycles catalyzed by its thioredoxin domains. In addition, it has been documented that PDI may also act as a true chaperone (see [2] for review), a function which has been recently suggested to be mediated by 'inactive' thioredoxin modules serving as peptide binding domains during folding [3,4]. PDI has been rediscovered several times during the identification of multisubunit enzymes of the ER. For example, it has been shown to represent the β -subunit of the $\alpha_2\beta_2$ heterotetrameric prolyl-4-hydroxylase [5] and a component of the triacylglycerol transfer protein [6,7]. Accompanying its identification, characterization and cloning in a growing number of species, it soon appeared that PDI and related proteins form a complex superfamily including at least four subfamilies: PDI, Erp60, Erp72

and P5 [2]. In most cases studied, PDI and related proteins behave as classical ER-resident enzymes. This steady-state distribution of PDI is the result of a combination of direct retention and retrieval of ER escapees [8–10]. Even though most of the PDI proteins present in the database bear at their C-termini a classical ER retrieval signal in the form of a KDEL or related motif [11], three exceptions to that rule have been reported: *Acanthamoeba castellanii* [12], alfalfa [13] and *Onchocerca volvulus* [14]. It is of great importance to establish whether such exotic members of the PDI superfamily also are ER-resident enzymes and, if so, to understand in molecular terms how such abundant proteins can be kept from exiting the ER compartment. Interactions of PDI with other proteins, in the form of either a reticular-like matrix with procollagen, as in human fibroblasts [15], or specific, stoichiometric complexes with calreticulin, as in pancreatic cells [16], have been reported. As these PDI bear a KDEL retrieval signal, the formation of multiprotein complexes may only represent an additional partial contribution to their efficient retention in the ER.

Here we report on the identification, molecular cloning and intracellular localization of a new member of the PDI superfamily from *Dictyostelium discoideum*. Interestingly, this protein, Dd-PDI, possesses many structural hallmarks of a true PDI, but lacks any identifiable KDEL-type motif. Phylogenetic analysis of 41 PDIs and related sequences suggests that Dd-PDI belongs to the P5 subfamily. This subfamily regroups the two other P5-related proteins lacking a classical ER retrieval signal. *D. discoideum* is a genetically tractable organism, which will offer the unique opportunity to investigate non-classical mechanisms of retrieval/retention of an abundant ER enzyme at the molecular level.

2. Materials and methods

2.1. Production of anti-Dd-PDI monoclonal antibodies

A crude phagosome fraction was prepared from wild type AX2 *D. discoideum* cells as described elsewhere [17] and used as an antigen to immunize mice. Five monoclonal antibodies (mAbs) were selected by Western blotting and immunofluorescence on *D. discoideum* cells and shown to recognize a polypeptide of about 40 kDa, and to stain a membranous structure that was reminiscent of ER. mAb 221-135-1 was further used to screen a cDNA library.

2.2. Molecular cloning of Dd-PDI

First, a lambda gt11 cDNA expression library of developed *D. discoideum* harvested 4 h after starvation was screened with mAb 221-135-1. From the 18 clones originally picked, 10 were subjected to PCR analysis and the four longest subsequently cloned and sequenced. To complete the cloning of this PDI cDNA towards the 5' end, a lambda ZAP-II library (generous gift of Dr. William Loomis, UCSD, USA) was screened using a DIG-labeled probe derived from

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Abbreviations: ER, endoplasmic reticulum; PDI, protein disulfide isomerase

the 5'-most 880 bp available. Among eight clones analyzed, we found one complete cDNA sequence.

2.3. Southern, Northern and Western blotting

D. discoideum genomic DNA was purified according to a standard procedure [18] and digested with the indicated restriction enzymes. For Northern blotting analysis, total RNAs were extracted by the method of Chomczynski [19], slightly modified for *D. discoideum*, from staged AX2 cells developed on starvation plates for the indicated times (Fig. 4). RNA gels were run and transferred to Hybond-N⁺ nylon membrane (Amersham Life Science) according to standard protocols [20]. The Boehringer DIG-labeling system was used to produce probes from the 5' and 3' halves of the PDI cDNA. Hybridizations and detection were carried out according to the manufacturer's instructions (Boehringer Mannheim). Western blotting was carried out using standard protocols (Amersham Life Science) on extracts from staged *D. discoideum* cells developed on starvation plates for the indicated times. Five different mAbs were used and revealed the same 40 kDa protein.

2.4. Construction and expression of a GFP-HDEL chimera

An improved version of GFP, GFPmut2 [21], was introduced in a *D. discoideum* expression vector [22] provided by Dr. Christophe Raymond (University of Lausanne, Switzerland) through a Material Transfer Agreement. The GFP sequence was fused in frame with the signal sequence of csA [22,23], and followed by a small polylinker and the sequence coding for the HDEL retrieval signal (Met-csA SP-Pro-Trp-Val-Pro-Cys-GFPmut2-Arg-Ser-Lys-Ala-Tyr-Ala-Ser-His-A-sp-Glu-Leu). AX2 cells were transfected by electroporation [24] and stable transformants selected by resistance to G418.

2.5. Immunofluorescence and microscopy

Cells expressing GFP-HDEL were allowed to adhere for 30 min to glass coverslips and fixed either by a standard picric acid/paraformaldehyde fixative [25] or by a rapid ethane freezing/methanol fixation procedure that will be described elsewhere (Neuhaus, E.-M., Horstmann, H., Almers, W., Maniak, M. and Soldati, T., manuscript submitted). Secondary antibodies conjugated to Cy3 (BioTrend) were used to detect binding of the primary monoclonal antibodies, anti-PDI and anti-calreticulin (generous gifts of Drs. B. Knoblach and R. Mutzel, Konstanz University, Germany). Confocal laser scanning microscopy was carried out on a Leica TCS.

3. Results

3.1. Molecular cloning of Dd-PDI

A full length cDNA encoding a *D. discoideum* PDI was cloned as described in Section 2. Briefly, the sequence as sub-

mitted to GenBank (accession number AF019112) has a small untranslated 5' end, the context around the AUG start codon matches the consensus found in protozoa [26] and the 3' untranslated region contains multiple poly(A) addition signals (not shown). The deduced protein sequence (Fig. 1) begins with a signal peptide flanked by an N-terminal positive charge and a C-terminal negative charge which directly follows the predicted cleavage site (Husar software package, DKFZ, Heidelberg, Germany). The mature polypeptide has a calculated mass of 37.80 kDa and bears strong homologies to members of the PDI superfamily particularly in two clearly identifiable thioredoxin domains. An alignment with the three other most related proteins, all belonging to the P5 subfamily, is presented in Fig. 1. The closest relative of *D. discoideum* PDI is the alfalfa protein, which also lacks the KDEL-type motif [11]. The conservation is highest in and around the thioredoxin boxes, but is also highly relevant outside these regions. A pairwise comparison of these four proteins indicates that they are about 40–45% identical and 60–65% similar. The degrees of identity and similarity outside this subfamily decreased to around 30% and 50% respectively with the other members of the P5 class, down to around 25% and 50% when compared to classical PDIs.

3.2. Phylogenetic analysis

In order to compare this PDI to the molecules isolated from other species, phylogenetic analysis was performed using the Husar software package (DKFZ, Heidelberg, Germany). Alignments and computation of the tree were carried out with ClustalW (Fig. 2). The analysis of 41 PDIs and related sequences resulted in a classification similar to the one presented by Freedman [2] and suggested that the P5 subfamily contains two subgroups, one comprising three out of the four eukaryotic PDIs lacking a KDEL-type retention signal. A compilation of some basic biochemical characteristics (size, pI and charge) of the members of the PDI superfamily strongly supports this classification. Perhaps most indicative is the charge, which broadly varies between +20 and −57, and is around −30 for canonical PDIs but is close to neutral for members of the mentioned P5 subgroup (Fig. 2).

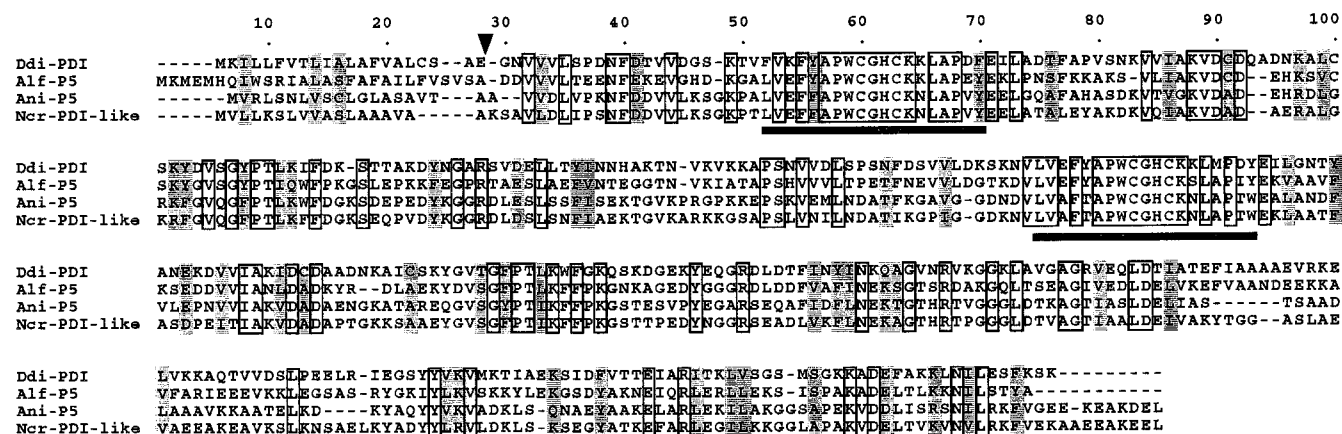


Fig. 1. Sequence alignment of Dd-PDI with the most closely related members of the P5 subfamily. The sequence alignment was computed with the ClustalW software. Identical residues are boxed and conservative changes shadowed. The two 19 residue thioredoxin motifs are underlined. All four proteins have a similarly sized signal peptide (predicted cleavage site indicated by an arrowhead), lack the intervening b or b' inactive thioredoxin module [4], and have no C-terminal acidic domain. In addition, despite the lack of -DEL-type retrieval motifs in the top two sequences all four bear strong homologies in the last 60 C-terminal residues.

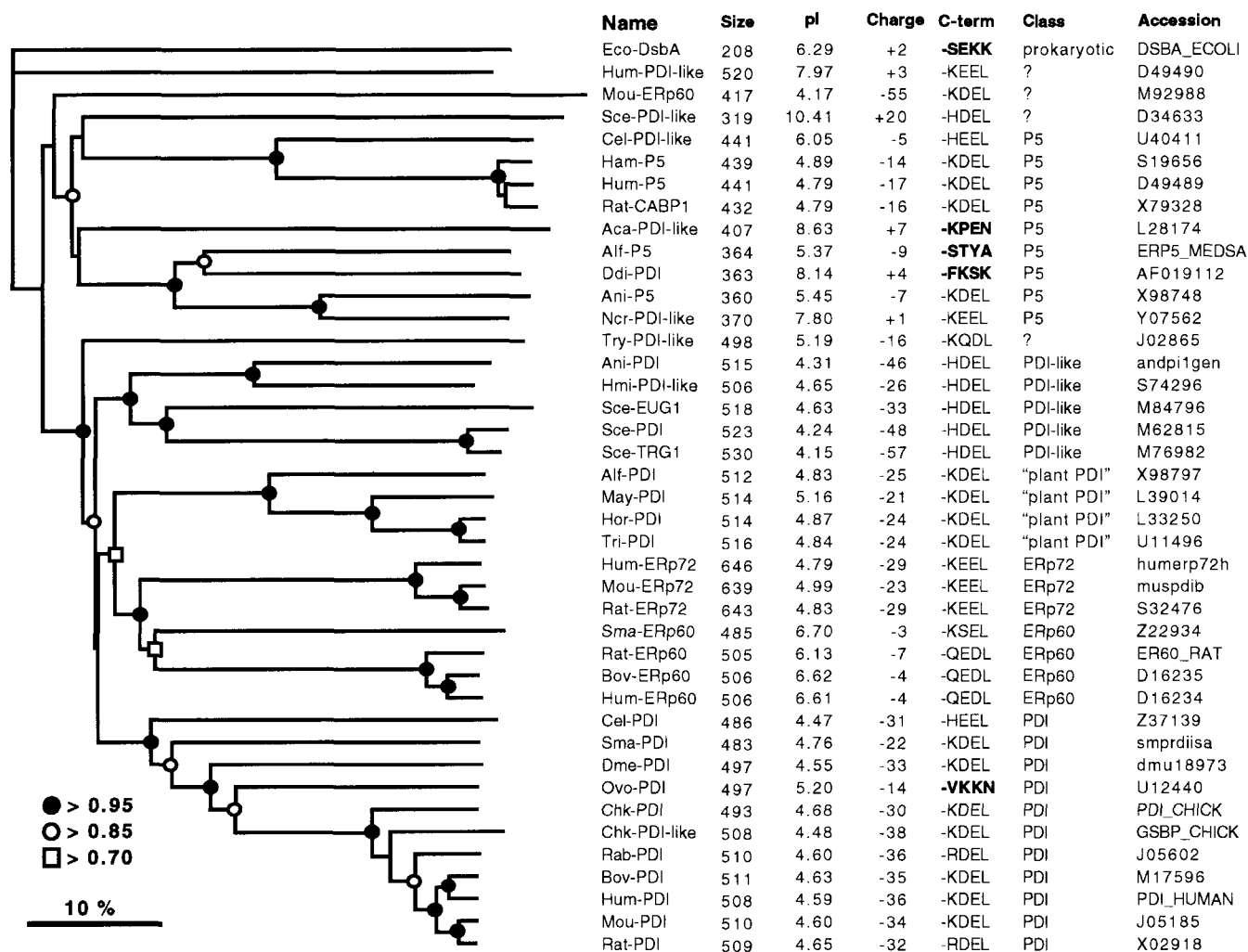


Fig. 2. Classification of the members of the PDI superfamily. Phylogenetic classification and some biochemical characteristics of the members of the PDI superfamily. The size indicated is in amino acids and includes the signal peptide; pI is the isoelectric point; the net charge is calculated for pH 7.0. The C-terminal four residues are given, and the four sequences that differ from a KDEL-like motif are outlined in bold. The class assignment is mainly derived from the literature [2]. The accession numbers for the different databases are indicated. The tree was generated using the ClustalW program (and visualized with TreeView) with 1000 bootstrapping iterations, and the symbols on some forks indicate the probability of a given branching. The *E. coli* DsbA was used as outgroup to root the tree. The scale bar indicates a 10% divergence. The PDI molecules from different species are abbreviated as follows: Eco: *Escherichia coli*; Hum: human; Mou: mouse; Sce: *Saccharomyces cerevisiae*; Cel: *Caenorhabditis elegans*; Ham: hamster; Rat: rat; Aca: *Acanthamoeba castellanii*; Alf: alfalfa; Ddi: *Dictyostelium discoideum*; Ani: *Aspergillus niger*; Ncr: *Neurospora crassa*; Try: *Trypanosoma brucei*; Hmi: *Humicola*; May: maize; Hor: *Hordeum*; Tri: *Triticum*; Sma: *Schistosoma mansoni*; Bov: bovine; Dme: *Drosophila melanogaster*; Ovo: *Onchocerca volvulus*; Chk: chicken; Rab: rabbit.

3.3. Dd-PDI gene and expression

In order to determine the number of copies of PDI genes in *D. discoideum*, Southern blotting analysis was used. Genomic DNA was digested with the indicated enzymes and, after electrophoresis and transfer to nylon membrane, hybridized with DNA probes derived from our PDI sequence (Fig. 3). The signals detected correlate with the presence of a single sequence. Nevertheless, hybridization experiments performed with the 5' half of PDI cDNA at low stringency suggested the presence of another faint band (data not shown). This signal could correspond to the recent data obtained from the EST sequencing project of the *Dictyostelium* genome, which revealed the existence of another PDI-related sequence sharing only weak homology with our Dd-PDI.

PDI expression at the mRNA and protein levels was studied at different stages of the developmental cycle of *D. discoideum* (Fig. 4). The mRNA level appears to be slightly

increased early in development (starvation and aggregation), then decreases somewhat (slug formation) and finally increases again towards the end of the cycle (culmination, spore differentiation) (Fig. 4A). The steady-state level of the protein is nearly constant throughout the developmental cycle. This is in agreement with a 'housekeeping' function of PDI (Fig. 4B).

3.4. Intracellular localization

To identify unambiguously the tubular-vesicular structure labeled by the anti-Dd-PDI antibodies, we decided to carry out the localization in two steps. First, we constructed a chimera of GFPmut2 bearing a signal peptide and a HDEL C-terminal motif to label the ER. The fluorescent signal of the fusion protein coincided with the staining of monoclonal antibodies against a recently identified calreticulin (Knoblach, B. and Mutzel, R., manuscript in preparation), which bears a C-terminal HDEL motif analogous to the *Saccharomyces ce-*

revisiae ER retrieval signal [27] (Fig. 5A). In a second step, we investigated the distribution of Dd-PDI in cells with GFP-labeled ER (Fig. 5B). GFP fluorescence colocalized with both proteins, as is most evident in the perinuclear staining of the nuclear envelope, but also in extensions of the fine reticulum towards the cell periphery.

4. Discussion

The *D. discoideum* PDI-like molecule that we describe here as the first member of the PDI superfamily in this organism has the strongest homology to a PDI-related protein from alfalfa. These two proteins share many structural features and are members of the P5 subclass of the PDI superfamily. They both have two thioredoxin boxes, but appear to lack one of the intervening domains sometimes referred to as the b and b' domains [2] and recently suggested to adopt an inactive thioredoxin fold [4]. Three of the P5 proteins lack a KDEL-type retrieval motif. They also lack a C-terminal acidic domain which has been implicated in the Ca^{2+} binding capacity of PDI and related proteins; its absence from these P5 (and some other subclasses) suggests that they are not involved in calcium homeostasis, an important additional function ascribed to many ER-resident proteins, including calreticulin.

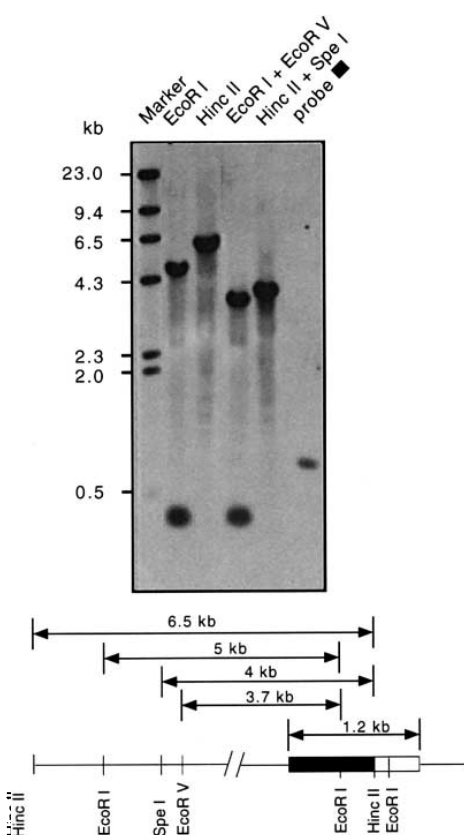


Fig. 3. Southern blotting analysis of the Dd-PDI gene. Genomic DNA from *D. discoideum* was restricted with the indicated enzymes. Hybridization was performed with a DIG-labeled probe derived from the 5' terminal moiety of the Dd-PDI cDNA as indicated on the small restriction map. The pattern of signals obtained is consistent with the information derived from the cloned cDNA. The bar represents the cDNA, the probe being highlighted in black, and the line represents flanking genomic sequences.

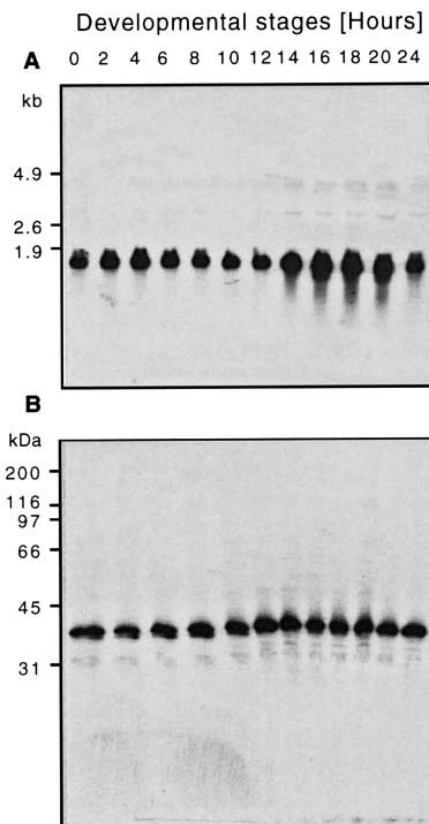


Fig. 4. Expression of Dd-PDI during the developmental cycle. Cells were allowed to develop on starvation plates, staged by visual inspection, collected at the indicated times and processed for extraction of total RNA or production of a total protein extract. The following stages into the developmental cycle (hours) correspond to: 0, vegetative cells; 2/4, loose, starving cells; 6, formation of 'domains'; 8, streaming; 10, loose aggregate; 12, tight aggregate, start of tip formation; 14, tipped aggregate; 16, fingers, falling slugs; 18, migrating slugs; 20/22, 'Mexican hats', culminants; 24, fruiting bodies. A: Northern blotting analysis of the expression of Dd-PDI mRNA. 5 µg of total RNA was loaded in each lane. Quality of RNAs and equal loading were checked by ethidium bromide staining of the gel. The probe used for hybridization is described in Fig. 3. The signal corresponds to the size of a 1.2 kb mRNA. B: Western blotting analysis of the expression of Dd-PDI. 20 µg of total cell protein was loaded in each lane. Detection was carried out with the monoclonal antibody 221-135-1 directed against the 40 kDa Dd-PDI.

In addition, from a phylogenetic point of view, it is interesting that the closest homologue is found in a plant, as the social amoeba *D. discoideum* very likely diverged right before the separation of plants and animals [28]. Finally, computer analysis of 41 PDI sequences including the *D. discoideum* P5 protein emphasizes the almost complete absence of predicted *N*-glycosylation sites (with the exception of the PDI and related proteins from *S. cerevisiae* and *Trypanosoma brucei*) and the presence of a high number of potential phosphorylation sites which may correlate with the identification of PDI as a major phospho-protein of the ER [29].

In most if not all organisms studied so far, there is more than one gene for PDI or PDI-related proteins. Surprisingly, we did not find strong evidence for a second *D. discoideum* PDI; Western, Northern and Southern blotting analyses failed to consistently reveal the presence of a second isoform. Nevertheless, a PDI-related sequence was recently identified in

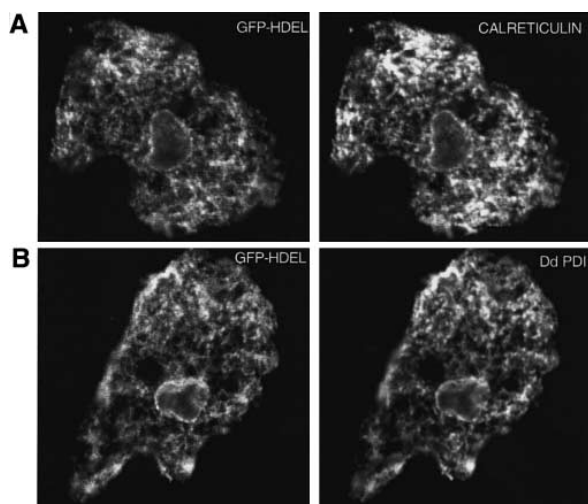


Fig. 5. Intracellular localization of Dd-PDI. Immunofluorescence of *D. discoideum* cells expressing a GFP-HDEL construct. A: The localization of the ER-resident protein calreticulin together with the intrinsic fluorescence of GFP-HDEL. B: The localization of the GFP-HDEL chimera together with Dd-PDI. In each pair of pictures note the colocalization of the GFP and either calreticulin or Dd-PDI, both in the nuclear envelope and in the peripheral tubulo-reticular system.

the course of the *D. discoideum* EST sequencing project (Tsukuba cDNA Project, Japan). Gene disruption experiments in progress may both bring a confirmation of the existence of a second PDI in *D. discoideum*, as well as information on their multiple proposed and essential functions. In yeast one classical PDI and two PDI-related sequences have been identified. The inviability caused by *PDI1* depletion [30–32] can be suppressed by overexpression of *EUG1* or *MPDI* genes [33,34].

It has been reported that in tissues that are highly secretory, such as the pancreas, PDI can escape the KDEL retrieval machinery and be found in secretory vesicles [35]. In addition, PDI and PDI activity have been detected at the plasma membrane of exocrine pancreatic cells [36], platelets [37] and hepatocytes [38]. Because of the absence of a classical retrieval signal on Dd-PDI, it appeared important to unambiguously ascertain the location of the protein to the endoplasmic reticulum.

The main aim of future investigations will be the dissection of ER retrieval/retention mechanisms in *D. discoideum*. This cellular amoeba probably possesses a HDEL/HDEL receptor system, as indicated by the HDEL motif of calreticulin and by the ER localization of the GFP-HDEL chimera. One can speculate that Dd-PDI may be retained in the ER by interacting with other, HDEL bearing proteins, analogous to interaction of PDI with calreticulin in pancreatic microsomes [16]. Even though not unique, the existence of a PDI lacking a KDEL-type motif is the first such case reported in a genetically tractable organism, and this should allow us to shed light on some aspects of the interplay and balance between retrieval and retention mechanisms in the physiology of the ER.

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