The Isolation and Characterization of a cDNA Encoding
Phospholipid-specific Inositol Polyphosphate 5-Phosphatase*

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We report the cDNA cloning and characterization of a novel human inositol polyphosphate 5-phosphatase (5-phosphatase) that has substrate specificity unlike previously described members of this large gene family. All previously described members hydrolyze water soluble inositol phosphates. This enzyme hydrolyzes only lipid substrates, phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 4,5-bisphosphate. The cDNA isolated comprises 3110 base pairs and predicts a protein product of 644 amino acids and \( M_r = 70,023 \). We designate this 5-phosphatase as type IV. It is a highly basic protein (\( pI = 8.8 \)) and has the greatest affinity toward phosphatidylinositol 3,4,5-trisphosphate of known 5-phosphatases. The \( K_m \) is 0.65 \( \mu M \), 1/10 that of SHIP (5.95 \( \mu M \)), another 5-phosphatase that hydrolyzes phosphatidylinositol 3,4,5-trisphosphate. The activity of 5-phosphatase type IV is sensitive to the presence of detergents in the in vitro assay. Thus the enzyme hydrolyzes lipid substrates in the absence of detergents or in the presence of \( n \)-octyl \( \beta \)-glucopyranoside or Triton X-100, but not in the presence of cetyltrimethylammonium bromide, the detergent that has been used in other studies of the hydrolysis of phosphatidylinositol 4,5-bisphosphate. Remarkably SHIP, a 5-phosphatase previously characterized as hydrolyzing only substrates with \( \beta-3 \) phosphates, also readily hydrolyzed phosphatidylinositol 4,5-bisphosphate in the presence of \( n \)-octyl \( \beta \)-glucopyranoside but not cetyltrimethylammonium bromide. We used antibodies prepared against a peptide predicted by the cDNA to identify the 5-phosphatase type IV enzyme in human tissues and find that it is highly expressed in the brain as determined by Western blotting. We also performed Western blotting of mouse tissues and found high levels of expression in the brain, testes, and heart with lower levels of expression in other tissues. mRNA was detected in many tissues and cell lines as determined by Northern blotting.

Inositol polyphosphate 5-phosphatases (5-phosphatases)\(^ 1 \)

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\(^*\) The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\(^ \dagger \) EBI Data Bank with accession number(s) AF187891.

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\(^\S\) The abbreviations used are: 5-phosphatase, inositol polyphosphate 5-phosphatase; PtdIns 3,4,5-P\(_3\), phosphatidylinositol 3,4,5-trisphosphate; PtdIns 4,5-P\(_2\), phosphatidylinositol 4,5-bisphosphate; EST, expressed sequence tag; kb, kilobase(s); RACE, rapid amplification of cDNA ends; HPLC, high pressure liquid chromatography; ITAM, immunoreceptor tyrosine-based activation motif; PK, pleckstrin homology; Btk, Bruton’s tyrosine kinase; PtdIns, phosphatidylinositol; GPI (or GroPIns), glycerophosphorylinositols; CTAB, cetyltrimethylammonium bromide.

EXPERIMENTAL PROCEDURES

cDNA Cloning—The consensus sequences that define 5-phosphatases were used to search the sequence data bases using the BLAST algorithm. An EST from human infant brain, accession number H10559, was identified as a putative 5-phosphatase. The EST was obtained from ATCC and sequenced. The 1.8-kb EST insert was used to screen a human fetal brain \( \lambda \) ZapII cDNA library (Stratagene) to obtain an additional sequence. 5’-RACE of human fetal brain Marathon-Ready cDNA (CLONTECH) was performed according to the manufacturer’s instructions to obtain the 5’-sequence. However, the 5’-region of 5-phosphatase type IV contains a very high percentage of GC residues, including long stretches of multiple GC repeats, leading to secondary structure.

Phosphate; PtdIns 4,5-P\(_3\), phosphatidylinositol 4,5-bisphosphate; EST, expressed sequence tag; kb, kilobase(s); RACE, rapid amplification of cDNA ends; HPLC, high pressure liquid chromatography; TTAM, immunoreceptor tyrosine-based activation motif; PK, pleckstrin homology; Btk, Bruton’s tyrosine kinase; PtdIns, phosphatidylinositol; GPI (or GroPIns), glycerophosphorylinositols; CTAB, cetyltrimethylammonium bromide.

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tured liquid scintillation counter. Scrapings of radiolabeled phospholipids were counted in a Beckman (v/v)). Phospholipid standards were stained with iodine. Radiolabeled overnight. TLC plates were developed using a solvent mixture of chloroform/octanol buffers.

Expression and Purification—The Bac-to-Bac expression system (Life Technologies, Inc.) was used for high level expression of 5-phosphatase type IV. In cultured insect cells, cDNA (nucleotides 1-2135) was subcloned into the pFastBac1 vector to express full-length 5-phosphatase type IV protein. For purification, 5-phosphatase type IV was subcloned into the pFastBac HT vector. Cell extracts were harvested 48 h postinfection, and the protein was purified on Ni2+-NTA resin according to the manufacturer’s instructions, with the addition of 5 mM MgCl2 in all buffers.

TLC—Silica Gel 60 TLC plates were treated with a solution of 1% potassium oxalate in 50% ethanol. The plates were baked at 90 °C overnight. TLC plates were developed using a solvent mixture of chloroform/acetone/methanol/glacial acetic acid/water (80:30:26:24:14 (v/v)). Phospholipid standards were stained with iodine. Radiolabeled phospholipids were detected by a Phosphomager or autoradiography. Scracings of radiolabeled phospholipids were counted in a Beckman liquid scintillation counter.

PtdIns 3,4,5-P3 Hydrolysis—PtdIns 3[32P]4,5-P2 was synthesized essentially as described previously (10). 0.5 mg of PtdIns 4,5-P2 or 0.5 mg of phosphatidylinosine were dried under nitrogen and resuspended in 1 ml containing 20 mM HEPES, pH 7.6, 2 mM EDTA, and 5 mM MgCl2. After brief sonication, the phosphoinositide 3-kinase immunoprecipitate was added followed by [γ-32P]ATP (2 μCi, 6000 Ci/mmol). After a 1-h incubation, the lipids were extracted twice with chloroform/methanol and 1% NaCl. The lipids were separated on TLC, and radiolaabeled PtdIns 3,4,5-P2 was scraped from the plate and extracted from silica gel with chloroform/methanol.

For enzyme assay, radiolabeled lipids were dried under nitrogen and resuspended in buffer containing 50 mM Tris-HCl, pH 7.5, 3 mM MgCl2, and 0.3% n-octyl β-D-glucopyranoside and briefly sonicated on ice. The assay of activity was performed as described previously (10, 11).

PtdIns 4,5-P2 Hydrolysis—[3H]PtdIns 4,5-P2 (3000 cpm/assay) was dried under nitrogen and resuspended in buffer containing 50 mM Tris-HCl, pH 7.5, 3 mM MgCl2, 150 mM NaCl, and 2 mM CaCl2 followed by brief sonication. Alternatively, the dried radiolabeled [3H]PtdIns 4,5-P2 was resuspended in buffer containing 50 mM Tris-HCl, pH 7.5, 3 mM MgCl2, 50 mM NaCl, and 0.03% n-octyl β-D-glucopyranoside. Reactions were started by the addition of the enzyme and carried out at 37 °C. Extraction of the lipids and analysis of the reaction products were as above.

Proof of Product—Deacylation of inositol lipids was as described previously (12). GroPls derivatives were separated by HPLC on a Partisphere SAX column (Whatman) with the gradient of 0-1.25 mM NaH2PO4, pH 4.5. The gradient consisted of a 0-5 min linear rise to 29% for pump B, a 5-35 min linear rise to 60% B, and a 35-45 min linear rise to 100% B followed by a 5-min wash with 100% B. Radiolaabeled deacylated phosphorylcholine lipids were detected by β-RAM Flow-Through System (In/US Systems, Inc., Tampa, Florida).

RESULTS AND DISCUSSION

cDNA Cloning of Type IV Phosphatidylinositol Polyphosphate 5-Phosphatase—Inositol polyphosphate 5-phosphatases are defined by two essential domains, with the consensus sequences, FWGDFNS/F(Y)R and R/NPSA/W/C/T/DR/F/V/L. Our method for screening for additional members of the family was a search of the human EST data base with the consensus sequences. One EST clone identified in the GenBank™ data base with the BLAST search was clone H10559, with a reported sequence, FWGDFNFR that corresponded to the consensus sequence of the 5-phosphatase domain I. The clone was sequenced and shown to encode a partial open reading frame that contained both 5-phosphatase domains.

The 1.8-kb EST insert was used to screen a human fetal brain azapII cDNA library (Stratagene). However, all clones isolated contained only partial coding sequence. To obtain additional N-terminal sequence, we used a 5‘-RACE protocol and human fetal brain Marathon-ready cDNA (CLONTECH). However, because of a very high GC content of the cDNA, the protocol was modified as described under “Experimental Procedures” (8, 9). An additional 300 base pairs were isolated containing a methionine codon. Although the full-length mRNA is 3.4 kb according to the Northern blot (see below), we believe that we have identified the starting methionine, because the putative initiation site is well in a perfect Kozak consensus sequence (13) and there is an in-frame stop codon 5‘ of it.

The composite cDNA of 3110 base pairs encodes an open reading frame of 644 amino acids (Fig. 1A). It contains two 5-phosphatase domains, FWGDFNFR and QKRTPSY-DRVLY (invariant consensus residues are in bold). The sequence of domain II is unusual; it encodes tyrosine instead of an invariable tryptophan residue in the consensus motif. Only one other putative 5-phosphatase gene from C. elegans has the same tryptophan to tyrosine substitution. This gene has not been characterized at the protein level. The four C-terminal residues CSVS represent an S-farnesylation signal. Of other mammalian 5-phosphatases, types I and II also have post-translation lipid modifications at their C termini. 5-Phosphatase type I has a CVQV C-terminal isoprenylation site and is a substrate for purified farnesyltransferase (14). 5ptase type II has a CNPL prenylation motif and is likely to be geranyl-geranylated (15, 16).

5-Phosphatase type IV has relatively low similarity to other known mammalian 5-phosphatases (Fig. 1B). There is only 10–20% amino acid identity with the closest identity of 19% to SHIP. There are two mouse EST clones found in the sequence data base that encode the partial sequence of the 5-phosphatase type IV homologue. We sequenced both of them, and the deduced amino acid sequences are 87% identical to the human protein in the coding portion of the clones. The 3‘-untranslated regions have no similarity.

5-Phosphatase type IV has a very high content of proline residues (9.5%), particularly in the N-terminal portion (19%). The N-terminal region of the enzyme contains a Pro-rich domain class I (17) and thirteen P sequences, a potential SH3 binding core. Surprisingly, 5-phosphatase type IV also contains a sequence VYLLSSAAGVLYML that corresponds to YXXL (6-8) YXXL consensus, which is the immunoreceptor tyrosine-based activation motif (ITAM). ITAM modules function to link cell surface receptors to signaling effector molecules and are found in the cytoplasmic tails of T- and B-cell receptors, CD3, high affinity Fc receptors for IgE and IgA, and FcγRIIA. This is the first case where an ITAM sequence has been reported in a nonreceptor molecule. One of the clones isolated in the screen of the human brain library had a 102-nucleotide deletion spanning this region. It is likely to represent an alternatively spliced transcript, resulting in deletion of 34 amino acids including the putative ITAM domain. It will be of interest to study the functional role of this protein motif in 5-phosphatase type IV. Another member of the 5-phosphatase family, SHIP, is known to interact with the immunoreceptor tyrosine-based inhibition motif of FcγRIIB in B cells. SHIP 1 is recruited to the phosphorylated immunoreceptor tyrosine-based inhibition motif via its SH2 domain, providing the inactivation of the signal presumably by hydrolysis of PtdIns 3,4,5-P3 and inositol 1,3,4,5-tetrakisphosphate-signalng molecules (18). Because 5-phosphatase type IV is also a PtdIns 3,4,5-P3 phosphatase, it may serve as a “double inhibitor,” both hydrolyzing phosphatidylinositol signaling molecules and competing with immunoreceptors as a scaffolding base.

Tissue Distribution of Phosphatidylinositol 5-Phosphatase Type IV—Expression of 5-phosphatase type IV was analyzed by
Northern blot analysis. In human tissues, 5-phosphatase type IV mRNA was present at highest levels in brain, heart, spleen, pancreas, and testis (Fig. 2, A and B). A major band of 3.6 kb was detected in all tissues; however, additional species of 4.9 and 9.5 kb are present as well and are most pronounced in testis. Other members of the 5-phosphatase family are known to exist as multiple splice variants. Multiple splice forms of SHIP, SHIP2, synaptojanin, and 5-phosphatase type II have been reported (16, 19). Different splice variants may have different tissue and/or intracellular distribution as in the case of 5-phosphatase type II (16) and synaptojanin 1 and synaptojanin 2 (20, 21). They may also lack a protein module, SH2 domain in the case of SIP110, a splice variant of SHIP (19). A splice variant of synaptojanin 1 without an N-terminal SAC domain has been reported also (22). Both synaptojanin 1 and synaptojanin 2 exist as multiple splice forms. The majority of synaptojanin 1 in brain is a 145-kDa isoform, whereas a 175-kDa isoform is ubiquitously expressed. The 175-kDa isoform is tightly bound to membranes compared with the 145-kDa isoform. Interestingly, these two isoforms arise from a small (27 nucleotide) deletion that deletes the stop codon and results in splicing of a long open reading frame found in the 3'-untrans-
5-phosphatase type IV mRNA expression in human tissues (A and B), cancer cell lines (C), and mouse tissues (D). Multitissue blots (CLONTECH) were probed with the 3′-nontranslated region of human (A, B, and C) and mouse (D) 5-phosphatase type IV cDNA. The staining was specific based on the cDNA sequence. The staining was specific molecular mass correlates well with the predicted mass of 70 kDa based on the cDNA sequence. The staining was specific.

We expressed the protein in Sf9 cells to study the catalytic activity of the enzyme. First, the full-length construct was expressed to confirm that the starting methionine supports initiation of protein synthesis. In accordance with the predicted molecular mass, a band of 70 kDa was detected in insect cells 48 h following infection with 5-phosphatase type IV baculovirus (Fig. 3C, lane 3). To obtain a highly purified enzyme, we expressed His-tagged 5-phosphatase type IV followed by affinity purification on a Ni2+–NTA resin, silver staining: Lane 2, immunoblotting of the same sample with 5-phosphatase-specific polyclonal antibodies. Lane 3, immunoblotting of Sf9 cell soluble extract 48 h postinfection with the virus encoding full-length human 5-phosphatase type IV (not His-tagged).

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PtdIns 3,4,5-P3 Hydrolysis and the Effect of Detergents—Known inositol polyphosphate 5-phosphatases can potentially utilize four different substrates, soluble inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrasphosphate, as well as lipids, PtdIns 4,5-P2, and PtdIns 3,4,5-P3. Substrate preference is the basis of classification of known 5-phosphatases into four groups as described above. We examined the ability of the type IV enzyme to hydrolyze all four potential substrates. It was unable to hydrolyze either of the soluble substrates, making it the first known 5-phosphatase that is not capable of metabolizing soluble substrates. In these experiments we used 10–50 times...
as much recombinant enzyme as in the experiments using lipid substrates, incubated for up to 40 min, and found no hydrolysis. Thus if the enzyme were able to hydrolyze at even 1% of the rates seen with lipids, we would have detected it. When lipid substrates were used, type IV enzyme readily utilized PtdIns 3,4,5-P$_3$ converting it to PtdIns-P$_2$ in a time- and concentration-dependent manner as shown below. To identify the product of the reaction, the substrate and the product of the reaction were separated by HPLC (Fig. 4). The GroPIns-P$_2$ product migrated in the position corresponding to PtdIns 3,4-P$_2$, confirming that the novel enzyme is an inositol 5-phosphatase.

Of the known 5-phosphatases, SHIP is the most efficient enzyme that hydrolyzes PtdIns 3,4,5-P$_3$. It is thought to be the primary enzyme that controls the level of PtdIns 3,4,5-P$_3$ in cells during activation of tyrosine kinase receptors. We compared the enzymatic characteristics of 5-phosphatase type IV and SHIP.

**Fig. 4. Identification of the product of PtdIns 3,4,5-P$_3$ hydrolysis by phosphatidylinositol 5-phosphatase type IV.** PtdIns 3,4,5-P$_3$ was prepared in mixed micelles and incubated in 50 mM Tris-HCl, pH 7.5, 3 mM MgCl$_2$, and 0.03% n-octyl β-glucopyranoside for 30 min at 37 °C in the absence (A) or presence (B) of 5 ng of phosphatidylinositol 5-phosphatase type IV. Both reactions were deacylated and separated by HPLC on a PartiSphere SAX column as described under “Experimental Procedures.” [3H]yeast-derived GroPIns internal standards were added to each sample prior to injection. 32P-labeled deacylated products of phosphatase reactions are shown by black circles; [3H]yeast-derived GroPIns standards are shown by open circles. The position of glycerophosphate phospholipid derivatives (GPIP$_n$) is marked with arrows.

**Fig. 5. Enzymatic properties of phosphatidylinositol 5-phosphatase type IV.** A, Lineweaver-Burk plot of purified recombinant His-tagged phosphatidylinositol 5-phosphatase type IV (1.65 ng) hydrolyzing PtdIns 3,4,5-P$_3$. Results shown are representative of two experiments. B, enzymatic characteristics of 5-phosphatase type IV and SHIP.

The ability of type IV enzyme to hydrolyze PtdIns 3,4,5-P$_3$ is very sensitive to the presence of detergents in the assay. Whereas the enzyme is capable of hydrolyzing PtdIns 3,4,5-P$_3$ presented in mixed vesicles without added detergent and in the presence of 0.03% n-octyl β-glucopyranoside or Triton X-100, its activity was completely inhibited in the presence of 2 mM CTAB (Fig. 7A). The later detergent is present in standard assays for hydrolysis of PtdIns 4,5-P$_2$. Thus, we re-evaluated the ability of type IV enzyme to hydrolyze PtdIns 4,5-P$_2$ under different assay conditions.

**PtdIns 4,5-P$_2$ Hydrolysis—** We compared the ability of three enzymes, OCRL, SHIP, and 5-phosphatase type IV to hydrolyze PtdIns 4,5-P$_2$ with either n-octyl β-glucopyranoside or CTAB in the assay. Under standard assay conditions, with 2 mM CTAB, only OCRL effectively converted PtdIns 4,5-P$_2$ to PtdIns 4-P, with no activity observed using either SHIP or type IV enzyme (Fig. 7C). However, both type IV enzyme and, surprisingly, SHIP were capable of PtdIns 4,5-P$_2$ hydrolysis when n-octyl β-glucopyranoside but not CTAB was added to the assay (Fig. 7B). The ability of SHIP to utilize PtdIns 4,5-P$_2$ was unexpected, because in all previously reported studies SHIP was able to utilize only the D-3 phosphate-containing substrates. We compared the three 5-phosphatases for their ability to

**Experimental Procedures.**
hydrolyze PtdIns 4,5-P₂. The first order rate constants of OCRL, 5-phosphatase type IV, and SHIP were 63.05 ± 10.54 min⁻¹ μg⁻¹, 41.99 ± 25.07 min⁻¹ μg⁻¹, and 29.9 ± 18.75 min⁻¹ μg⁻¹. Thus the classification of SHIP as an enzyme specific for γ-3 phosphate containing substrates is incorrect as it readily cleaves PtdIns 4,5-P₂ under appropriate conditions. Which substrates are most important for either SHIP or type IV enzymes in vivo cannot be determined by these in vitro assays.

Selective sensitivity to detergents has been reported for yeast inositol polyphosphate 5-phosphatases (23). PtdIns 4,5-P₂-hydrolyzing activity of Inp51p is completely abolished in the presence of 0.2% Triton X-100, whereas 5-phosphatase activity of the two other gene products, Inp52p and Inp53p, is detergent-insensitive. At the same time, polyphosphoinositide 3/4/5-phosphatase activities associated with the SACI-like domain of Inp52p and Inp53p is inhibited by detergent. It is possible that detergent sensitivity reflects the sensitivity of the enzymes to their lipid micro-environment in vivo.

Recent years mark tremendous progress in understanding the important role that phosphatidylinositol polyphosphates play in cellular signaling. A role for PtdIns 4,5-P₂ in the regulation of the actin cytoskeleton and vesicular trafficking is well established (reviewed in Ref. 7). There is also a growing body of evidence that PtdIns 3,4,5-P₃ is an important second messenger (reviewed in Ref. 6). Although practically absent in quiescent cells, PtdIns 3,4,5-P₃ is produced in response to cell stimulation predominately by phosphoinositide 3-kinase from PtdIns 4,5-P₂, or by recently characterized phosphatidylinositol 4/5-kinase from PtdIns 3-P by a concerted reaction (24, 25).

PtdIns 3,4,5-P₃ binds with high affinity to PH domains of several proteins, including ARNO, a guanine nucleotide exchange factor for ARF, Grp1 and cytohesin-1, Bruton’s tyrosine kinase (Btk), PLCγ, Akt/PTP and it’s kinase PDK. PH domains of proteins have varying degrees of binding preferences between PtdIns 3,4,5-P₃, PtdIns 4,5-P₂ and, in case of Akt/PTB, PtdIns 4,5-P₂. The PH domain of Btk is the most PtdIns 3,4,5-P₃-specific and binds PtdIns 3,4,5-P₃ with a Kᵩ of less than 1 μM, about 10 times less than PtdIns 4,5-P₂. (26). This preferential binding to PtdIns 3,4,5-P₃ must be physiologically important, because a mutation in the Btk PH domain that results in a significant reduction of the selectivity for PtdIns 3,4,5-P₃ binding also causes agammaglobulinemia. An intact PH do-
main of Btk and PtdIns 3,4,5-P$_3$ production enhance phosphorylation and activation of Akt. One of the proposed downstream targets of Btk, PLC$_\gamma$, also preferentially binds PtdIns 3,4,5-P$_3$ over PtdIns 4,5-P$_2$ via its PH domain. Thus, PtdIns 3,4,5-P$_3$ binding by PH domains of both Btk and PLC$_\gamma$ may lead to simultaneous translocation of the proteins to the plasma membrane and activation of PLC$_\gamma$. A somewhat similar dual role of PtdIns 3,4,5-P$_3$ has been proposed in the activation of Akt in response to mitogenic stimuli (27). PtdIns 3,4,5-P$_3$ binding to PH domains of Akt and PDK1 brings both proteins to the plasma membrane. In addition, PtdIns 3,4,5-P$_3$ binding to the PH domain of Akt exposes the Thr-308 residue, which is critical for phosphorylation and activation of Akt.

PtdIns 3,4,5-P$_3$ can also bind to SH2 domains, competing with phosphoryrosine. PtdIns 3,4,5-P$_3$ binding to the SH2 domain of PLC$_\gamma$ leads to the enhanced ability of PLC$_\gamma$ to hydrolyze PtdIns 4,5-P$_2$, providing a link between the levels of these two phosphoinositol lipids (28, 29).

As with all signaling molecules, PtdIns 4,5-P$_2$ and PtdIns 3,4,5-P$_3$ need to be present in the right place at a precise time, so it is of great interest to identify enzymes that control their intracellular levels. One way of control is removal of phosphate at the D-5 position by members of the inositol polyphosphate 5-phosphatase family of enzymes. Synaptotagmin, a group II 5-phosphatase, is the key enzyme that mediates vesicular trafficking at the synapses via control of the PtdIns 4,5-P$_2$ and, possibly, PtdIns 3,4,5-P$_3$ levels. SHIP, a group III 5-phosphatase, is a negative regulator of signaling initiated by growth factor receptors in hematopoietic cells and FcyRIIB negative signaling in B cells, where it regulates the PtdIns 3,4,5-P$_3$ levels. SHIP2, which is ubiquitously expressed, is likely to play a similar role in nonhematopoietic cells. Cloning and identification of a novel 5-phosphatase with a unique substrate specificity toward phosphatidylinositol polyphosphates will help to elucidate complex regulation of PtdIns 3,4,5-P$_3$ and PtdIns 4,5-P$_2$ levels in the cell.

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