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PrP^C interacts with potassium channel tetramerization domain containing 1 (KCTD1) protein through the PrP_{51-136} region containing octapeptide repeats

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ABSTRACT

To identify molecular interaction partners of the cellular prion protein (PrP^C), we applied a yeast two-hybrid screen on a bovine brain cDNA expression library and identified the potassium channel tetramerization domain containing 1 (KCTD1) as a PrP^C interacting protein. Deletion mapping showed that PrP^C specifically binds KCTD1 through the unstructured PrP_{51-136} region. We further confirmed the interaction between PrP^C and KCDT1 protein by co-immunoprecipitation *in vivo* and by a biosensor assay *in vitro*. Interestingly, the binding of an insertion mutant PrP^{8OR} to KCTD1 is higher than that of wild-type PrP^C, suggesting an important role for an unstructured region harboring octapeptide repeats in the KCTD1-PrP^C interaction. Our results identify a novel PrP^C-interacting protein and suggest a new approach to investigating the unidentified physiological cellular function of PrP^C.

1. Introduction

Prion diseases, or transmissible spongiform encephalopathies (TSEs), are a group of fatal neurodegenerative disorders that can be sporadic, inherited, and infectious. These diseases result from the conformational conversion of a normal cell-surface glycoprotein (PrP^C) into a pathogenic isoform (PrP^{Sc}) that appears to be infectious in the absence of nucleic acid. The conformational conversion of PrP^C into the misfolded state, PrP^{Sc}, has been implicated in all animal prion diseases including bovine spongiform encephalopathy in cattle, Creutzfeldt–Jacob disease in humans, scrapie in sheep, and chronic wasting disease in deer and elk [1].

Although a great deal is known about the role of pathogenic PrP^{Sc} in diseases, the normal, physiological function of the widely expressed nonpathogenic PrP^c has remained a mystery. A powerful strategy for elucidating the physiological function of PrP^C would be to identify other cellular proteins that interact with PrP^C. Over the years, a number of candidates have been identified as potential PrP-binding partners using conventional yeast two-hybrid screening, co-immunoprecipitation, biosensor analysis, and other methods. Although a variety of molecules have been identified as binding to PrP^C, it is as yet unknown whether PrP^C functions via binding to these proteins, and whether one or more of these binding proteins affects its conformational conversion to PrP^{Sc}. Thus, attempts have been made to identify new PrP^C binding proteins that may provide insights into its physiological/pathological functions and conformational conversion.

We employed a yeast two-hybrid method to search for new potential cellular prion protein interacting partners using a brain expression library. A novel PrP^C interacting protein, potassium channel tetramerization domain containing 1 (KCTD1), was identified. KCTD1, which contains a BTB/POZ domain, may be involved in many possible interactions with itself, other domains of the full-length channel, or other proteins. Our results indicate that PrP^C interacts with KCTD1 through the unstructured peptide region PrP_{51-136}, which contains the octapeptide repeat sequence (OR), charge cluster 2, and hydrophobic core domains, and suggest that this complex might be relevant in some as yet unidentified physiological cellular function.

2. Materials and methods

2.1. Vector construction

Bovine brain cDNA library fragments (Unit-ZAP XR Library, Stratagene, La Jolla, CA) were ligated into the pGADT7 vector (Clontech, Mountain View, CA) at the *Eco*R I-*Xho* I sites, to construct the pGADT7-cDNA library for use as the prey. The gene encoding mature bovine PrP^C (25–242) was amplified and then cloned into the pGBDKT7 vector (Clontech) via the *Ned* I and *EcoR* I restriction

Abbreviations: TSEs, transmissible spongiform encephalopathies; BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt–Jacob disease; KCTD1, potassium channel tetramerization domain containing 1.

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sites, yielding the pGBDKT7-PrP^C construct for use as the bait. For the pET30a-KCTD1 construct, the KCTD1 coding region was amplified and cloned into the pET30a plasmid using the *Eco*R I and *BamH* I restriction sites. The pET30a-PrP^C construct was created as described previously [2]. A human pathogenic insertion mutant of PrP^{8OR}, which contains three extra octapeptide repeats, was constructed using the human prion insertion mutant DNA template, and named as pET30a-PrP^{8OR} [3]. A deletion mutant of PrP^{90}, which has a deletion of the octapeptide sequence and contains only the PrP^{Sc} core domain (residues 90–231), designated as PrP^{90}, was constructed from pET30a-PrP^C using the QuikChange® site-directed mutagenesis kit (Invitrogen, Carlsbad, CA).

2.2. Yeast two-hybrid screen

The yeast two-hybrid Gal4 interaction trap assay was done as previously described [2]. *Saccharomyces cerevisiae* strain PJ69-4A/4α cells (Clontech) were cotransformed with the pGADT7-cDNA library and the pGBDKT7-PrP^C bait plasmid, Approximately 5×10^6 yeast transformants were screened and false-positive clones were excluded by retransforming the prey DNA into PJ69-4A/4α cells. Positive clones were verified by using X-galactosidase filter assays and then sequenced in both directions.

2.3. Co-immunoprecipitation

The use of animals in this investigation was in compliance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996). In addition, the study protocol involving animal use was approved by China Hubei Provincial Science & Technology Department (SCXK 2003-0013). Fresh brain tissue from adult Kunmin mice was homogenized on ice with a non-denaturating buffer containing 20 mM Tris, pH 7.5, 10% glycerol, 2 mM ethylene glycol tetraacetic acid, 150 mM sodium chloride, 5 mM sodium fluoride, 1 mM sodium pyrophosphate, and 1 mM phenylmethylsulfonyl fluoride. Proteins were quantified by the Bradford method (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA). Proteins were incubated overnight with 2 μg monoclonal antibody at 4 °C with gentle agitation. A slurry of Protein A Sepharose beads was added to the reaction mix, and the mixture was incubated for 2–3 h at 4 °C with gentle agitation. Finally, the beads were mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer and resolved by SDS–PAGE. Membranes were immunoblotted with the indicated antibodies. Blots were revealed using the enhanced chemiluminescence reagent (Pierce, Waltham, MA) using the Fusion FX7 image acquisition system (France).

2.4. Protein preparation

The four constructs, pET30a-KCTD1, pET30a-PrP^C, pET30a-PrP^{8OR}, and pET30a-PrP^{90}, were individually transformed into *Escherichia coli* BL21(DE3). The expression and purification of pET30a-PrP^C were performed as described previously [4]. The expression and purification of pET30a-PrP^{8OR} and pET30a-PrP^{90} were carried out as for pET30a-PrP^C. The expressed KCTD1 was purified using immobilized metal affinity chromatography with Ni–NTA His Bind Resin (Novagen, Gibbstown, NJ) according to the manufacturer's instructions.

The purified proteins were verified by SDS–PAGE and concentrations were measured using the Bio-Rad protein assay kit (Bio-Rad) with bovine serum albumin as a standard according to the manufacturer's instructions. We also monitored the secondary structures of the two mutant proteins using circular dichroism [5] spectra acquired on a chiroptical spectrometer (Jasco, Tokyo, Japan).

2.5. Surface plasmon resonance (SPR) analysis

SPR analysis was performed using a BIAcore 3000 instrument (GE Healthcare, Buckinghamshire, UK) equipped with a CM5 sensor chip. All assays were carried out at 25 °C with HEPES buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% v/v surfactant P20) used as the running buffer. Following a standard 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide protocol, approximately 850 resonance units of KCTD1 protein were immobilized on the sample flow cell via the amine group. PrP protein was diluted into the running buffer at different concentrations and injected over both sample and reference flow cells at a flow rate of 40 μl/min. After a 60-s association phase followed by a 90-s dissociation phase, the chip surface was regenerated by the injection of regeneration buffer (1 M NaCl, 10 mM NaOH). Kinetic analyses were performed using BIAevaluation software version 4.1 (GE Healthcare).

3. Results

To identify proteins that physically interact with the prion protein, we performed a yeast two-hybrid screen using a bovine brain library. The two-hybrid system confirmed that the bait plasmid (pGBDKT7-PrP^C), as well as the empty vectors (pGBKT7 and pGADT7), when individually tested for self-activation of the reporter cassette in the absence of interacting partners, produced no activation of reporter gene expression. Thus, a positive signal in the two-hybrid assay confirmed the presence of specific interactions between PrP^C and the proteins tested. The pGADT7-cDNA positive plasmids isolated from the screened clones were transformed into *E. coli* for amplification and DNA purification, and their interaction with PrP^C was reconfirmed by the yeast two-hybrid assay. The reconfirmed positive clones were selected and sequenced. The sequences of the isolated positive plasmids showed that several of these positive clones encoded KCTD1, suggesting an interaction between PrP^c and KCTD1. KCTD1 contains a highly conserved BTB domain in its N-terminus and mediates homomeric or heterogenous protein–protein interactions. Interestingly, the bovine and human KCTD1 protein sequences show 99% protein sequence identity, suggesting that the function of KCTD1 is extremely well conserved across species.

Having identified KCTD1 as a interaction partner of the prion protein, we next investigated the specific domain of the prion protein with which it interacts. To map the KCTD1 interaction domain in the prion protein, deletion mutants of PrP^C were made based on the secondary structural characteristics of the prion protein, and tested for interaction using the yeast two-hybrid assay (Fig. 1A). The results indicate that KCTD1 binds to the peptide fragment $PrP_{51\text{-}136}$ of PrP^C, which contains the octapeptide domain within the largely unstructured N-terminal domain (Fig. 1B).

To further confirm whether PrP^C and KCTD1 interact with each other in mammalian cells, we carried out an immunoprecipitation assay. Endogenous PrP^C was immunoprecipitated using a polyclonal anti-PrP^C antibody, and the immunoprecipitated proteins were resolved on SDS–PAGE gels and cross-detected by Western blot using a polyclonal anti-KCTD1 antibody. As shown in Fig. 2, endogenous KCTD1 selectively co-immunoprecipitated with endogenous PrP^C, demonstrating that KCTD1 binds to PrP^C in mammalian brain tissue.

Next, we used SPR to analyze the dynamic interaction between the two proteins in real time *in vitro*. The SPR binding curves shown in Fig. 3 illustrate the specific binding of PrP^C to immobilized KCTD1. The association (k_{ass}) and dissociation (k_{diss}) constants were calculated based on the K_{on} value for different concentrations of PrP^C (12.5, 25, 50, 100, and 200 nM) using the BIAevaluation

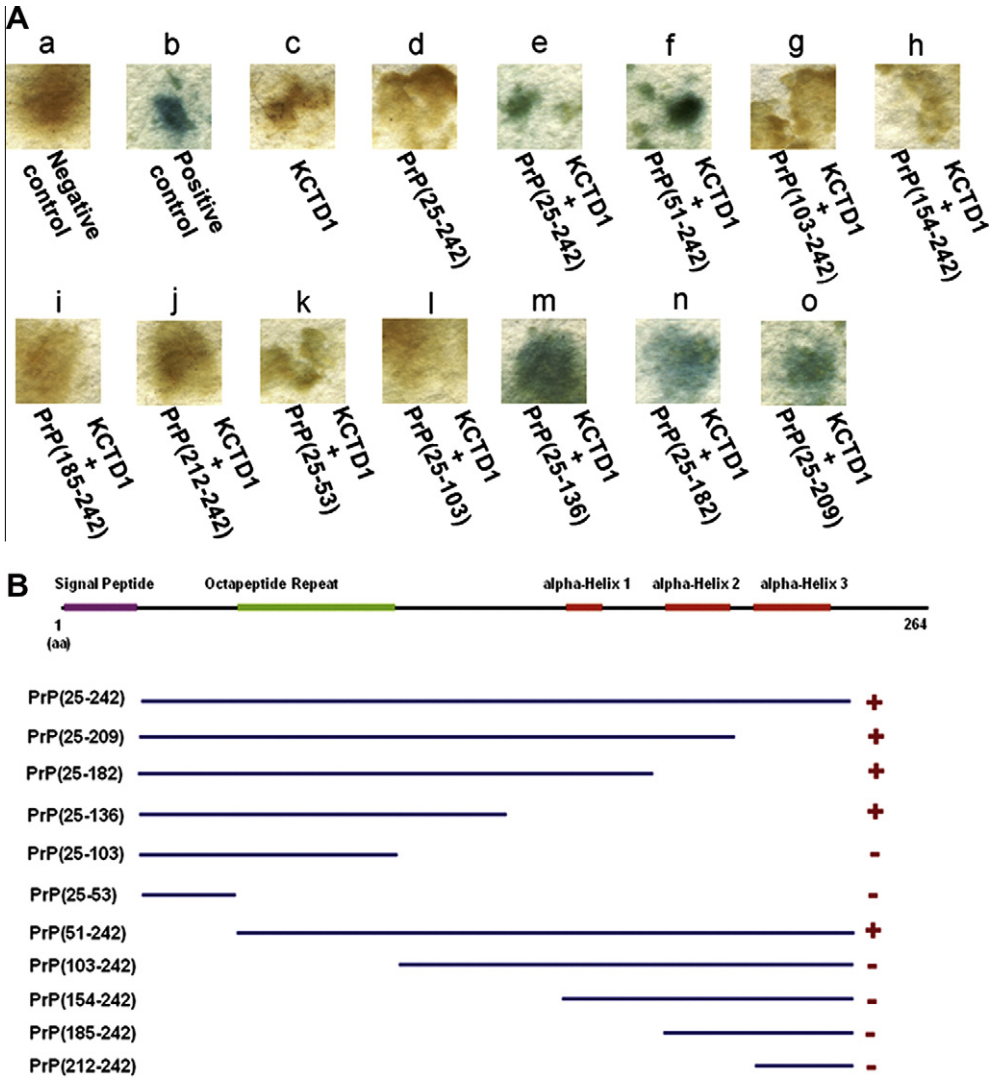

Fig. 1. Interaction of PrP^C with KCTD1 in the yeast two-hybrid assay. (A) β-Galactosidase activity assay. (a) The negative control, human lamin C did not interact with the SV40 large T-antigen. (b) The positive control, murine P53, interacted with the SV40 large T-antigen. (c and d) No auto-activity was found in pGADT7-KCTD1 and pGBKT7-PrP^C. (e–o) The interaction of various deletion mutants of PrP with KCTD1 was tested, and a positive signal was obtained only in panels e, f, m, n, and o. (B) Schematic representation of the interaction of KCTD1 with PrP^C deletion mutants that express various PrP^c fragments spanning amino acid residues 25–242. +, positive interaction; –, no interaction. These results show that PrP^C binds to KCTD1 through the peptide region $PrP_{51\text{-}136}$.

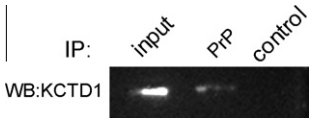

Fig. 2. Co-immunoprecipitation of endogenous PrP^C and KCTD1 proteins. Proteins isolated from fresh mouse brain tissue in non-denaturing solution were precipitated by a polyclonal anti-PrP antibody, electrophoretically separated by SDS–PAGE, and probed with a polyclonal anti-KCTD1 antibody (indicated by +). Pre-immunization serum was used as a negative control (indicated by –) for the immunoprecipitation and a fraction of the input preparation was used in the SDS–PAGE electrophoresis and Western blot probing (indicated by Input). The blots were revealed using the enhanced chemiluminescence reagent and recorded using an image acquisition system.

software. The k_{ass} and k_{diss} were found to be $4.47 \times 10^5\ M^{-1}\,S^{-1}$ and $0.0821\ S^{-1}$ respectively. From the ratio of the respective rate constants, the K_D (1.84×10^{-7} M) and K_A ($5.44 \times 10^6\ M^{-1}$) values were determined. These data suggested a high-affinity specific interaction between PrP^C and KCTD1.

To examine the importance of the N-terminal unstructured domain of PrP^C in the interaction between PrP^C and KCTD1, we constructed a deletion mutant (PrP^{90}) and a mutant containing three octapeptide insertions (PrP^{8OR}), and expressed and purified the two mutant proteins (Fig. 4A). We analyzed the binding of the mutant proteins to KCTD1 by SPR. As expected, the deletion mutant PrP^{90}, which contained a deletion of part of the KCTD1 interaction domain, did not show any interaction with KCTD1. Interestingly, the binding of the insertion mutant PrP^{8OR} to KCTD1 was found to be higher than that of wild-type PrP^C (Fig. 4B), suggesting that the octapeptide repeats play an important role in the KCTD1-PrP^C interaction.

4. Discussion

Accumulation of abnormal or misfolded proteins is a common hallmark of neurodegenerative disorders such as Alzheimer's, Parkinson's, or prion diseases [6–8]. Despite two decades of research, the physiological role of PrP^C, the biochemical nature of the conversion of normal PrP^C to pathological PrP^{Sc}, and the mechanism of PrP^C infection remain enigmatic. It has been proposed that PrP^C is part of a multiprotein complex that modulates various cellular functions. Our results show that PrP^C directly interacts with KCTD1

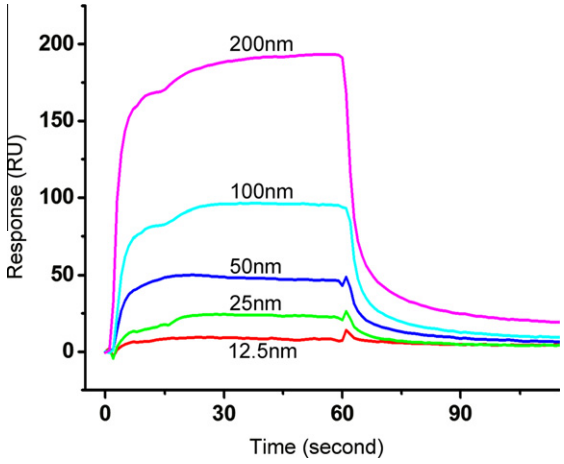

Fig. 3. Analysis of PrP^C-KCTD1 interaction dynamics by SPR. KCTD1 was immobilized on a CM5 sensor chip. Various concentrations of PrP^C were injected onto the chip equilibrated with a running buffer, and signals representing the binding of the two proteins were monitored. The surface was regenerated with 1 M NaCl and 10 mM NaOH, and the experiment was repeated. Data were analyzed by the BIAevaluation software. $K_D = 1.84 \times 10^{-7}$ M and $K_A = 5.44 \times 10^6$ M^{-1}.

through binding to the $PrP_{51\text{-}136}$ region, which is a highly unstructured sequence and contains the OR, charge cluster 2, and hydrophobic core domains.

It should be noted that the N-terminal segment of PrP^C, from residues 23 to 126, is largely unstructured [9–11]. The N-terminal region that spans residues 60–91 of the human prion protein contains five octapeptide repeats of the conserved sequence PHGGGWGQ, and this region plays a role in efficient prion transformation and propagation. Hornshaw et al. confirmed that this region is the major component of the amyloid fibrils in Gerstmann–Straussler–Scheinker disease [5,12,13]. Interestingly, the binding affinity of the insertion mutant PrP^{8OR} to KCTD1 is higher than that of wild-type PrP^C, suggesting that the octapeptide repeats play an important role in the KCTD1-PrP^C interaction. Salmona et al. suggested that the sequence spanning residues 106–126 has the highest propensity to adopt a stable β-sheet structure and assemble into amyloid fibrils that are ultra-structurally similar to those observed in Gerstmann–Straussler–Scheinker patients [14]. Selvaggini et al. also proposed that the region of PrP spanning residues 106–126 displays certain biochemical properties typical of PrP^{Sc} (aggregation, protease resistance), and its mechanism of toxicity has been proposed to be similar to that of PrP^{Sc} [15]. Consistent with these toxicity data, a variant of PrP containing a deletion of amino acid residues 105–125 was found to be lethal in engineered transgenic mice. Harris et al. speculated that the deletion of the 105–125 region of PrP alters its binding interactions, and that the region may also play a role in generating neurotoxic signals during prion infection [16]. Baumann et al. confirmed that the expression of a PrP variant lacking 40 central residues (94–134) induced lethal recessive myelin toxicity [17]. Based on these findings, we believe that the binding interaction between KCTD1 and the region spanning residues 51–136 of PrP^C may play a role in prion propagation and neurotoxicity.

The highly conserved BTB domain of the KCTD protein can mediate homomeric or heterogenous protein–protein interactions. The BTB/POZ domain proteins have been shown to participate in many cellular functions including cellular proliferation, apoptosis, ion channel assembly, protein degradation, and human cancer. Ding et al. initially identified the interaction of KCTD1 with transcriptional repressors of the AP-2 family, especially AP-2a [18]. Recent work on protein degradation of KCTD yields a surprisingly unifying view: a large number of KCTD proteins perform the analogous tasks of recruiting target proteins to cullin-based E3 ubiquitin ligases [19–21]. The question therefore arises whether KCTD1 recruits the prion protein to cullin-based E3 ubiquitin ligases to facilitate prion protein degradation. Other studies have also shown that the KCTD1 homologous proteins KCTD8, 12, 12b, and 16 associate tightly with the carboxyl terminus of gamma aminobutyric acid $(GABA)_{B2}$ receptors as tetramers, and suggest that KCTD proteins may extensively modify G-protein signaling as well as alter agonist potency at $GABA_B$ receptors [22]. Additionally, several authors have reported that the $GABA_A$ receptor and $GABA_A$-mediated receptors are co-regulated in Prnp−/− and PrP^C-overexpressing Tg20 mice [23–26]. Therefore, the finding that PrP interacts with KCTD1 raises the possibility that this interaction may play a role in the TSE signaling pathway via GABA receptors. Whether the association between PrP^C and KCTD1 can affect GABA receptors to influence signaling pathways and prion protein transformation requires further study.

In conclusion, we have shown that KCTD1 interacts with PrP^C *in vitro* and *in vivo* and that the octapeptide repeats within the unstructured region of the PrP^C play an important role in this interaction. Because the unstructured region of PrP^C is known to play an important role in prion aggregation and neurotoxicity, it will be interesting to further investigate whether the interaction between KCTD1 and PrP^C affects prion aggregation or propagation; this would provide a new potential approach towards the prevention of prion diseases. On the other hand, this interaction of PrP^C with

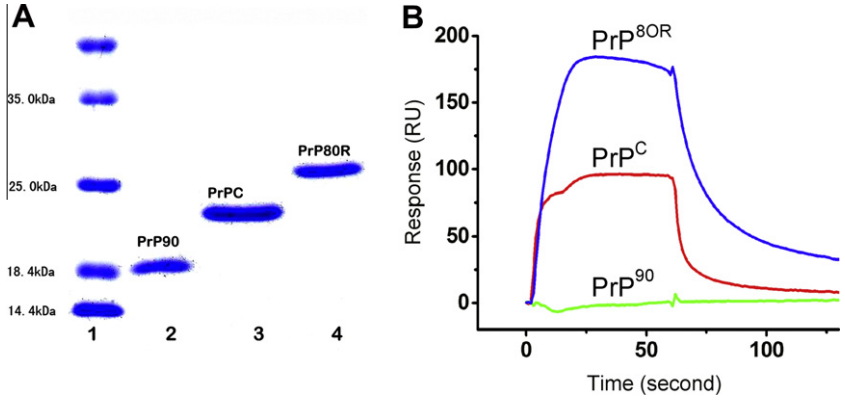

Fig. 4. Interaction dynamics of PrP^C, PrP^{90}, and PrP^{80R} with KCTD1 analyzed by SPR. (A) Expression and purification of mutant PrP^{90} and PrP^{80R}, visualized by SDS–PAGE. Lane 1, molecular weight standard; lane 2, deletion mutant (PrP^{90}); lane 3, wild type (PrP^C); lane 4 insertion mutant (PrP^{80R}). (B) The same concentration (100 nM) of PrP^C, PrP^{90}, and PrP^{80R} were separately injected onto the chip equilibrated with running buffer. Binding analysis revealed that PrP^{90} did not bind KCTD1, while PrP^{80R} had a higher affinity than PrP^C for KCTD1.

KCTD1, a member of a protein family whose members are capable of recruiting proteins to cullin-based E3 ubiquitin ligases, may suggest a potential mechanism for PrP^C degradation *in vivo*.

Acknowledgments

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