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The signal peptide NPFSD fused to ricin A chain enhances cell uptake and cytotoxicity in *Candida albicans*☆

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Abstract

Microorganisms possess stringent cell membranes which limit the cellular uptake of antimicrobials. One strategy to overcome these barriers is to attach drugs or research reagents to carrier peptides that enter cells by passive permeation or active uptake. Here the short endocytosis signal peptide NPFSD was found to efficiently deliver both FITC and GFP into *Saccharomyces cerevisiae* and *Candida albicans* with uptake into the majority of cells in a population. The NPFSD signal is itself non-toxic, but when fused to the ricin A chain toxin (RTA) the peptide enhanced both cell uptake and toxicity against *C. albicans*, which like other yeasts is resistant to naked RTA. Cell entry required at least 1 h incubation, temperatures above 4 °C, and an energy source, and uptake was outcompeted with free peptide. Therefore, the NPFSD peptide can carry a range of compounds into yeasts and this delivery route holds promise to enhance the activity of antifungals.

Keywords: Peptide; Antifungal; *Candida albicans*; Endocytosis

There are a large number of antimicrobials used in medicine; however, these products are made up of only a few distinct chemical classes having similar mechanisms of action. New classes of antimicrobials are needed to help combat resistance to conventional drugs. Unfortunately, it has proven very difficult to develop effective and safe new antimicrobials. One problem is poor drug uptake past multilayered microbial cell barriers, which stringently exclude most foreign molecules [1]. Also, many antimicrobial resistance traits further restrict uptake through cell wall modifications or drug efflux mechanism [2]. Therefore, improved delivery technology would expand the repertoire of antimicrobials [3–6].

A variety of natural peptides can enter microorganisms and mammalian cells by passive and active uptake mechanisms, and such peptides can be used to improve substance delivery. The best known carrier peptides are the so called cell-penetrating peptides (CPPs), which can deliver large molecules into eukaryotic cells through passive cell permeation [4,5]. It may seem surprising that peptides can permeate into cells and even carry attached cargo molecules; however, there is a growing list of natural and synthetic CPPs and an impressive range of applications [7]. Interestingly, peptides also can permeate into bacteria. Several well known natural antimicrobial peptides have intracellular targets [8], and the membrane activity of the $(KFF)_3K$ peptide [9] has been exploited to improve the delivery of peptide nucleic acids (PNA) into *Escherichia coli* [10]. Therefore, cell permeating peptides offer exciting possibilities to improve substance delivery into microorganisms.

The most apparent route for peptide-mediated delivery into microbial cells is via permeases involved in nutritional substrate uptake [11]. Unfortunately, most drugs are not accommodated by permeases and resistance can develop quickly against drugs that enter cells through this route [12]. A more practical approach may be to use endocytosis, as it is involved in a wide range of substrate trafficking, such as continuous sampling of the

☆ *Abbreviations:* FACS, fluorescence-activated cell sorting; GFP, green fluorescence protein; FITC, fluorescein isothiocyanate; RTA, ricin toxin A chain; CPP, cell-penetrating peptide.

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external environment and control of plasma membrane proteins [13,14]. Indeed, there is increasing evidence for absorptive endocytosis as a partial explanation of the in vivo uptake properties of CPPs [15,16]. While the signals that trigger the various forms of endocytosis are poorly understood, and many routes lead to degradation, several endocytosis signal peptides have been identified which lead to cytoplasmic delivery [3]. As evidence of the potential usefulness of this route into the cytoplasm, several natural cytotoxins appear to exploit endocytosis to gain cytoplasmic entry [17].

The NPFSD signal sequence can trigger cell entry into yeasts [8,18] and here we aimed to confirm that this signal sequence can deliver both large and small molecules into *Saccharomyces cerevisiae* and the pathogenic fungi *Candida albicans*. The attached signal sequence improved the uptake of distinctly different cargo molecules and improved the activity of the cytotoxin ricin A chain against *Candida*. The results open possibilities to use the NPFSD signal sequence and related peptides to improve the delivery of research reagents and antifungal substances having intracellular mechanisms of action.

Methods

Strains and culture conditions. *Saccharomyces cerevisiae* (BY4741) and *C. albicans* (CA14) were grown in YPD medium (1% yeast extract, 2% bacterial peptone, and 2% glucose) or minimal medium with or without uracil (25 mg/L) at 30 °C with shaking at 200 rpm. Prior to use, overnight cultures were collected by centrifugation at 3000*g*, rinsed, and suspended in 1× PBS.

Peptides. The peptides VLTNENPFSDP and FITC-CVLTNEN PFSDP were synthesised and HPLC purified by Saveen Biotech AB (SE).

RTA expression. The plasmid pAKG plasmid, a gift from Dr. Robert Weaver (University of Kansas), was used as a template to amplify DNA encoding RTA and NPFSD-RTA. The upstream primer contained an *Eco*RI site followed by a sequence encoding the signal peptide and the first six RTA amino acids (cggaattcatggtgctgaccaac gaaaacccgttttctgatccggctagcatattccccaaacaatac). The downstream primer contained a *Sma*I restriction site and encoded the C-terminal six codons of RTA (tcccccgccaaactgtgacgatggtgg). The PCR fragment was cloned into pKK223-GFP [8].

Fusion protein expression and purification. The GFP fusion proteins were partially purified. Bacteria expressing GFP, NPFSD-GFP, RTA-GFP or NPFSD-RTA-GFP were collected by centrifugation and suspended in Tris buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 5 mM EDTA). The cells were disrupted by sonication in an ice-water bath for 15 min and the insoluble material was removed by centrifugation at 16,000*g*, 4 °C for 15 min. Triethanolamine base and dry ammonium persulfate were added to the bright green supernatant to a final concentration of 100 mM and 40% saturation, respectively. After 1 h of incubation on ice, precipitated proteins were removed by centrifugation for 20 min at 6000*g*. Dry ammonium persulfate was added to a final concentration of 70% saturation. After 1 h, the peptide-GFP fusion proteins were collected by centrifugation as above and dialysed against 1× PBS buffer. Protein concentration was estimated by using BioRad Protein Assay Dye binding reagent.

Ricin concentrations within protein preparations were determined by comparative ELISA. Samples (100 μl) in PBS buffer were added to wells in a standard adsorbent microtiter plate (NUNC immunoplate) and incubated for 2 h at room temperature. The plates were washed with PBS containing 0.05% Tween 20 and 1% BSA. The Primary antibody, a gift from Dr. Orith Leitner, Weizmann Institute of Science, Rehovot, Israel, was diluted to 1:100 for use. The secondary antibody was anti-mouse IgG-HRP (Sigma), diluted to 1:2000 for use. The substrate was 2,2-Azino-Bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma). The plates were washed several times with PBS between antibody and substrate addition. Blue green colour development was measured at 405 nm. A standard curve was prepared from pure RTA (Sigma).

Fluorescence microscopy and image analysis. Freshly grown yeast cells were suspended in minimal medium supplemented with 25 mg/ml uracil and 5 mM $MgCl_2$, incubated with NPFSD-FITC (5 μM) or NPFSD-GFP (10 μg/ml total protein) at room temperature for 1–3 h, centrifuged at 2000 rpm for 5 min, and suspended in 1× PBS. Fluorescence microscopy was performed with a Leica DMRXA microscope with a cooled frame CCD camera with excitation and emission wavelengths of 488 and 507 nm for GFP and 490 and 520 nm for FITC and Openlab image analysis software.

Fluorescence-activated cell sorting analysis. Cells were prepared for FACS analysis as described for microscopy and analyzed with a FACStar (BD) instrument equipped with an argon laser set to excite samples at 488 nm and light emission was detected at 530/35 nm. Mean fluorescence intensities were calculated using Cell Quest software (BD).

Cytotoxicity assay. *Candida* cells were grown overnight at 30 °C in YPD medium. The cells were grown in 96-well plates at 1×10^4 cells/well initial density in 50% YPD medium, adjusted to 1 M sorbitol to prevent cell settling during incubation. Wild-type RTA and NPFSD-RTA were added at 4 μg/ml total RTA. Cultures were incubated at 30 °C and every 2 min the plates were shaken and the turbidity was measured at 550 nm for 30 h.

Results

Cellular delivery of NPFSD-FITC and NPFSD-GFP

The NPFSD sequence is an endocytosis signal involved in receptor recycling [18]. Also, this peptide was identified as a promising carrier for the delivery of foreign molecules into yeasts [8]. Here, we aimed to test whether the NPFSD peptide could deliver a range of cargo molecules into the cytoplasm. First, the 11 amino acid VLTNENPFSDP sequence was attached to the small fluorophore (FITC) during solid phase synthesis and fused with GFP within the *E. coli* expression vector (pKK223-3) (Fig. 1; [8]). The two conjugate molecules were partially purified and then incubated with *S. cerevisiae* and *C. albicans*. After incubation for 3 h, the cells were rinsed and examined by fluorescence microscopy. For both NPFSD-FITC and NPFSD-GFP, strong intracellular fluorescence was indicated with both *S. cerevisiae* and *C. albicans* (Fig. 2). The pattern of fluorescence seen in both yeasts is clearly indicative of cytoplasmic delivery, with little apparent distribution to the nucleus (Fig. 2). To better quantify the proportion of cells containing NPFSD-GFP after incubation, delivery was assessed by FACS analysis. Again, cells incubated with NPFSD-GFP showed pronounced fluorescence (Figs. 2 and 3). Therefore, the results from FACS

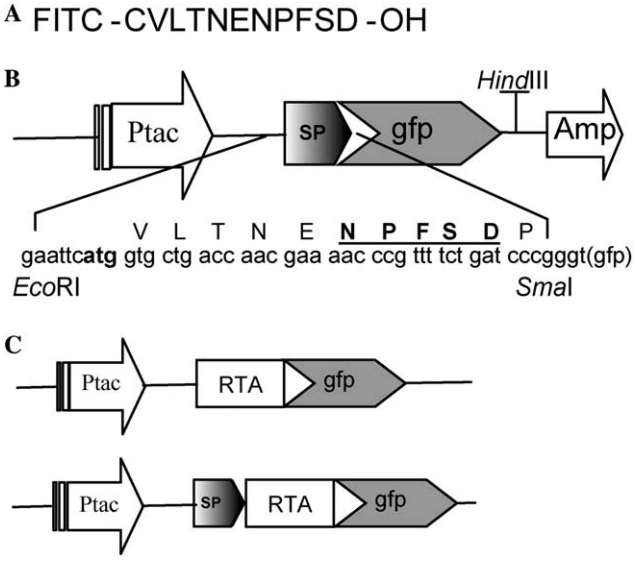

Fig. 1. Illustration of the NPFSD containing molecules used in this study. (A) The NPFSD-FITC construction. (B) The NPFSD-GFP expression plasmid, with the sequence encoding NPFSD-GFP controlled by the P*tac* promoter, followed by the signal peptide sequence (underlined) and GFP gene. The *Sma*I restriction site is indicated and the start codon is in bold. (C) The RTA-GFP and NPFSD-RTA-GFP constructions.

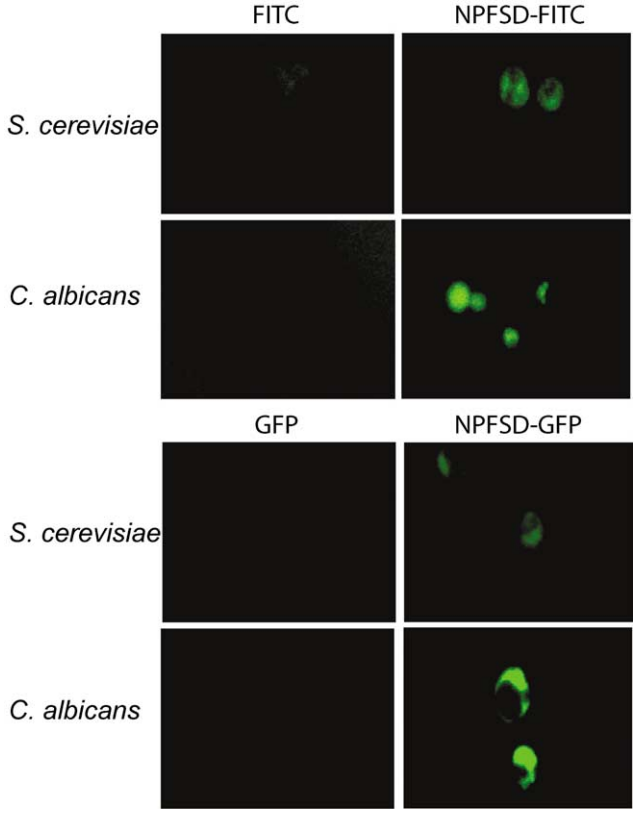

Fig. 2. Microscopic analysis of peptide-fluorophore entry into yeasts. Cells were grown overnight, harvested, and incubated with FITC-CVLTNENPFSDP or VLTNENPFSDP-GFP (signal sequence underlined) for 3 h at a concentration of 5 μM. The cells were harvested, washed, and examined for FITC and GFP fluorescence. The images were captured with a CCD camera, 1000× total magnification.

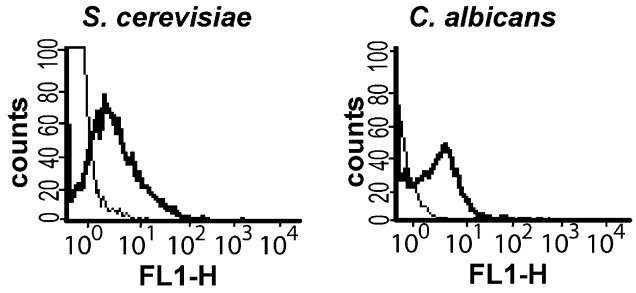

Fig. 3. FACS analysis of NPFSD-GFP entry into yeasts. Cells were grown overnight and then incubated with GFP (thin lines) or VLTNENPFSDP-GFP (thick lines) for 3 h at 10 μg/ml total protein. Fluorescence intensities for 1×10^4 events are indicated.

analyses correspond to those from microscopy and indicate that the NPFSD-GFP entered the majority of cells in a population.

The results from fluorescence microscopy and FACS analysis are consistent with uptake mediated by the NPFSD signal sequence [8,18]. Nevertheless, there are alternative explanations for the results. To ensure that the results were not due to a form of cell toxicity or membrane damage, cell growth rate and morphology were monitored. In the presence of NPFSD-GFP, both yeasts grew at a normal pace and appeared normal when viewed by light microscopy. Therefore, increased autofluorescence associated with cell death does not appear to be a factor. Also, to ensure that fluorophore uptake was not due to some inherent property of FITC or GFP, free FITC and GFP were included in the experiments involving fluorescence microscopy and FACS analyses. In all cases, yeasts treated with the free fluorophore showed only background levels of fluorescence (Figs. 2 and 3). Therefore, the NPFSD peptide carried both FITC and GFP into *S. cerevisiae* and *C. albicans*.

NPFSD enhances RTA toxicity against C. albicans

Given that the NPFSD peptide can deliver a wide range of substances into yeasts and possibly other fungi, it should be possible to use this delivery approach to improve the activity of substances that suffer from poor cellular uptake. To evaluate this possibility the NPFSD peptide was fused to the N-terminus of ricin A chain—a cytotoxin that destroys ribosomes in the cytoplasm. As an indicator of cell uptake, GFP was incorporated at the C-terminus of the fusion protein. The RTA-GFP and NPFSD-RTA-GFP constructs were expressed in *E. coli* and the fusion proteins were partially purified. The concentration of RTA in the protein preparations was measured by ELISA and aliquots were added to provide equal ricin concentrations. When present at 4 μg/ml with respect to RTA, the NPFSD-RTA-GFP fusion was inhibitory over a 30 h period, indicating long lasting inhibitory effects (Fig. 4). The control RTA-GFP fusion

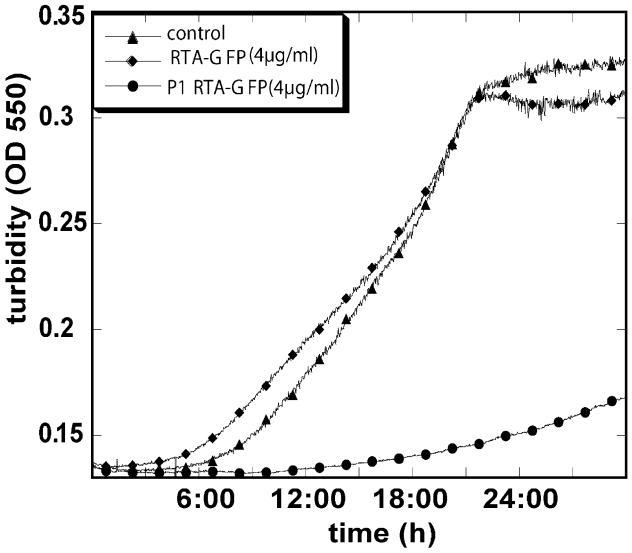

Fig. 4. Effect of RTA-GFP and NPFSD-RTA-GFP on *C. albicans* growth. Cells were grown overnight, harvested, and incubated at 1×10^4 cells/ml with RTA-GFP or VLTNENPFSDP-RTA-GFP, at 4 µg/ml RTA concentration (signal sequence underlined).

was not inhibitory at the same concentration. Therefore, enhanced toxicity against *C. albicans* suggests that the peptide improved the cytoplasmic delivery of the cytotoxin RTA, demonstrating that this uptake route can be used to deliver substances that enter cells only poorly.

The NPFSD sequence mediates uptake of RTA-GFP

The results described above are consistent with cytoplasmic delivery, as described for this signal peptide [18]. Nevertheless, we asked whether the relatively large NPFSD-RTA-GFP construct retained the cell entry properties found for the naked peptide [18]. When cells were treated with NPFSD-RTA-GFP and examined by fluorescence microscopy clear intracellular fluorescence was apparent, suggesting that this construct retained its reported cell entry mechanism [18]. However, in this case the cells appeared smaller in size and less symmetrical in shape, presumably due to damage caused by the internalised toxin. Therefore, the enhanced toxicity and intracellular distribution observed for NPFSD-RTA-GFP indicate that the NPFSD peptide mediated cytoplasmic delivery.

To test further whether uptake was mediated by the NPFSD signal sequence and active uptake, the requirement for an energy source was assessed by incubating cells in the presence and absence of glucose. Glucose at 1% concentration clearly enhanced uptake (Fig. 5). Also, temperatures above 4 °C were required and uptake was more efficient with longer incubation periods and higher substrate concentrations (data not shown). Therefore, the time, temperature, and energy requirements observed are consistent with NPFSD-

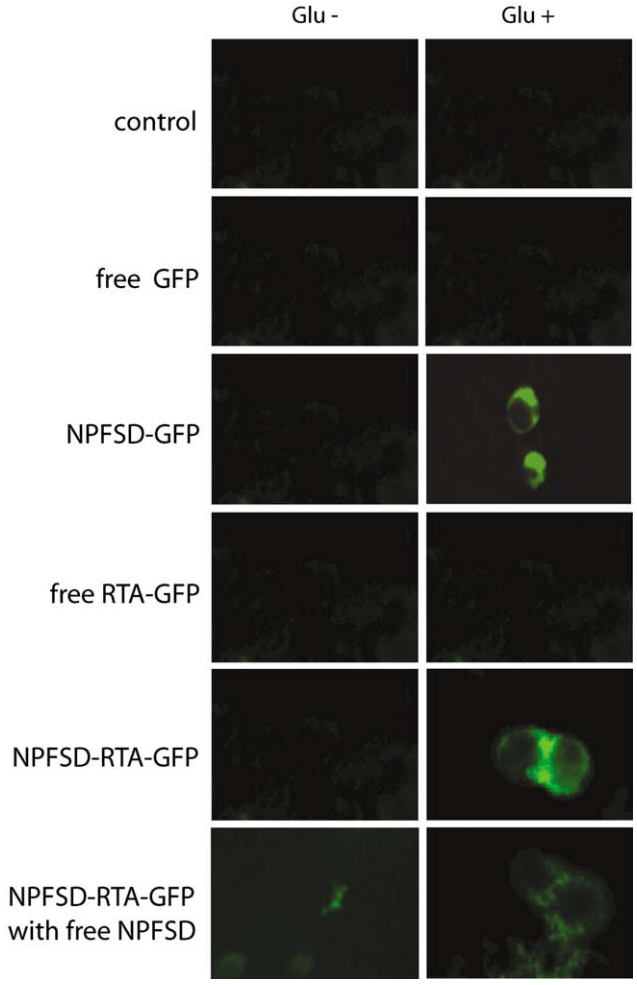

Fig. 5. Microscopic analyses of RTA-GFP and NPFSD-RTA-GFP into yeasts in the presence and absence of added glucose. Cells were grown overnight, harvested and incubated with either RTA-GFP or VLTNENPFSDP-RTA-GFP with controls for 3 h (signal sequence underlined). The cells were washed and examined for GFP fluorescence; the images were captured with a CCD camera. Control GFP, RTA-GFP and VLTNENPFSDP-RTA-GFP were incubated in the presence and absence of glucose added to 1% in YE medium, and also in the presence and absence of free VLTNENPFSDP peptide at (5 µM), viewed at 1000× total magnification.

RTA-GFP uptake by endocytosis or another form of active uptake. Also, the requirement for glucose excludes surface binding as an explanation for the observed cellular fluorescence.

To evaluate the need for the NPFSD sequence in another way, a competition experiment was conducted using added free NPFSD peptide. If it is true that the fused peptide mediates uptake by receptor binding and endocytosis, it should be possible to limit uptake through competition with added free peptide. *Candida* cells were incubated with NPFSD-RTA-GFP in the presence and absence of 5 µM VLTNENPFSDP peptide. Decreased accumulation of NPFSD-RTA-GFP was observed in the presence of the free peptide, indi-

cating receptor mediated entry into *Candida* cells (Fig. 5). Therefore, the NPFSD-RTA-GFP fusion protein appears to enter cells via a receptor mediated and active route that requires NPFSD.

Discussion

Peptide-mediated delivery offers possibilities to improve the cellular delivery of large molecular weight substances into microorganisms through both passive permeabilisation and active cell uptake. Here we focused on the endocytosis signal sequence NPFSD fused to large and small fluorophores and the ricin A chain cytotoxin. Ricin was selected as a toxin which has a well defined mechanism of action against ribosomes localised in the cytoplasm [19]. While ricin is a potent cytotoxin against mammalian cells, externally added ricin does not intoxicate yeast, as fungi lack galactosylated surface components needed for cell binding [20,21]. Therefore, enhanced ricin toxicity against yeast can indicate enhanced cytoplasmic delivery.

In this study, the results show that the NPFSD peptide improved delivery of a range of cargo molecules into *C. albicans* and the carrier peptide enhanced the cytotoxic activity of RTA when fused to the N-terminus of the toxin. Although several natural toxins enter cells through endocytosis [3,6], we are not aware of other results where endocytosis signal peptides were used to enhance delivery into yeasts. Moreover, it was not clear to us that this mechanism could be exploited to enhance antifungal activity. Nevertheless, relative to cell permeating peptides, endocytosis signal peptides appear to offer several advantages as carriers. While CPPs may be a broad class of carrier peptides, most appear to require denatured cargo proteins during membrane passage [5]. Also, CPPs typically show activity against many cell types, which excludes cell-type-specific delivery. In contrast, receptor mediated endocytosis requires recognition that may enable cell-type-specific delivery, and the uptake mechanism appears to accommodate structurally distinct substrates.

Certain endocytosis signals and delivery routes lead to cytoplasmic delivery, and in some cases specific signal sequence recognition appears to trigger uptake [3,22,23]. Relatively little is known about the NPFSD signal studied here, however, the evidence indicates that it reaches the cytoplasm following uptake [8,18], and related sequences appear to be involved in a variety of protein uptake and trafficking mechanisms [18,24,25]. The present results demonstrate that the NPFSD signal sequence can mediate efficient cytoplasmic delivery of two diverse fluorophores into *S. cerevisiae* and *C. albicans*. Furthermore, when attached to a cytotoxin that does not normally enter yeast the signal sequence enhanced cytotoxicity against *C. albicans*. The uptake properties are consistent with receptor mediated endocytosis triggered by NPFSD [18]; however, other routes of cell entry cannot be excluded. The most attractive future possibility is to use carrier peptides to enhance the activity of antifungal agents. Also, it may be possible to use endocytosis signal peptides to deliver proteins into a range of fungal species for functional studies that cannot be achieved using standard genetics.

Acknowledgments

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