Phg1p Is a Nine-transmembrane Protein Superfamily Member Involved in Dictyostelium Adhesion and Phagocytosis*

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Phagocytosis is the process by which cells internalize large particles (typically >1 μm diameter), such as bacteria or cell debris. In higher eucaryotes, phagocytic cells are essential players of the host defense against invading pathogens and tissue remodeling (for review see Ref. 1). Phagocytosis involves adhesion of the phagocytic cell to the particle, and reorganization of the actin cytoskeleton to allow engulfment. A number of receptors required for the recognition of particles to be phagocytosed have been identified in mammalian cells, e.g., Fc receptors involved in the phagocytosis of opsonized particles (2). These receptors presumably transduce a local activation signal upon recognition of their ligand, leading to reorganization of the actin cytoskeleton. Protein kinases such as Syk (3) as well as GTP-binding proteins of the Rho family (4) have been implicated in the transduction of the activation signal.

To identify the molecular mechanisms involved in phagocytosis, we generated random insertion mutants of Dictyostelium discoideum and selected two mutants defective for phagocytosis. Both represented insertions in the same gene, named PHG1. This gene encodes a polytopic membrane protein with an N-terminal luminal domain and nine potential transmembrane segments. Homologous genes can be identified in many species; however, their function is yet to be elucidated. Disruption of PHG1 caused a selective defect in phagocytosis of latex beads and Escherichia coli, but not Klebsiella aerogenes bacteria. This defect in phagocytosis was caused by a decrease in the adhesion of mutant cells to phagocytosed particles. These results indicate that the Phg1 protein is involved in the adhesion of Dictyostelium to various substrates, a crucial event of phagocytosis and demonstrate the usefulness of a genetic approach to dissect the molecular events involved in the phagocytic process.

EXPERIMENTAL PROCEDURES

Cell Culture and Internalization Assays—Wild-type cells used in this study are DH1–10 cells, a subclone of DH1 cells. They were grown at 21 °C in HL5 medium (8) and subcultured twice a week. Cells were typically not allowed to reach a density of more than 2 × 10^6 cells/ml.

To obtain rhodamine-labeled bacteria, an overnight culture was centrifuged and resuspended in PBS. Bacteria were boiled 30 min in a water bath under mild stirring conditions, and then washed four times with PBS and once with SB (2 mM NaHPO₄, 14.7 mM KH₂PO₄, pH 6.0). Cells were then resuspended in a solution of 50 mM NaHPO₄, pH 9.2, 5 mg of rhodamine-isothiocyanate (ICN Biomedicals Inc.) at 2 × 10⁶ cells/ml and incubated for 30 min under mild agitation. They were then washed twice in SB plus 40 mM NH₄Cl, twice in SB, and frozen in aliquots.

To assess internalization, 10⁶ cells were transferred in 1 ml of fresh HL5 medium containing: 1 μl of 1 μM diameter fluorescein isothiocyanate (FITC) dextran (Molecular Probes, Eugene, OR), or 5 μg/ml FITC-dextran (Molecular Probes, Eugene, OR), or 5 × 10⁵ rhodamine-labeled bacteria. The cells were incubated with or without shaking (200 rpm) for 1 h, then washed twice with ice-cold HL5 and analyzed using a fluorescence spectrophotometer (FACScan, Beckton Dickinson, San Jose, CA).

Isolation of Phagocytosis Mutants—Cells were transformed with the pUCBrΔBamHI vector by the restriction enzyme-mediated integration procedure essentially as described (9–11). Briefly, cells were washed once in sterile ice-cold electroporation buffer (10 mM NaPO₄, pH 6.1, 50 mM sucrose), mixed with 10 μg of BamHI-linearized vector and 10 units of DpnI restriction enzyme and electroporated using a Bio-Rad Gene

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Pulser (0.4-cm cuvettes, 1 kV, 3 microfarads), and then resuspended in 30 ml of HL5 medium. Bacterial S hydrochloride (10 μg/ml; ICN Biomedicals Inc., Aurora, OH) was added 24 h later. After 10 days of selection, cells were grouped into five pools, incubated with fluorescent latex beads in HL5 medium for 1 h, and the cells having phagocytosed no beads were washed once in a fluorescent-associated scintillator (FACS; FACStar plus). Cells were subjected to a second round of selection 7 days later and cloned in 96-well plates after the FACS. Finally, individual clones were restested for their ability to phagocytose fluorescent latex beads. Forty-seven individual clones were identified as deficient for phagocytosis. Three of them corresponded to phg1-1 mutants, and one clone was named phg1-2 (17).

Genomic DNA was extracted as described (12), digested with various enzymes, and analyzed by Southern blot as described previously (9), using radiolabeled pUCBsrABamHI as a probe. Genomic DNA from phg1 mutants (4 μg) was digested with ClaI, precipitated, and resuspended in 100 μl of distilled H2O. Fifteen microliters of DNA were then ligated overnight at 16 °C in a final volume of 100 μl, in the presence of 400 units of ligase (New England Biolabs, Beverly, MA). Ligation products were precipitated and used to transform DH10B bacteria by electroporation. Plasmids recovered by plasmid rescue of phg1 mutants (10 μg) were linearized with ClaI and electroporated into DH1-10 cells (see above). After bacterial lysis, the insert of plasmid at the homologous site in the genome was confirmed by Southern blot. Disruption mutants were obtained as described in a separate publication (17). Briefly, cells were spread evenly on a glass plate and allowed to settle for 15 min in the indicated buffer. A flat stainless steel disc pierced in its center was placed above. Medium was flowed at a constant rate for 5 min through the central orifice of the disc, before removal of the disc and microscopic examination of the remaining radius. The radius at which 50% of the cells were detached was determined (r050), and the stress at this distance to the center was σ050 = 3Dνm2r050, where D is the flow rate, e the distance between the plate and the disc (0.21 mm for experiments in SB buffer, 0.56 in HL5 buffer), and ν the fluid viscosity (10−3 Pa s).

To visualize adherent cells by scanning electron microscopy, cells were seeded in HL5 medium at low density on sterile glass coverslips and incubated for 3 days. The coverslips were transferred directly to HL5 containing 1% glutaraldehyde, and fixed for 30 min at room temperature. They were then rinsed with PBS and dehydrated by successive 10-min incubations with increasing concentrations of ethanol (30%, 50%, 70%, 90%, and 100%). Dehydrated cells were vacuum-coated with gold and photographed with a Siemens AuToscan scanning microscope.

Western Blot Analysis—Cells were washed once in PBS, resuspended at 106 cells/20 μl in sample buffer (0.103 g/ml sucrose, 5 × 10−2 M Tris, pH 6.8, 5 × 10−3 M EDTA, 0.5 mg/ml bromphenol blue, 2% SDS), and 20 μl of each sample were run on a 9% acrylamide gel in nonreducing conditions. The gel was then transferred onto a nitrocellulose BA 85 membrane (Schleicher & Schuell, Dassel, Germany). The membrane was incubated sequentially with an antipeptide antisera (YCI, 1/2000) directed to a sequence in the lumenal domain of the Phg1 protein (YKKVENWKGDTGGDC), and with a horseradish peroxidase-coupled donkey anti-rabbit Ig (Amersham Pharmacia Biotech), washed, and revealed by ECL. Monoclonal antibody to protein-disulfide isomerase (221-135-1) was a kind gift of M. Maniak (16).

Purification of Phagoosomes—Approximately 7 × 108 exponentially growing cells were allowed to internalize latex beads for 90 min in HL5 medium, washed twice with HL5 and once with ice-cold homogenization buffer (250 mM sucrose, 5 mM Hepes, 3 mM imidazole, pH 7.4, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 100 μM phenylmethylsulfonyl fluoride). Cells were resuspended in 2 ml of homogenization buffer, and disrupted by 25 passages in a ball-bearing cell cracker. Intact cells were removed by centrifugation at 1000 × g, and the phagoosomal cell fraction was isolated as already described by flotation on a sucrose step gradient (17). The phagosomal fraction was collected at the interface of the 10% and 25% sucrose solutions, and submitted to a second round of flotation, a procedure that was found to reduce significantly the contamination by non-phagosomal markers. The phagosomal membranes were finally diluted with PBS and pelleted by centrifugation. Total protein content was tested by Micro BCA protein assay (Pierce) in the cellular lysate and in the purified phagosomal fraction, and equivalent amounts of proteins (300 ng) were loaded on SDS-polyacrylamide gels and analyzed by Western blot as described above.

RESULTS

Isolation of phg1 Mutants Defective for Phagocytosis—The isolation of mutants defective for phagocytosis followed the classical method for the generation of mutants by restriction enzyme-mediated integration (11). Briefly, a plasmid containing a selectable marker (resistance to blasticidin) was introduced into cells by electrocroration together with the DpnII restriction enzyme. This resulted in random integration of the plasmid at one of the many DpnII sites present in the genome of Dictyostelium. The blasticidin-resistant cells were then incubated for 1 h in the presence of fluorescent latex beads and washed, and nonfluorescent cells were sorted with a FACS. The procedure was repeated 1 week later, and the negative cells cloned into individual wells. The resulting clones were then individually tested, and those found to be defective for phagocytosis were identified. One of these clones represented an insertion in the myosin VII gene, a gene recently reported to be implicated in phagocytosis (18). Two other clones (phg1-1 and phg1-2) were selected for further analysis.

Digestion of the genomic DNA of these two clones with ClaI yielded an identical plasmid-containing fragment of approximately 9 kilobase pairs (data not shown). Genomic DNA digested with ClaI was allowed to recircularize, then introduced into bacteria. This resulted in the selection of bacteria containing the original plasmid with the flanking sequences of the integration site. Upon sequencing, the two clones were found to represent distinct insertions in the same gene, named PHG1 (Fig. 1A). To ascertain that the observed phenotype was caused solely by the insertion in the PHG1 gene, plasmids corresponding to the phg1-1 and phg1-2 insertions were used for targeted gene inactivation in wild-type cells. In both cases the insertion of the plasmid in PHG1 gave rise to a strain with a strong defect in phagocytosis.

The Phg1 Protein Belongs to a New Family of Polytopic Membrane Proteins—The genomic DNA of PHG1 was sequenced, as well as a full-length cDNA clone recovered from the Dictyostelium cdna library (SSL630; Ref. 13). The Phg1 protein (642 amino acid residues) exhibits a potential N-terminal signal sequence followed by a luminal domain and nine potential membrane-spanning segments (Fig. 1C). The alignment of the Phg1 protein with its closest homologue in human (KIAA0255 = D78444) is shown in Fig. 1B.

Numerous genes previously sequenced in other species show a high homology to the PHG1 gene. All of them exhibit a similar overall structure, with a rather variable potential luminal domain followed by a more conserved membrane domain with nine putative transmembrane domains. Three homologues of PHG1 have been fully sequenced in human (D78444 = KIAA0255, U51006 and U94831; Refs. 19 and 20), three in Saccharomyces cerevisiae (U53980, Z45758, U18916), two in Caenorhabditis elegans (Z79759, AF026213) and three in Arabidopsis thaliana (AC005867, AC006592, U95973). Expression sequence tag data bases also reveal additional related genes in Dictyostelium and human, and homologues in other species (Drosophila melanogaster, Mus musculus). No function has been determined for any of these previously sequenced genes. On the basis of their primary structure, they have been named 9TM proteins.

In wild-type cells, the Phg1 protein migrates at an apparent molecular mass of approximately 65 kDa, as observed by im

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FIG. 1. Structure of PHG1 gene. A, schematic drawing of the PHG1 gene. Sequencing of the genomic DNA and cDNA revealed the presence of one intron, the position of which is indicated (amino acid 98). The position of the vector insertion site in mutants phg1-1 (amino acid 479) and phg1-2 (amino acid 329) is also indicated. B, the amino acid sequences of the Phg1 protein and its closest human homologue (KIAA0255 = D87444; 44.1% identity) were aligned using the ALIGN program. C, hydrophobicity plot (Kyte-Doolittle) of the Phg1 protein.
the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot.Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. 

A phg1 mutant protein. Molecular mass standards are indicated (kDa). Truncated forms of Phg1 are visible in mutant cells as a band of about 57 kDa for phg1-1 cells and 50 kDa for phg1-2 cells. B, cells were incubated for 1 h in the presence of fluorescent latex bead. The results are expressed as a percentage of the internalization by wild-type (WT) cells in the same experiment. Strains used are as follows: 1, wild-type; 2, phg1-1; 3, phg1-1 + PHG1; 4, phg1-2; 5, phg1-2 + PHG1.

**Fig. 2.** The phenotype of phg1 mutants is reversed by the expression of the Phg1 protein. A, expression of the Phg1 protein was assessed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot. Cells were lysed in sample buffer, and the equivalent of 10^6 cells was loaded on each lane and analyzed by Western blot.

**Fig. 3.** Phagocytosis defect of phg1 mutant cells. Wild-type (WT, thin line) or phg1-1 mutant (thick line) cells were incubated for 1 h in the presence of fluorescent latex beads (A) or FITC-dextran (B). The amount of internalized fluorescence was analyzed using a fluorescence-activated cell sorter (FACS). The fluorescence corresponding to 0, 1, 2, or approximately 30 internalized beads is indicated in A. C, cells were incubated with indicated substrates for 1 h with or without shaking (200 rpm), then washed and analyzed by FACS. The results are expressed as a percentage of the internalization by wild-type cells in the same experiment. K.a., K. aerogenes. Each bar represents the mean of three independent clones. Black bar, phg1-1 cells; hatched bar, phg1-2 cells.

**Phagocytosis Mutant in Dictyostelium.**

**phg1 Mutants Exhibit Specific Defects in Phagocytosis—**To determine more precisely the nature of the internalization defect in phg1 mutants, we tested the ability of the mutant cells to internalize various types of particles. As seen during the isolation of the mutants, phg1 mutant cells exhibited a strong inability to phagocytose latex beads (Fig. 3A). On the contrary, the internalization of the fluid phase markers FITC-dextran (Fig. 3B) or lucifer yellow (data not shown) was comparable to that of wild-type cells. Interestingly, although a significant phagocytosis defect was observed for E. coli, phagocytosis of Klebsiella aerogenes bacteria was much less affected (Fig. 3C). Phagocytosis of various bacteria by phg1 cells showed that the defect was minor only for K. aerogenes and K. aerogenes KP21 (25) (80.1% and 48.0% percentage of internalization, respectively, compared with 10.4%, 12.1%, 10.5%, 15.3%, and 28.8% for E. coli, Pseudomonas aeruginosa, Legionella pneumophila, Salmonella typhimurium, and Streptococcus bovis, respectively).

Previous publications have indicated that the (unidentified) receptor responsible for phagocytosis of most substrates in HL5 medium is not necessary for phagocytosis in phosphate buffer (SB) (see “Discussion”). When phagocytosis experiments were performed in SB medium, very little defect was observed for phg1 cells (34%, 24%, and 37% decrease for the phagocytosis of latex beads, K. aerogenes, and E. coli, respectively, compared with wild-type cells).

**Phagocytosis Mutant Are Defective for Adhesion to Certain Substrates—**Fluid phase uptake occurs essentially by macropinocytosis in Dictyostelium (21), a process involving the actin cytoskeleton in a manner similar to phagocytosis. The fact that fluid phase uptake was not affected in phg1 mutant cells suggested that the basic machinery responsible for formation of endocytic vacuoles was intact in these cells. The inability of the mutant cells to adhere to certain phagocytic substrates could be responsible for this selective phenotype. It has been previously shown that talin mutants defective for adhesion exhibit an enhanced defect in phagocytosis when the suspension of cells and particles is shaken (22). Thus, adhesion of the cell to the particle is even more crucial for phagocytosis of particles in a shaken suspension. This also appeared to be the case for phg1 mutants (Fig. 3C). This result suggests that the phenotype of...
phg1 cells could primarily be manifested in the adhesion properties of mutant cells.

Whereas wild-type cells in culture adhered efficiently to the surface of the culture plate, phg1 mutant cells were often observed floating in the medium, suggesting that they exhibit reduced adhesion to the substrate (data not shown). To quantitate this, the cells were allowed to adhere to a glass plate, and the hydrodynamic stress necessary to detach 50% of the cells was determined as described under “Experimental Procedures.” The adhesion of phg1 mutant cells in HL5 medium was markedly decreased compared with wild-type cells, as revealed by the smaller value of the hydrodynamic stress measured for phg1 cells (<0.025 Pa) compared with wild-type cells (0.5 ± 0.1 Pa). In SB medium, phg1 cells did not show a reduced adherence to the substrate according to the similar hydrodynamic stress values measured for wild-type and phg1 cells (2.5 ± 0.2 and 2.4 ± 0.6 Pa, respectively).

Upon more prolonged culture in HL5 medium, phg1 mutant cells did adhere to their substrate. However, examination of the cells by scanning electron microscopy revealed distinct differences between adherent wild-type and mutant cells. Whereas wild-type cells adhered tightly to the glass coverslip, phg1 cells did not spread as extensively and could be seen to detach locally from the substrate (Fig. 4).

Together, these results indicate that the primary defect of phg1 mutant cells is a decrease in their adhesion capacity.

The Phg1 Protein Is Present in Phagosomes—The implication of the Phg1 protein in the early steps of phagocytosis suggests that it should be present at the cell surface and in the phagocytic compartments. Previous publications suggest that other members of the family might be present in the endocytic pathway (20, 23). Unfortunately, all the antisera generated in our laboratory only recognized the denatured Phg1 protein onto nitrocellulose and did not allow us to assay the presence of Phg1 at the cell surface by immunofluorescence or immunoprecipitation. The abundance of naturally biotinylated proteins in Dictyostelium cells also prevented the isolation of surface proteins by surface biotinylation followed by adsorption to avidin (24). To test for the presence of the Phg1 protein in phagosomes, we allowed cells to phagocytose latex beads and purified phagosomes by flotation on a sucrose gradient as described previously (17). The purified phagosomal fractions contained only minor amounts of protein-disulfide isomerase, a marker of the endoplasmic reticulum, in contrast to high amounts of Phg1 protein (Fig. 5). The presence of the Phg1 protein in the phagosomal pathway is compatible with a role in adhesion to phagocytosed particles.

DISCUSSION

Our work allowed the identification of Phg1p, a protein essential for adhesion to and phagocytosis of a range of substrates. How does inactivation of PHG1 result in decreased adhesiveness of mutant cells? The fact that phagocytosis of various particles is affected differentially in phg1 mutant cells suggests some element of specificity in the function of the Phg1 protein, namely that Phg1 might act as a receptor for the phagocytosis of certain substrates (latex beads, E. coli). The presence of Phg1 in the phagocytic pathway is compatible with this hypothesis.

Previous publications (5) have indicated that in HL5 medium a so-called nonspecific receptor is responsible for the phagocytosis of most particles. This work is based on the analysis of a mutant very similar to the phg1 mutants described here, but the corresponding gene could not be identified. In a series of very elegant experiments, it was shown that this receptor recognizes highly hydrophilic particles: bacteria or latex beads coated with HL5 components. In phosphate buffer (SB), the situation is radically different. The very hydrophobic surface of noncoated latex beads ensures their binding to cells and their phagocytosis in a receptor-independent manner. An additional (unidentified) lectin-type receptor can also operate in SB medium. In HL5 medium the lectin is inhibited by the maltose present in the medium, while in SB it ensures the phagocytosis of bacteria. Thus phagocytosis of latex beads as well as bacteria is dependent on the nonspecific receptor in HL5 medium, but not in SB medium.

Strikingly, both the phagocytosis and the adhesion defect of phg1 mutants are observed in HL5 medium, but not in phosphate (SB) medium. It is tempting to speculate that Phg1p might be the previously described nonspecific receptor of Dictyostelium, necessary for phagocytosis of a range of hydrophilic substrates in HL5 medium (5).

In this view, Phg1 proteins could constitute a family of receptors with a variable extracellular domain involved in recognition of various substrates, while the conserved membrane domain would ensure interaction with cellular components. However, other interpretations are compatible with our observations. In particular, Phg1 protein(s) might act as a modulator of another as yet unidentified receptor, either by regulating its adhesive properties, or by controlling its surface expression.

The genetic approach described in this study allows random isolation and functional characterization of new genes involved in the phagocytic process. In the future, it will hopefully allow a more systematic dissection of the molecular events involved at different stages of the phagocytic process.

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