*Drosophila Egghead* Encodes a β1,4Mannosyltransferase Predicted to Form the Immediate Precursor Glycosphingolipid Substrate for Brainiac.

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This work was supported by: Human Science Frontier Program (RGP0063/2002-C), the Velux Foundation, the Danish Medical Research Council, the NIH Resource Center for Biomedical Complex Carbohydrates (NIH P41 RR05351), European Community Marie Curie Fellowship, IHP HPMF-CT-2000-01083, and the Biological Research Infrastructure Network-Center for Structural Biology (NIH P20 RR16459).

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**Key words:** Egghead/brainiac/glycosyltransferase/glycosphingolipid/glycosylation/receptor modulation/lipid rafts

**Category:** Glycobiology and Extracellular Matrices

**Running Title:** Egghead Encodes a β1,4Mannosyltransferase
Summary

The neurogenic Drosophila genes brainiac and egghead are essential for epithelial development in the embryo and in oogenesis. Analysis of egghead and brainiac mutants has led to the suggestion that the two genes function in a common signaling pathway. Recently, brainiac was shown to encode a UDP-N-acetylglucosamine:βMan β1,3-N-acetylglucosaminytransferase (β3GlcNAc-transferase) tentatively assigned a key role in biosynthesis of arthrolines glycosphingolipids and forming the trihexosylceramide, GlcNAcβ1-3Manβ1-4Glcβ1-1Cer. In the present study we demonstrate that egghead encodes a Golgi-located GDP-mannose:βGlc β1,4-mannosyltransferase tentatively assigned a biosynthetic role to form the precursor arthrolines glycosphingolipid substrate for brainiac, Manβ1-4Glcβ1-1Cer. Egghead is unique among eukaryotic glycosyltransferase genes in that homologous genes are limited to invertebrates, which correlates with the exclusive existence of arthrolines glycolipids in invertebrates. We propose that brainiac and egghead function in a common biosynthetic pathway, and that inactivating mutations in either lead to sufficiently early termination of glycolipid biosynthesis to inactivate essential functions mediated by glycosphingolipids.

Introduction

The Drosophila genes brainiac and egghead play essential roles in epithelial development in the embryo and during oogenesis (1;2). Brainiac and egghead encode proteins that are required in the germline to allow for normal interaction between germ-line and somatic cells in the developing ovary (2). In the absence of brainiac or egghead in the germ-line defects are observed in the overlying follicular epithelium, which is of somatic origin (1;2). On one hand, these follicular epithelial defects resemble defects in EGF receptor signaling between germ-line and follicle cell layers. On the other
hand, they resemble a subset of the follicular defects associated with Notch mutants (1-3). Defects in female fertility have also been described (4). The diversity of defects caused by brainiac and egghead mutants suggests that they may be involved in communication between cells at a fundamental level and that they can affect multiple signaling pathways.

Brainiac and egghead mutants exhibit similar and non-additive phenotypes, leading to the proposal that they function in a common signaling pathway. Based on sequence analysis, Yuan et al. (5) originally proposed that brainiac together with the distant homologous gene fringe encoded glycosyltransferases. This hypothesis has subsequently proved correct and both represent glycosyltransferases with functionally conserved mammalian homologs (6-9). Brainiac encodes a UDP-N-acetylglucosamine: βMan β1,3-N-acetylglucosaminy1transferase1 (β3GlcNAc-T) with a predicted function in biosynthesis of arthrosesial glycosphingolipids in the Drosophila (8;9). Brainiac was shown to catalyze addition of the third monosaccharide residue to form the trihexosylceramide glycolipid, GlcNAcβ1-3Manβ1-4Glcβ1-1Cer. Arthrosesial glycolipids have only been found in invertebrates and differ fundamentally from mammalian glycolipids by having a core disaccharide structure based on Manβ1-4Glcβ1-Cer (MacCer) rather than Galβ1-4Glcβ1-Cer (LacCer) (10). Interestingly, brainiac was found to transfer β1-3 linked GlcNAc to both MacCer and LacCer, while mammalian homologs only transfer to LacCer (8;9;11). Sequence analysis of egghead indicates that it could encode a type II transmembrane glycosyltransferase. Homologous genes appear limited to invertebrates, and no similar genes are found in the mammalian databases. In the present study we tested the hypothesis that egghead encodes a unique invertebrate glycosyltransferase activity in the same biosynthetic pathway as brainiac, and present evidence that egghead indeed encodes a β1,4-mannosyltransferase predicted to form the MacCer precursor glycolipid substrate for brainiac.
Experimental Procedures

Expression of Egghead in Insect - An expression construct of the full coding region of egghead was prepared by RT-PCR using \textit{D. melanogaster} mRNA and the sense primer Egh001 (5’-AGCAGATCTCAAGATGAACTCCACCACAAG -3’) with a \textit{Bg}II restriction site and the anti-sense primer Egh002 (5’-AATAGTCTAGACGTCCTCCAGTACGCG -3’) with a \textit{Xba}I restriction site. The resulting 1.1 Kb fragment was cloned into the \textit{Bg}II/\textit{Xba}I sites of pVL1393 (PharMingen) and pVL1393-MYC. Baculo-virus expression constructs, pVL-egghead-full and pVL-egghead-Myc-full, were co-transfected with Baculo-Gold\textsuperscript{TM} DNA (Pharmingen) in Sf9 cells as described (12). Control constructs included pVL-GalNAc-T4-full (13) and pVL-brainiac-full (8). Standard enzyme assays were performed in 50 μl reaction mixtures containing 25 mM HEPES-KOH (pH 7.4), 10 mM MgCl\textsubscript{2}, 0.1% \textit{n}-octylgalactoside (Sigma), and 100 μM GDP-[\textsuperscript{14C}]Man (2,000 cpm/nmol) (Amersham), and varying concentration of acceptor substrates (purchased from Fluka, Merck, Sigma and Toronto Research Chemicals Inc; see Table I for structures). Assays with brainiac were carried out in the same reaction mixture except for addition of UDP-[\textsuperscript{14C}]GlcNAc (3,000 cpm/nmol) (Amersham) and MnCl\textsubscript{2}. Enzyme sources were microsomal fractions of baculo-virus infected Sf9 and High Five\textsuperscript{TM} cells prepared essentially as described (14). Briefly, cells were lysed in lysis buffer (25 mM Tris-HCl pH 7.4, 250 mM Sucrose), after incubation 30 min on ice cells were homogenized and lysate centrifuged at 1000 x G. Glycerol were added to 20% and membrane pellets were obtained by 100,000 × g. Pellets were used at 10 mg/ml (Protein concentration determined by BCA, Pierce). Reaction products of soluble acceptors were quantified by chromatography on Dowex AG1-X8 (Sigma). Assays with glycosphingolipids included 5 mM 2-Acetamido-2-deoxy-D-Glucono-1,5-lactone (inhibitor of hexosaminidase activity), and products were purified on octadecyl-silica cartridges (Supelco) and analyzed by high performance thin-layer chromatography followed by autoradiography.
Expression of Egghead in CHO cells – The 1.1 Kb fragment used for baculo constructs was cloned into the BamH1/Xba1 sites of pcDNA3(+). CHO-K1 cells were stably transfected with the pcDNA3-egghead-Myc-full as described previously, and clones selected with anti-Myc antibodies (13). Cells were grown to subconfluency and fixed with 3% paraformaldehyde and immunostained with anti-Myc monoclonal antibody (Invitrogen). Transferase assays were performed in standard reaction mixtures with cell lysates.

Isolation and analysis of a Product Formed by Egghead – The product formed with n-octyl-glucoside (1 mg) was purified on octadecyl-silica cartridges (Bakerbond, J.T. Baker), followed by stepwise elution with increasing concentrations of methanol in water. The purified glycolipid was deuterium exchanged by repeated addition of CDCl3-CD3OD 2:1, sonication, and evaporation under nitrogen, then dissolved in 0.5 mL DMSO-d6/2% D2O (containing 0.03% tetramethylsilane as chemical shift reference) for NMR analysis. 1-D 1H, 2-D 1H-1H gCOSY, TOCSY, and ROESY NMR spectra were acquired on a Varian Inova 500 MHz spectrometer at 35°C, with solvent suppression by pre-saturation pulse, using standard pulse programs included in the Varian vNMR software package.

Results

tBLASTn searches performed with D. melanogaster egghead coding region (GenBank accession number NM_080313) of the National Center for Biotechnology Information (NCBI) database and the whole genome database GadFly released by the Berkeley Drosophila Genome Project (BDGP), revealed genes with significant similarity in flies (diptera) and nematodes, including C.elegans. Low sequence similarity was found to the putative cellulose synthetase CelA (GenBank accession number AAC41435) from Agrobacterium tumefaciens as well as other bacterial genes predicted to be glycosyltransferases (GenBank accession number NP_348317 [Clostridium acetobutylicum], NP_531181[Agrobacterium tumefaciens str. C58]). No significant similarity was found with
mammalian genes. Egghead is predicted to encode a protein of 457 amino acids with a putative N-terminal signal sequence and a putative hydrophobic transmembrane retention signal (3), which is typical for Golgi located glycosyltransferases. SDS-PAGE western blot analysis with anti-Myc antibodies of lysates of baculo-virus infected High Five cells or a stable CHO egghead transfectant revealed a single protein migrating with an apparent molecular weight of 52 Kd (not shown).

Subcellular localization of egghead was analyzed by immunofluorescense staining of a stable CHO egghead transfectant, where immunoreactivity was limited to a supranuclear pattern characteristic for Golgi localization (not shown). A similar staining pattern was found for a stable CHO transfectant with human \( \beta 3 \)GnT2 (not shown), as well as transfectants with other human glycosyltransferases (13). The GadFly database predicts that egghead contains a sugar nucleotide donor substrate binding site with potential DXD/E binding motifs (15).

**Egghead encodes a GDP-Man: \( \beta \)Glc \( \beta 1,4 \)Mannosyltransferase** – Initial assays of activity included a screen with high concentrations of monosaccharide substrates and different donor substrates as described previously (6;8). Microsomal fractions of infected High Five cells expressing the full coding region of *egghead* exhibited a marked increase in GDP-Man transferase activity with D-Glucose (Fig. 1). Egghead exhibited strict donor substrate specificity for GDP-Mannose and did not utilize other donor sugar nucleotides tested (UDP-Gal, UDP-GalNAc, UDP-GlcNAc). Analysis of a panel of mono- and disaccharide derivatives showed that egghead exhibits strong preference for substrates containing terminal \( \beta \)-linked glucose (\( \beta \)-Glc) (Table I). Interestingly, some \( \beta \)Man monosaccharide derivatives also served as efficient substrates, however, no activity was found with the disaccharides Man\( \beta 1-4 \)GlcNAc and Man\( \beta 1-4 \)Glc\( \beta 1-n \)-Oct. Analysis of apparent \( K_m \) for the most active substrates identified showed that \( n \)-octyl-\( \beta \)-Glc was the preferred acceptor substrate (apparent \( K_m \) 0.67 ± 0.08 mM) with Glc\( \beta 1-p \)Nph (apparent \( K_m \) 1.10 ± 0.3 mM) being comparable and Man\( \beta 1-p \)Nph (apparent \( K_m \) 2.30 ± 0.5 mM) less preferred. The apparent \( K_m \) for GDP-Man with \( n \)-octyl-\( \beta \)-Glc acceptor substrate was 58.0 ±6.2 \( \mu \)M.
Optimization of the enzyme assay using microsomal membranes demonstrated that Triton X-100, Triton CF-54 and Nonidet P-40 inhibited egghead activity at 0.1 %, while \textit{n}-octylgalactoside at 3.4 mM (0.1 %) and to a lesser extent CHAPS activated the enzyme. The pH optimum of egghead activity was pH 7-8. Addition of 5 to 10 mM MgCl$_2$ and MnCl$_2$ activated enzyme activity (Mg$^{++}$ being better than Mn$^{++}$) and CaCl$_2$ had no effect, while addition of 10 mM EDTA destroyed the activity.

Analysis of egghead activity in the established CHO transfectant cells showed the same properties as when egghead is expressed in insect cells (not shown). Attempts to visualize \textit{in vivo} formed products by lectin staining with Vicia Faba (Sigma) was unsuccessful, and further characterization of the products formed await large scale production of cells for chemical analysis of glycolipids.

\textbf{Egghead functions in glycosphingolipid biosynthesis:} Glycosphingolipids of the fruit fly are based on the arthroseries GlcNAc\textbeta$1$-3Man\textbeta$1$-4Glc\textbeta$1$-1Cer core (10). The finding that egghead exhibits \textit{\beta}-mannosyltransferase activity with \textit{\beta}Glc acceptor substrates strongly suggested that egghead transfers Man to Glc\textbeta$1$-1Cer to form MacCer. As shown in Figure 2 egghead utilizes Glc\textbeta$1$-1Cer as an acceptor substrate, whereas LacCer does not serve as substrate. In addition, Gal\textbeta$1$-1Cer was found not to serve as a substrate (not shown). Based on this result it was predicted that egghead functions as the MacCer synthase. Evidence in support hereof was provided by showing, that brainiac utilizes the product formed by egghead (Fig. 3). This assay was carried out with \textit{n}-octyl-\textit{\beta}-Glc as initial acceptor substrate because it served as a better substrate than GlcCer under the assay conditions used.

\textbf{Structural characterization of product formed by egghead -} A 1-D $^1$H NMR spectrum of the diglycosyl product formed with \textit{n}-octyl-\textit{\beta}-glucoside exhibited resonances consistent with approximately 55% conversion to Man\textbeta$1$-4Glc\textbeta$1$-1\textit{n}Octyl, i.e., anomeric signals at 4.477 and 4.143
ppm ($^3J_{1,2} = \sim 1$ and 7.9 Hz, respectively), corresponding to H-1 of Manβ1-4 and Glcβ1-1 residues of this glycolipid. H-1 of unreacted Glcβ1-1 is observed at 4.080 ppm ($^3J_{1,2} = 7.6$ Hz) (Fig. 4). Following complete assignment of $^1$H resonances from all three monosaccharide spin systems present (see Table II) by 2-D $^1$H-$^1$H gCOSY and TOCSY experiments (not shown), the connectivity between the β-Man and the more abundant β-Glc (spin system originating from the H-1 at 4.143 ppm) was established as a 1→4 linkage by a 2-D ROESY experiment; which showed a dipolar cross-relaxation correlation (Overhauser enhancement) between β-Man H-1 and β-Glc H-4. This is consistent with the substantial downfield shift of H-4 compared with that observed for unreacted n-octyl-β-glucoside (3.350 vs 3.016). Although other β-Glc resonances are affected by the glycosylation, H-4 is shifted downfield by the largest increment ($\Delta \delta_{1,4} = 0.334$ ppm; $\Delta \delta_{1,3} = 0.244$ ppm; $\Delta \delta_{1,5} = 0.151$ ppm).

**Discussion**

The original prediction that the neurogenic genes *brainiac* and *egghead* encoded proteins serving functions in a common pathway has been verified by demonstrating that both genes encode glycosyltransferases, and that egghead can synthesize the immediate precursor glycolipid substrate for brainiac. The two enzymes function very early in glycosphingolipid biosynthesis at the second and third steps in build-up of the oligosaccharides of glycosphingolipids, and it is likely that this reflects the severe phenotypes associated with inactivation of these genes. Glycosphingolipids of *Drosophila* have been reported to be based on the arthroseries and exist as extended oligosaccharide structures such as the hexosylceramide Galβ1-3GalNAcα1-4GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ1-1Cer, which can be terminated by glucuronic acids and modified with phosphoethanolamine chains to give charged and zwitterionic glycolipids (10;16). Specific biological functions of distinct glycolipid structures have not been elucidated in *Drosophila*, but it is conceivable that termination of glycolipid biosynthesis at GlcCer and at MacCer could block biological activity of glycolipids to similar effect.
Genetic approaches to studying glycosphingolipid functions in mammals have so far provided some insight into defined biological activities. In contrast to invertebrate glycolipids that appear to be based on one class, mammalian glycolipids are based on multiple classes. Mice deficient in ganglioseries glycolipids build on GalNAcβ1-4Galβ1-4Glcβ1-Cer have yielded significant information (17-21). Globoseries glycolipids built on Galα1-4Galβ1-4Glcβ1-Cer are dispensable in man as evidenced from the rare P^k and p blood groups (22;23). While the biosynthesis of ganglioseries and globoseries glycolipids are carried out by unique single copy genes, each step in the biosynthesis of lacto- and neolactoseries glycolipids build on GlcNAcβ1-3Galβ1-4Glcβ1-Cer is carried out by multiple isoenzymes, many of which serve functions in the synthesis of glycoproteins as well (24). Drosophila and C.elegans may in this respect constitute simpler systems for studies of functions of glycolipids. Recently, the β4GalNAc-transferase acting in sequence after brainiac to form GalNAcβ1-4GlcNAcβ1-3Manβ1-4Glcβ1-1Cer was characterized (25). An interesting application of this β4GalNAc-transferase was to override β4galactosylation in mammalian cells, which despite a high degree of redundancy of β4galactosyltransferases is a strategy to study the function of β4galactosylation. Availability of brainiac and egghead glycosyltransferases now opens possibilities for similar studies. LacCer synthesis may be overridden with MacCer using egghead, as it is predicted that MacCer is not elongated by mammalian glycosyltransferases including β3GlcNAc-transferases. Brainiac is capable of using both MacCer and LacCer and can presumably be used to selectively restore (neo)lactoseries glycolipid expression in mice. Conversely, Drosophila egghead mutants may be rescued by a mammalian lactosylceramide synthase such as β4Gal-T5 or -T6 (26;27).

An increasing number of genes involved in biosynthesis of glycoconjugates have been identified as essential or important for normal development of flies and nematodes. Genes involved in proteoglycan
biosynthesis are particularly prevalent among these (28;29). A number of genes involved in biosynthesis of the proteoglycan core region were identified through an elegant screen for defects in vulval invagination of *C.elegans* (29), and these include glycosyltransferases functioning in precursor–product relationships and relevant sugar nucleotide transporters. *Fringe*, a distant homolog of *brainiac*, was found to encode a key enzyme controlling elongation of O-linked fucose directly on Notch (6;7), and precursor–product relationships with glycosyltransferases functioning after fringe have also been implicated (30). To our knowledge, *egghead* and *brainiac* are currently the only available examples of essential genes in *Drosophila* with functions in the biosynthesis of glycolipids. Glycolipids are known to serve important biological functions in mammals including modulation of receptor functions (31). Modulation may be mediated through direct lectin-carbohydrate interactions between the receptor and glycolipids (32), or through organization of lipid rafts which are known to be enriched in MacCer in *Drosophila* (33). *Egghead* and *brainiac* offers new tools to decipher mechanisms of receptor modulation through glycolipids.

**Footnotes**

1 The abbreviations used are: β3GlcNAc-transferase, UDP-β-N-acetylglucosamine: acceptor β1,3-β-N-acetylglucosaminyltransferase; Cer, ceramide; LacCer, lactosylceramide; MacCer, mactosylceramide; PCR, polymerase chain reaction; TOCSY, total correlation spectroscopy; gCOSY, gradient-enhanced correlation spectroscopy; ROESY, rotating frame Overhauser spectroscopy.
Tables

Table I. Substrate specificities of egghead β1-4-mannosyltransferase.

Table II. $^1$H, $^{13}$C chemical shifts (ppm) and $^3J_{1,2}$ coupling constants (Hz, in parenthesis) for N-Octyl-βGlucoside substrate and biosynthetic Manβ1-4Glc-Octyl product.

Figure Legends

Fig. 1. Egghead exhibits GDP-Man: βGlc mannosyltransferase activity with monosaccharides.


Fig. 2. Egghead transfer Man to Glcβ1-1Cer. Microsomal fraction of egghead (Egh) and GalNAc-T4 (GT4) were incubated with Glcβ1-1Cer, LacCer, or no glycolipid and GDP-Man as described in Experimental Procedures. Autoradiography of thin-layer chromatography of reaction products (4 h). Plate was run in chloroform-methanol-water (60/38/10, v/v/v). Migration of standard glycolipids is indicated with arrows.

Fig. 3. The product formed by egghead with n-octyl-βGlc serves as a substrate for brainiac.
High performance thin layer chromatography analysis of product developments (2 h) with combinations of microsomal fractions of egghead (Egh), polypeptide GalNAc-T4 (GT4), and brainiac (Brn) expressing High Five cells, and combinations of sugar nucleotides GDP-Man and UDP-GlcNAc. Upper panel is stained with orcinol and the lower panel represents an autoradiography. Plates were run in chloroform-methanol-water (60/30/8, v/v/v), and the migration of n-octyl-βGlc (NOG) and the disaccharide and trisaccharide products hereof are indicated in the margins. Man-Glc-Oct is formed only in the presence of egghead and GDP-Man, and GlcNAc-Man-Glc-Oct is formed only in the presence both of egghead and brainiac as well as GDP-Man and UDP-GlcNAc. In lane 7, the asterisks indicate that the autoradiography assay was carried out with non-labeled GDP-Man to confirm that the initial added sugar was Man.

Fig. 4. Downfield region of 500-MHz ¹H-NMR spectrum (DMSO- d₆/2% D₂O, 35°C) of the Manβ1-4Glcβ1-1nOctyl product of egghead. Arabic numerals refer to ring protons of residues designated by standard three-letter monosaccharide nomenclature in the corresponding structure; P = product; S = substrate. Impurity peaks are marked by asterisks.
Reference List


Table I

Substrate specificities of Egghead β1-4-mannosyltransferase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Egghead&lt;sup&gt;a&lt;/sup&gt; nmol / h / mg</th>
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<tr>
<td></td>
<td>1 mM</td>
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<tr>
<td>Glcβ1-MeUmb&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<tr>
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<sup>a</sup> Enzyme sources were microsomal preparations of pVL-Egghead infected High Five™ cells (see “Experimental Procedures”). Background values obtained with microsomes of cells infected with an irrelevant construct (GalNAc-T4) were subtracted.

<sup>b</sup>; Bzl, benzyl; MeUmb, 4-methyl-umbelliferyl; Nph, nitrophenyl; ND, not determined.
Table II

$^1$H chemical shifts (ppm) and $^3$J$_{1,2}$ coupling constants (Hz, in parenthesis) for Glcβ1nOctyl substrate and biosynthetic Manβ4Glcβ1nOctyl product.

<table>
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<th>Glcβ1nOctyl$^a$</th>
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<tr>
<td></td>
<td>Manβ4</td>
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<td>H-1</td>
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<td>4.143</td>
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<tr>
<td>$^3$J$_{1,2}$</td>
<td>(~1)</td>
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<td>3.695</td>
<td>3.597</td>
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<td>H-8 (CH₃)</td>
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</table>

$^a$ Data were obtained in DMSO-$d_6$/2% D₂O at 35°C. Chemical shifts are referenced to internal TMS (set to 0.000 ppm).
Wandall et al. Fig.1.
Manβ1→4Glcβ1→1nOctyl (P) + Glcβ1→1nOctyl (S)
Drosophila egghead encodes a b1,4mannosyltransferase predicted to form the immediate precursor glycosphingolipid substrate for Brainiac
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J. Biol. Chem. published online November 25, 2002

Access the most updated version of this article at doi: 10.1074/jbc.C200619200

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