Glycolipid Intermembrane Transfer Is Accelerated by HET-C2, a Filamentous Fungus Gene Product Involved in the Cell−Cell Incompatibility Response[†]

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ABSTRACT: Among filamentous fungi capable of mycelial growth, *het* genes play crucial roles by regulating heterokaryon formation between different individuals. When fusion occurs between fungal mycelia that differ genetically at their *het* loci, the resulting heterokaryotic cells are quickly destroyed. It is unclear how *het* gene products of *Podospora anserina* trigger heterokaryon incompatibility. One unexplored possibility is that glycosphingolipids play a role because the *het-c2* gene encodes a protein that displays 32% sequence identity and an additional 30% similarity to the mammalian glycolipid transfer protein. Here, *P. anserina* protoplasts containing wild-type *het-c2* genes were shown to have greater glycosphingolipid transfer activity than protoplasts with disrupted *het-c2* genes, a condition previously linked to altered cell compatibility following hyphal fusion. The observed glycolipid transfer activity could not be accounted for by nonspecific lipid transfer protein activity. Direct assessment showed that purified, recombinant HET-C2 accelerates the intermembrane transfer of glycolipid in vitro, but that the HET-C2 activity is mitigated much less by negatively charged membranes than the mammalian glycolipid transfer protein. The findings are discussed within the context of HET-C2 being a member of an emerging family of ancestral sphingolipid transfer proteins that play important roles in cell proliferation and accelerated death.

Self−nonself recognition among somatic cells is a universally important process among eukaryotic cells. In fungi, somatic incompatibility is referred to as vegetative or heterokaryon incompatibility where it regulates hyphal fusion that permits the exchange of cytoplasm and nuclei during the assimilative phase of growth. Under conditions of vegetative incompatibility, the resulting heterokaryon undergoes a self-destructive process that leads to cell death (*1*−*3*). Vegetative incompatibility reduces the risks of transmission of infectious cytoplasmic elements, such as virus-like dsRNAs, and of exploitation by aggressive genotypes. In filamentous fungi such as *Podospora anserina*, genes at the *het* loci play a key role in controlling heterokaryon incompatibility. The *het* loci show evolutionary features that are in common with loci that mediate self/nonself recognition in other eukaryotes, such as the MHC complex in humans and the *S* locus in plants (*3*, *4*). *Het* gene products regulate the compatibility and stability of heterokaryons during hyphal fusion between different strains. Although *het* genes have been studied and characterized in *P. anserina* and *Neurospora crassa* (*5*−*10*), it is not clear how these genes and their products trigger vegetative incompatibility. Because of the observation that the *P. anserina het-c2* gene product, HET-C2[1] (208 a.a.),[2] displays 32% sequence identity and an additional 30% sequence similarity to the porcine glycolipid transfer protein [(GLTP); 209 a.a.][3] (*5*), one unexplored possibility is a role for glycosphingolipid interactions with *het* gene products.

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[1] Abbreviations: HET-C2, *Podospora anserina het-c2* gene product; GLTP, glycolipid transfer protein; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; AV-GalCer, *N*-[(11*E*)-12-(9-anthryl)-11-dodecenoyl]-1-*O*-*β*-galactosylsphingosine; AV−PC, 1-acyl-2-[(11*E*)-12-(9-anthryl)-11-dodecenoyl]-*sn*-glycero-3-phosphocholine; AV-SPM, *N*-[(11*E*)-12-(9-anthryl)-11-dodecenoyl]-sphingosine-1-phosphocholine; Per-TG, *rac*-1,2-di-oleoyl-3-[9-(3-perylenoyl)nonanoyl]glycerol; GalCer, galactosylceramide; GST, glutathione-*S*-transferase; FRET, fluorescence resonance energy transfer; AV, anthrylvinyl; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol.

[2] The nucleotide sequences for *P. anserina* HET-C2 (U05236), for bovine brain GLTP (AF209701 and NM016433), and for *Arabidopsis* ACD11 (AT2G34690) have been deposited in the GenBank database. The amino acid sequences can be accessed through the NCBI Protein Database (accession nos. AAA20542 and S59950 for HET-C2, AAF33207 and P17403 for bovine GLTP, and AT2G34690 and T05004 for ACD11).

[3] Compare GenBank database deposits for bovine, porcine, mouse, and human GLTPs (AF209701, AF209702, AF209703, and AF209704, respectively).

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Like other eukaryotic cells (*11*), filamentous fungi (multicellular) and unicellular yeasts possess a variety of glycosphingolipids in their membranes (*12*). These glycosphingolipids and associated metabolites participate in important cell functions such as regulation of calcium homeostasis, signal transduction associated with cell stress and death, and dimorphism associated with pathogenic infectivity of host cells (*13*−*15*). Fungi have two types of membrane sphingolipids. In one sphingolipid type, ceramide is linked to inositol phosphate (inositolphosphoceramides), whereas, in the other type, ceramide is linked to glucose or galactose to form monoglycosylceramides (*12*−*16*). The ability of such glycolipids to interact with *het* gene products has not been evaluated even though monoglycosylsphingolipids are known to be good "substrates" for GLTPs purified from various mammalian tissues (*17*−*22*). GLTPs facilitate the in vitro transfer of various glycolipids between membranes and are specific for glycolipids with sugar residues attached via β-linkages to the lipid hydrocarbon backbone (*19*−*21*). However, sequence similarity, such as that observed between HET-C2 and GLTP, does not guarantee functional homology. An *Arabidopsis* HET-C2/GLTP homolog, recently identified by Clustal W sequence alignment and involved in accelerated cell death triggered by avirulent stress (*23*), was not able to accelerate the intermembrane transfer of monoglycosylceramides, despite the presence of such glycolipids in plant cells (*24*).

Accordingly, the goals here were (a) to determine whether mycelial fungi with the capacity to undergo hyphal fusion (e.g., *P. anserina*) possess proteins that accelerate the intermembrane transfer of monoglycosylsphingolipids; (b) to assess the potential role of the *het-c2* gene product in such a glycolipid transfer process; and (c) to identify structural and functional similarities and differences between HET-C2 and GLTP. The findings are discussed within the context of the *het-c2* gene product being a member of an emerging family of ancestral sphingolipid transfer proteins that are likely to play important roles in cell proliferation and accelerated cell death.

EXPERIMENTAL PROCEDURES

Materials. 1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids (Alabaster, AL). *N*-[(11*E*)-12-(9-anthryl)-11-dodecenoyl]-1-*O*-β-galactosylsphingosine (AV-GalCer), *N*-[(11*E*)-12-(9-anthryl)-11-dodecenoyl]-sphingosine-1-phosphocholine (AV-SPM), 1-acyl-2-[(11*E*)-12-(9-anthryl)-11-dodecenoyl]-*sn*-glycero-3-phosphocholine (AV-PC), and *rac*-1,2-di-oleoyl-3-[9-(3-perylenoyl)nonanoyl]glycerol (Per-TG) were synthesized as described previously (*22*, *25*). [^{3}H]-bovine brain GalCer, labeled at carbon 6 or galactose, was prepared using galactose oxidase and tritiated sodium borohydride (*26*−*28*).

Glycolipid Transfer Activity. Two different assays were used to monitor the GLTP-mediated glycolipid transfer between membranes. A fluorescence resonance energy transfer (FRET) assay utilizing anthrylvinyl (AV) labeled glycolipid (1 mol %) and a nontransferable perylenoyl-labeled triglyceride (1.5 mol %) permitted continuous real time monitoring of GLTP activity (*22*, *29*, *30*). POPC donor vesicles containing the fluorescent lipids and the indicated amount of negatively charged phosphoglyceride were prepared by rapid ethanol injection as described previously (*29*). POPC acceptor vesicles were prepared by sonication. Calculation of the transfer rate was achieved by fitting to first order exponential behavior as previously described in detail (*22*, *29*, *30*).

Glycolipid transfer activity also was monitored using radiolabeled glycolipids. POPC donor vesicles containing 1 mol % [^{3}H]GalCer, 10 mol % negatively charged dipalmitoyl phosphatidic acid (DPPA), and a trace of nonexchangeable [^{14}C]tripalmitate were prepared by sonication in 10 mM sodium phosphate (pH 7.4), 1 mM DTT, 1 mM EDTA, and 0.02% NaN_3. POPC sonicated vesicles served as acceptor membranes. Following incubation with protein, the charged donor and neutral acceptor vesicles were separated by rapid elution over DEAE Sephacel minicolumns (*21*, *28*, *29*).

Preparation of Soluble Protein Extracts from P. anserina Protoplasts and Ascospores. Disruption of the *het-c2* gene was achieved as described previously (*5*). Briefly, the pCura plasmid containing the *het-c2* gene was disrupted in vitro by insertion of the *ura5* gene of *P. anserina* at the *Eco*RI site of the *het-c2* allele. To avoid expression of a truncated HET-C2, about 500 bp at the 5′ end of the *het-c2* gene were deleted from the pCura construct by digesting with *Cla*I and then with *Bal*-31. The resulting deletion overlapped the ATG start codon. This pCuraΔ construct was then used to transform a *het-c2 ura5*−*6* strain. Among 750 prototrophic transformants, one had lost the het-c2 phenotype and was compatible with strains containing the different *het-e* and *het-d* alleles. Disruption of the *het-c* locus in this Δhet-c2 strain was confirmed by Southern blot analysis. The lack of *het-c2* mRNA and of HET-C2 protein expression was confirmed by Northern and Western blot analyses, respectively.

Protoplasts were prepared and transformed as described previously (*31*). To recover the protoplast soluble protein fraction, cells were adjusted to 10^9 cells/mL in 0.1 M Tris-HCl (pH 7.5), 0.8 M sorbitol, centrifuged at 5000 rpm at 4 °C for 5 min, and resuspended in sodium phosphate (pH 7.4) containing 1 mM DTT, 1 mM EDTA, and 0.02% NaN_3. The cell suspension was sonicated with a Heat Systems-Ultrasonics probe sonifier three times for 30 s on ice. The lysed protoplasts were centrifuged at 4 °C (10 000 rpm for 10 min), and the supernatant containing the soluble protein was collected. To recover the ascospore soluble protein fraction, ascospores from 100 bunches of asci were lyophilized for 4 h and were broken by vigorously vortexing three times for 30 s together with glass beads (0.4−0.5 mm diameter) in sodium phosphate buffer. The suspension, without glass beads, was then sonicated three times for 30 s on ice using the probe sonifier. After centrifugation at 4 °C (10 000 rpm for 10 min), the supernatant was collected. The protein concentrations of the soluble fractions were determined using the Lowry method (*32*).

S. cerevisiae Spheroplast Production. Following inoculation of 2% lactate medium (pH 5.5), yeast were grown at 30 °C to an O. D. 0.3−0.6 at 600 nm, harvested by centrifugation at 4 °C, washed once with H_2O followed by 100 mM $TrisSO_4$ (pH 9.4) containing 10 mM DTT. Cells were washed and resuspended in 20 mM potassium phosphate (pH 7.4) containing 1.2 M sorbitol (SB) and incubated with gentle shaking for 1 h at 30 °C with zymolase (5 mg/g of wet cells). The resulting spheroplasts were harvested by

centrifugation, washed twice with SB, and resuspended in 20 mM HEPES (pH 7.4) containing 0.6 M mannitol and 1 mM PMSF prior to homogenizing at 4 °C with a Dounce homogenizer. The soluble protein was collected by centrifugation at 10 000 rpm for 10 min (4 °C).

Subcloning, Expression, and Affinity-Purification of HET-C2. The *het-c2* gene product, HET-C2, was previously cloned and expressed as a GST fusion protein in *Escherichia coli* using the pGEX-5X-1 expression vector (*5*). To facilitate purification, the *het-c2* cDNA open reading frame was subcloned into a pGEX-6P-1 vector using *Sma* I and *Not* I restriction enzymes (New England Biolabs). The GST cleavage site in pGEX-6P-1 is designed specifically for the PreScission protease, which itself is GST-fusion protein. Following cleavage of the GST-HET-C2, affinity purification removed not only GST and uncleaved GST-HET-C2, but also the PreScission protease, from the HET-C2. Recombinant bovine GLTP also was cloned and expressed as a GST-GLTP fusion protein in similar fashion (*18*).

SDS−PAGE and Isoelectric Focusing. Recombinant proteins were analyzed by electrophoresis under reducing conditions on 16% polyacrylamide gels containing 0.1% SDS and stained with Coomassie Brilliant Blue (*18*, *28*). Isoelectric focusing was carried out using the Pharmacia PhaseGel IEF system (Amersham Biosciences). The PhastGels IEF gels, with a pH range of 3−9, were run using PhastSystem SDS buffer strips according to the manufacturers instructions. The salt concentration of each sample was less than 0.20 M. The isoelectric points of HET-C2 and GLTP were determined by comparing their migration patterns with those of Pharmacia IEF standard proteins after Coomassie staining.

RESULTS

P. anserina Protoplasts, but not Ascospores, Possess Glycolipid Intervesicular Transfer Activity. To determine whether glycolipid transfer activity is present in *P. anserina* protoplasts and ascospores containing either the wild-type or a disrupted *het-c2* gene, soluble protein extracts were prepared and glycolipid intervesicular transfer rates were measured using a well-established fluorescence resonance energy transfer (FRET) approach (*22*, *29*, *30*). Figure 1A illustrates the general structures and the excitation−emission properties of the anthrylvinyl-labeled monoglycosylceramide (energy donors) and the 3-perylenoyl-labeled triglyceride (energy acceptor) used to assess glycolipid transfer activity. As the fluorescent monoglycosylceramide is transferred out of donor membrane vesicles that also contain nontransferable triglyceride labeled with the 3-perylenoyl fluorophore, the emission intensity of the glycolipid fluorophore at 425 nm increases as a function of time due a loss of resonance energy transfer as the two fluorphores become spatially separated (Figure 1B). The FRET approach permits kinetic assessment of the lipid transfer process (for details, see refs *22*, *29*, *30*). Figure 2 shows the calculated lipid transfer rates obtained by fitting the kinetic data to first order exponential behavior, as illustrated in Figure 1C. As illustrated in Figure 2B, significantly faster glycolipid intervesicular transfer rates were observed for protoplasts containing the wild-type *het-c2* gene (het-c2 strain) compared to protoplasts with a disrupted *het-c2* gene (Δhet-c strain). It is noteworthy, however, that protoplasts with the disrupted *het-c2* gene still

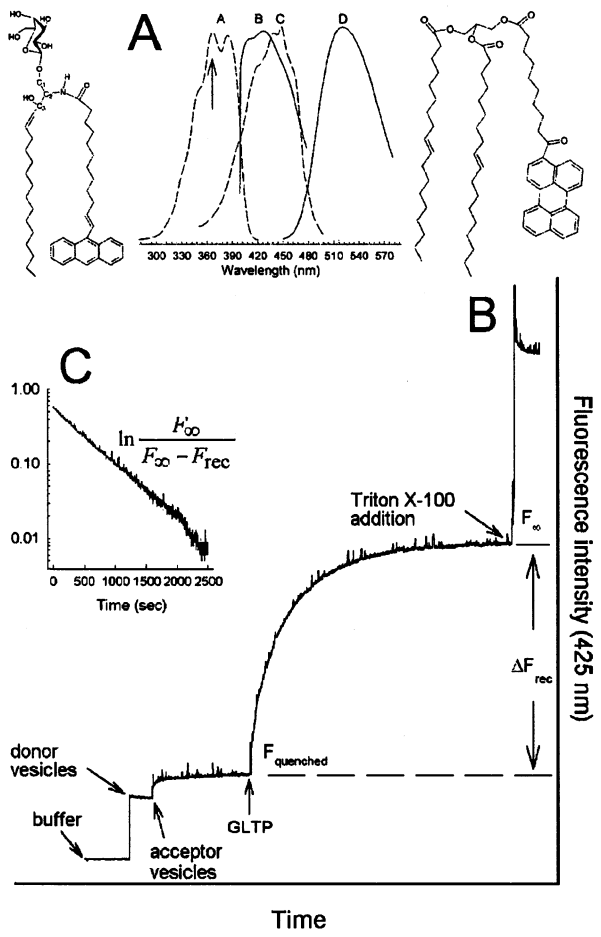

FIGURE 1: General structures and excitation/emission properties of fluorescent lipids and a typical response observed during glycolipid intervesicular transfer. Panel A shows the fluorescent monoglycosylceramide with attached anthrylvinyl group (upper left) and associated excitation (A) and emission (B) spectra, and the fluorescent triglyceride with the attached 3-perylenoyl group (upper right) and associated excitation (C) and emission (D) spectra. Panel B shows a typical response observed as the emission signal of the fluorescent glycolipid is recovered (ΔF_{rec}) when it is selectively transferred out of a donor vesicle that also contains the nontransferable fluorescent triglyceride. The kinetic response is first-order permitting a rate constant to be calculated, as shown in Panel C and described in detail in refs *22*, *29*, and *30*.

showed significantly higher glycolipid transfer activity than ascospores (Figure 2, panel B vs C). Only a trace of glycolipid transfer activity was detectable in ascospores even when the *het-c2* gene was not disrupted. Control experiments showed the activity in ascospores to be similar to the extremely slow spontaneous intervesicular transfer rate of the fluorescent glycolipid (AV−GalCer) observed in the absence of protein (Figure 2A). The results indicated that glycolipid intervesicular transfer activity is present in *P. anserina* protoplasts, but not in ascospores, and that disruption of the *het-c2* gene significantly lowers the glycolipid intervesicular transfer activity observed in protoplasts.

To determine whether the glycolipid intervesicular transfer activity observed in the Δhet-c protoplasts is due to a nonspecific lipid transfer protein (*33*−*35*) rather than a glycolipid-specific lipid transfer protein, additional lipid transfer assays were carried out using fluorescent phosphatidylcholine (AV−PC) instead of the fluorescent glycolipid. As shown in Figure 2E,F, similar levels of PC intervesicular

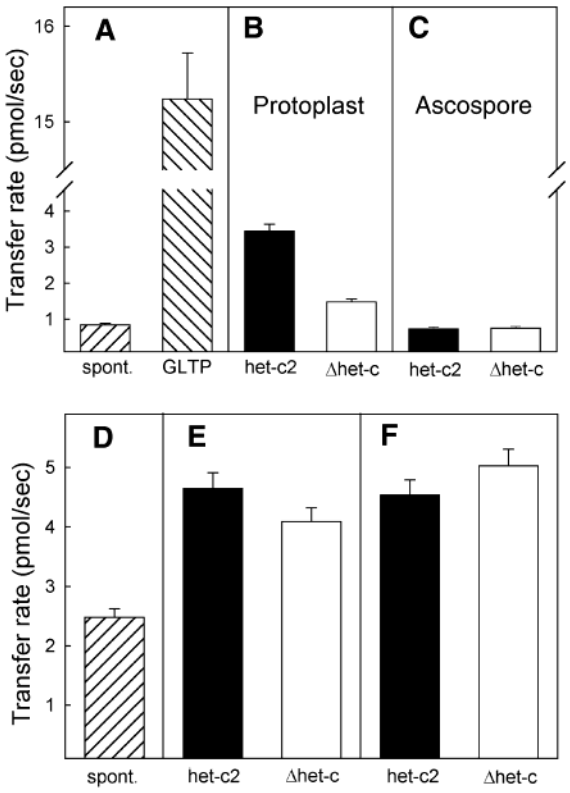

FIGURE 2: Glycolipid and phosphatidylcholine transfer activities of *P. anserina* protoplasts and ascospores expressing either wild-type or disrupted *het-c2* genes. A total of 10 μg of soluble protein extract was used in each experiment and values represent averages of three or more different experiments. het-c2, wild-type *het-c2*; Δhet-c, disrupted *het-c2* gene. Panels A−C. Glycolipid intermembrane transfer rates determined using fluorescent monoglycosylceramide (AV-GalCer) as "substrate". Spontaneous transfer (no protein) and GLTP-mediated transfer (10 μg) of AV-GalCer between neutral POPC donor and acceptor vesicles served as negative and positive controls, respectively (panel A). Panels D−F. Phosphatidylcholine intermembrane transfer rates determined using fluorescent phosphatidylcholine (AV-PC) as "substrate". Spontaneous transfer (no protein) of AV-PC between neutral POPC donor and acceptor vesicles served as a negative control, respectively (panel D).

transfer activity were observed in both protoplasts and ascospores. These levels of activity were significantly higher than the spontaneous intervesicular transfer rates observed for PC in the absence of protein (Figure 2D). More importantly, disruption of the *het-c2* gene did not alter the protein-mediated intervesicular transfer activities observed for PC (Figure 2E,F). The preceding results suggested that *P. anserina* protoplasts and ascospores contain either a PC-specific transfer protein or a nonspecific lipid transfer protein (*33*−*35*) that functions independently of the *het-c2* gene. The very similar level of PC transfer activity in the wild-type and the Δhet-c protoplasts and ascospores which displayed significantly different glycolipid transfer activities further suggested that the *het-c2* gene product, HET-C2, plays a significant role in the glycolipid transfer activity observed in the protoplasts.

Saccharomyces cerevisiae lacks glycolipid transfer activity, but not PC transfer activity. Because of the well-established role of the *het-c2* gene in the cell−cell incompatibility response of filamentous fungi, it was germane to determine whether unicellular fungi, that do not exhibit mycelial growth and hyphal fusion (e.g., *S. cerevisiae*),

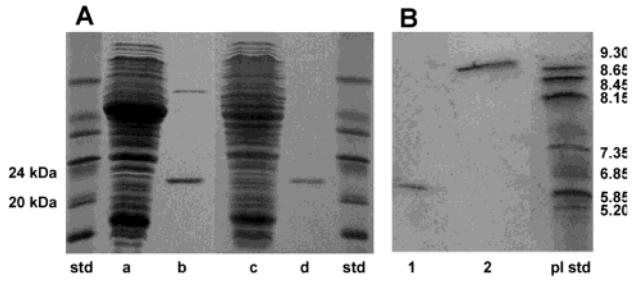

FIGURE 3: Panel A. SDS−polyacrylamide gel electrophoresis of HET-C2 and GLTP. Proteins were stained using Coomassie Brilliant Blue. Lane a, sonicated *E. coli* cells expressing GST-HET-C2 fusion protein; lane b, affinity-purified recombinant HET-C2 (~23.2 kDa); The thin upper band is due to "finger protein" (keratin) introduced when loading the gel. Lane c, sonicated *E. coli* cells expressing GST-GLTP fusion protein; lane d, affinity-purified recombinant GLTP. Panel B. Isoelectric focusing gel electrophoresis of HET-C2 and GLTP under nondenaturing conditions. Lane 1, HET-C2; lane 2, GLTP; lane 3, Pharmacia IEF standards.

possess glycolipid intervesicular transfer activity. Spheroplasts were produced and the 100000*g* supernatant was analyzed for glycolipid intervesicular transfer activity. No such activity was detected, regardless of whether galactosylceramide or glucosylceramide was used as a "substrate", even though both of these monoglycosylceramides are present in *S. cerevisiae* (*12*). Addition of the 100000*g* yeast supernatant did not inhibit the activity of control GLTP, suggesting that no endogenous inhibitors were present in the *S. cerevisiae* spheroplast supernatant. In agreement with previous reports (*33*, *35*, *36*), abundant PC transfer activity was detected.

HET-C2 Mediates Glycolipid Intervesicular Transfer. To directly assess whether HET-C2 was capable of accelerating the intermembrane transfer of monoglycosylceramides, recombinant HET-C2 was produced by subcloning the HET-C2 ORF into pGEX-6P-1 vector, heterologously expressing in *E. coli* as a GST-HET-C2 fusion protein, and then purifying as described in the Experimental Procedures. As shown in Figure 3A, SDS−PAGE analysis under reducing conditions (*18*, *28*) revealed the expected migration of HET-C2, which was similar to that of GLTP.

The capacity of HET-C2 to stimulate the intermembrane transfer of monoglycosylceramides was then monitored in real time using the FRET lipid transfer assay (*22*). Figure 4A shows the results obtained when fluorescent monoglycosylceramide (AV−GalCer) served as a "substrate" for HET-C2. Significant intervesicular transfer of the fluorescent glycolipid was observed [Figure 4A, trace b] relative to positive and negative controls that contained either GLTP [Figure 4A, trace a] or no protein at all [Figure 4A, trace c/d]. Including BSA (0.1 mg/mL), which has nonspecific lipid binding sites, did not affect the HET-C2 mediated transfer of AV-GalCer (data not shown). The HET-C2 transfer rate of AV-GalCer was more than 25-fold faster than the spontaneous transfer rate observed in the absence of protein. The extremely slow spontaneous intervesicular transfer of monoglycosylceramides is well-established (*37*).

To determine whether HET-C2 was selective for glycolipids, the fluorescent monoglycosylceramide in the donor vesicles was replaced with other fluorescent lipids. HET-C2 did not significantly accelerate the intervesicular transfers of sphingomyelin, phosphatidylcholine, or cholesterol above

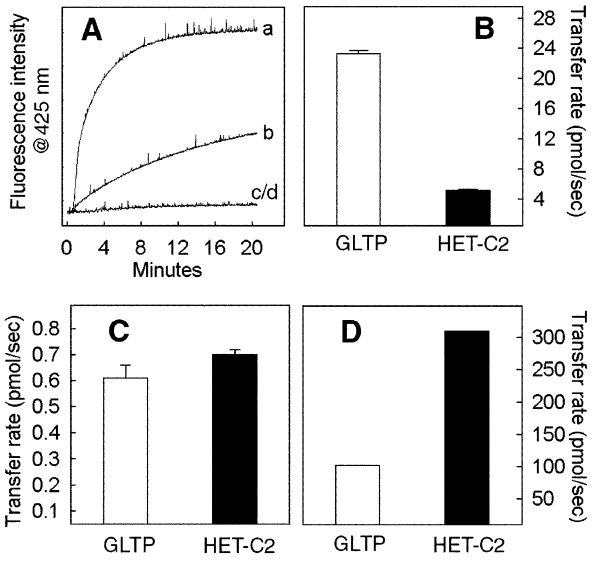

FIGURE 4: HET-C2- and GLTP-mediated glycolipid transfer of purified recombinant proteins as function of donor vesicle charge. Panel A. Monitoring of intervesicular lipid transfer as a function of time using 14.5 nM protein and POPC vesicles (λ_{ex} 370 nm, λ_{em} 425 nm); Trace a, GLTP-mediated transfer of AV-GalCer; Trace b, HET-C2 mediated transfer of AV-GalCer; Trace c/d, represents either spontaneous (no protein) transfer of AV-GalCer or HET-C2 mediated transfer of AV-SPM which yielded very similar kinetic responses. Panel B. Initial transfer rates of AV-GalCer mediated by GLTP or by HET-C2. Donor vesicles consisted of POPC and transfer rates (pmoles of glycolipid transferred per sec) were calculated as described in the Experimental Procedures. Panel C. Initial transfer rates of AV-GalCer for HET-C2 and GLTP (14.5 nM protein) from negatively charged donor vesicles containing 5 mol % POPG. Panel D. Initial transfer of [^{3}H-GalCer] GalCer for HET-C2 and GLTP (86.5 nM protein) from POPC donor vesicles containing 10% DPPA using the vesicle-ion exchange assay in which the total assay volume is 0.5 mL. Values are averages ± SD of at least three different experiments.

the spontaneous lipid transfer rates measured in the absence of protein. Data in Figure 4A (trace c/d) illustrate a typical response for fluorescent sphingomyelin (AV−SPM) which was similar to having no protein present.

To verify that the fluorescent probes did not significantly affect the results, we also used a well-established radioactive assay, in which donor vesicles contained tritiated glycolipid and a negatively charged phospholipid to enable separation from neutral acceptor vesicles by ion exchange chromatography (*21*, *28*, *29*). Tritiated monoglycosylceramide, such as [^{3}H]GalCer, was readily transferred. However, HET-C2 did not transfer [^{3}H]ceramide and only very slightly transferred [^{3}H]SPM. A complex glycolipid, [^{3}H] ganglioside GM1, also was readily transferred by HET-C2 (data not shown).

Effect of Negatively Charged Membranes on HET-C2 Glycolipid Transfer Activity. We previously showed that the transfer rate of AV-GalCer mediated by GLTP is sensitive to the membrane charge, probably due to electrostatic interaction between GLTP and the membrane interface (*29*). To determine to what extent negatively charged membranes modulate HET-C2 glycolipid transfer activity, we analyzed HET-C2's ability to facilitate AV-GalCer transfer from negatively charged donor vesicles. Figure 4C shows the effect of including 5 mol % POPG in the small unilamellar donor vesicles containing AV-GalCer. The HET-C2-mediated transfer of AV-GalCer was reduced about 6-fold compared to donors lacking the POPG (Figure 4, panel C vs B). However, the 6-fold reduction of HET-C2 mediated transfer activity was moderate compared to the 35-fold reduction in the transfer rate of AV-GalCer mediated by GLTP with donors containing 5 mol % POPG (Figure 4, panel C vs B). The end result was similar transfer rates (0.6−0.7 pmol/s of AV-GalCer) for HET-C2 and GLTP with the 5 mol % POPG donor vesicles (Figure 4C).

The effect of a different negatively charged lipid, DPPA, also was investigated because DPPA was routinely included in the radiolabeled lipid transfer assay. Figure 4D shows that a 3-fold faster transfer rate of [^{3}H]GalCer was observed with HET-C2 compared to GLTP when the donor vesicles contained 10% DPPA. The FRET lipid transfer assay gave a similar result, in that a 2-fold faster transfer rate of AV-GalCer was observed with HET-C2 compared to GLTP when the donor vesicles contained 10% DPPA (data not shown).

To show that HET-C2 was mediating glycolipid transfer and not fusion of donor and acceptor vesicles, control experiments were performed using the radiolabeled lipid transfer assay. Less than 5% of the [^{14}C]tripalmitin nontransferable marker in the negatively charged donor vesicles was recovered with acceptors following elution through the DEAE minicolumns. On the other hand, the tritiated material that did coelute with the acceptor vesicles behaved identically to authentic GalCer standard when analyzed by thin-layer chromatography ($CHCl_3/CH_3OH/H_2O$, 85:15:1.5 v/v). Thus, [^{3}H]GalCer redistribution was not due to enzymatic alteration of the glycolipid to water-soluble or spontaneously mobile radioactive products. Rather, the glycolipid itself was transferred between bilayer vesicles by HET-C2.

Isoelectric Focusing and Hydropathy Analysis. Because of the strikingly different response of HET-C2 and GLTP to membranes containing negatively charged phospholipid, the isoelectric points (pI) and hydropathy plots of the two recombinant lipid transfer proteins were determined. Experimental verification of the pIs was necessary because of differences noted in the experimental and theoretical pIs of wild-type GLTP. A pI near 9.0 was determined previously for bovine GLTP by FPLC chromatofocusing under nondenaturing conditions (*28*, *42*), whereas, theoretical predictions based on GLTP amino acid composition yielded a pI value of 7.68. Using the Pharmacia PhastSystem PhastGel IEF (Figure 3B), a pI value near 9.0 was found for recombinant bovine GLTP. In contrast, the pI of recombinant HET-C2 was found to be near 6.0 under nondenaturing conditions. This pI value of 6.0 agreed closely with a 5.9 value determined previously under denaturing conditions[4] and was slightly lower than the theoretical value of 6.34, predicted from HET-C2 amino acid composition.

To gain further insights into HET-C2 structure, the relative hydropathy of the amino acid sequence was analyzed by the Kyte-Doolittle approach (*38*). HET-C2 contained hydrophobic segments at its N-terminus, near amino acids 40−65, 110−130, 145−165, and near its C-terminus (Figure 5, solid black line). Except for the relatively hydrophobic N-terminus of HET-C2, the hydropathy pattern was mirrored in GLTP (Figure 5, dotted line), but with a 10−20 a.a. upstream shift that resulted in the hydrophobic segments being near amino

[4] Turcq et al., unpublished observations.

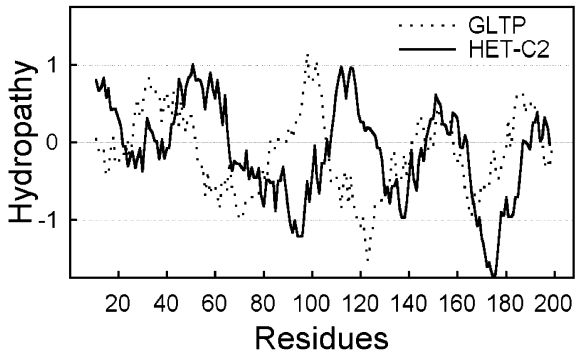

FIGURE 5: Hydropathy analysis of HET-C2 and GLTP. The Kyte and Doolittle approach (*38*) was used with an average window size of 19 amino acid residues plotted at one-residue intervals. Increasing hydrophobicity is indicated by an increasing positive value. HET-C2 (solid line); GLTP (dotted line).

acids 25−50, 90−110, 140−160, and 180−195. Also, a very polar region (a.a. 165−180), that occurred near the C-terminus of HET-C2, was nearer the midpoint of GLTP's sequence (a.a. 115−125).

DISCUSSION

HET-C2 Glycosphingolipid Transfer Activity. We have shown that the HET-C2 protein encoded by the *het-c2* gene in the filamentous fungus, *P. anserina*, has the ability to transfer glycosphingolipids between membranes in vitro. HET-C2 did not accelerate the intervesicular transfer of nonglycolipid "substrates" such as sphingomyelin, phosphatidylcholine, or cholesterol. A similar selectivity of bovine and porcine GLTPs for glycolipids is well-established (*19*−*21*). Although the net transfer rate displayed by HET-C2 toward monoglycosylceramides embedded in zwitterionic phosphoglyceride membranes is about 5-fold slower than that of mammalian GLTP, it is important to note that the glycolipid transfer rate mediated by HET-C2 represents at least a 25-fold acceleration over the spontaneous intervesicular transfer rate observed in the absence of protein. Moreover, glycolipid transfer mediated by HET-C2 is much less affected by negatively charged membranes than is bovine GLTP, which shows a dramatically reduced transfer capability when the monoglycosylceramide is embedded in a negatively charged phosphoglyceride membrane (*29*). This behavior appears to be related to the different isoelectric points determined for HET-C2 and GLTP. At neutral pH, HET-C2 (pI $\cong$ 6.0) is expected to show only a modest attraction for negatively charged vesicles, whereas GLTP (pI $\cong$ 9.0), with a net positive charge, is expected to display a stronger affinity for negatively charged donor membranes (*29*). Interestingly, although the presence of negatively charged phosphoglyceride slowed the HET-C2-mediated GalCer transfer rate much less than that of GLTP, the HET-C2-mediated glycolipid transfer was more strongly affected than GLTP by the type of anionic phosphoglyceride (e.g., POPG vs DPPA). We previously observed 20−25% greater reductions in the GLTP-mediated transfer rate of GalCer from donor vesicles having POPG (10 mol %) instead of DPPA (*29*). With HET-C2, much greater differences were observed between POPG and DPPA suggesting that the differing pIs of the two proteins cannot entirely account for the experimentally observed behavior.

Hydropathy analyses provided interesting initial insights into the structural features HET-C2 compared to GLTP (Figure 5). Although a similar pattern of peaks and valleys is evident in the hydropathy plots, a downstream displacement of about 10−20 amino acids occurs in HET-C2 compared to GLTP. The most striking differences occurred in three regions: (i) at the N-termini, where HET-C2 shows increased hydrophobicity, (ii) near the middle, where GLTP's polarity is highest (a.a. 115−130), and (iii) approaching the C-terminus, where the HET-C2 shows a very polar region (a.a. 165−180). These structural differences may play a role in the mitigated response of HET-C2 to negatively charged membranes. Clearly, further studies of HET-C2 and GLTP protein structure will be required to fully characterize the similarities and differences described here.

Physiological Implications. It is well established that *het* genes are highly involved in the growth properties and life cycle of filamentous fungi such as *Podospora anserina* where they participate in apical growth and ascospore formation. Our data suggest that the abnormal ascospore formation, previously observed when the *het-c2* gene is mutationally inactivated (*5*), is not directly linked to the glycosphingolipid transferring capability of HET-C2. Neither control cells nor *P. anserina* ascospores containing the disrupted *het-c2* gene displayed significant glycosphingolipid transfer activity in their cytosols, even though similar basal levels of phosphatidylcholine transfer activity were evident in both the controls and *het-c2* knockouts of the ascospores and protoplasts. In contrast, our data do suggest a link between the glycosphingolipid transferring capability of HET-C2 and the vegetative incompatibility response. Establishing that HET-C2 can selectively interact with monoglycosphingolipids embedded in phosphoglyceride membranes represents a potentially significant and intriguing step forward because of the well-established and widespread role that sphingolipid metabolites, such as ceramide, play in signaling processes linked to the stress response and to programmed cell death (*39*). In *P. anserina*, when fusion occurs between fungal individuals that differ genetically at their *het* loci, the resulting heterokaryotic cell is quickly destroyed in a lytic response. It is clear that *het-c* is not the only *het* gene involved in this response. The *het-e* gene, which encodes a polypeptide with a GTP binding site and WD40 repeats, appears to act antagonistically to the *het-c* gene. HET-E protein binds GTP in vitro, and mutations that abolish binding or affect WD40 repeats abrogate the incompatibility response (*40*). The GTP-binding property of HET-E suggests that it might play a role in signal transduction events. Recent data strongly suggest that regulation of HET-C and HET-E may involve formation of a protein complex (*41*). Taken together, a hypothetical scenario begins to emerge linking the interaction of the *het* gene products to a signaling system in which HET-C2 may act as a glycolipid sensor or local transporter involved in regulating the metabolic pool(s) of sphingolipid metabolites, which in turn participate in the stress and lytic responses linked to cell−cell interactions. Support for such a scenario is strengthened when the following observations are considered.

Saccharomyces cerevisiae, which exists in a unicellular state and exhibits neither mycelial growth nor hyphal fusion, shows no capacity to transfer monoglycosylceramides between membrane vesicles. On-line searches of the yeast genome databases provide no evidence for HET-C2, GLTP,

or related homologues in *S. cerevisiae*. These findings suggest that a transfer protein with selectivity for monoglycosylsphingolipids and sequence similarities to HET-C2 or GLTP is likely not to be present in *S. cerevisiae*.

It is also noteworthy that a HET-C2/GLTP homologue has recently been identified that significantly affects the stress and programmed cell death response in plants. The lethal, recessive *acd11* knockout in *Arabidopsis* exhibits cell death characteristics analogous to animal cell apoptosis, and constitutively expresses defense-related genes that accompany the hypersensitive response normally triggered by avirulent pathogens (*23*). This ACD11 null phenotype is caused by deletion of the *acd11* gene, encoding a protein homologous to the mammalian GLTP and to HET-C2. Alignment of the ACD11 amino acid sequence[2] with either the HET-C2 sequence or the bovine GLTP sequence using the Lalign algorithm shows 29 and 28% identity as well as 24 and 28% similarity, with 272 amino acid overlap. ACD11 accelerates the intermembrane transfer of sphingolipids in vitro. However, instead of glycosphingolipids, sphingosine appears to be a preferred "substrate". Cell death in the *acd11* knockout does not appear to proceed via the senescence-related pathway, but occurs via a programmed cell death pathway that is coordinated with the activation of defense responses (*23*). Although the in vivo function of ACD11 remains unclear, the in vitro transfer of sphingosine by ACD11 provides a potentially important link between ACD11 function and a key sphingolipid metabolite in the generation of related sphingolipid signals that are directly involved in eukaryotic apoptotic responses. Because of their homology to GLTP and analogous in vitro transfer activities, linking ACD11 and HET-C2 to the cell death response of multicellular eukaryotes is an exciting development and an important step in shedding light on the physiological role of the emerging family of sphingolipid transfer proteins.

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