Identification and Characterization of Taglin, a Mannose 6-Phosphate Binding, Trypsin-Activated Lectin from *Giardia lamblia*†

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ABSTRACT: We have previously reported the presence of a cell surface associated lectin activity in *Giardia lamblia*, a human protozoan parasite that is a significant cause of diarrheal disease worldwide [Lev, B., Ward, H., Keusch, G. T., & Pereira, M. E. A. (1986) *Science* (*Washington, D.C.*) *232*, 71–73]. This lectin is specifically activated in vitro by a host protease, trypsin, which is secreted in vivo at the site of infection. The activated lectin agglutinates cells to which the parasite adheres in vivo and binds specifically to isolated brush border membranes of these cells. These findings suggest that this lectin may be of importance in the host–parasite interaction. We now report the identification of this lectin, which we have named taglin (to denote trypsin-activated *Giardia* lectin), and describe some of its properties. A monoclonal antibody that inhibits the hemagglutinating activity of taglin recognizes a protein of 28 000/30 000 kdaltons in Western blots of *Giardia* lysates. This finding was confirmed by direct demonstration of lectin activity with the technique of erythrocyte binding to proteins electroblotted to nitrocellulose, which revealed specific red cell binding to giardial protein bands in the same molecular weight range as those recognized by the monoclonal antibody. This study also elucidates the binding of taglin to terminal phosphomannosyl residues. The involvement of cell surface phosphate in binding of taglin to erythrocytes is shown by the abolition of lectin activity by alkaline phosphatase treatment of the erythrocytes. Taglin also requires divalent cations, Ca^{2+} or Mn^{2+}, for hemagglutinating activity and is active within a narrow pH range of 6–7.

A number of inter- and intracellular functions of eukaryotic cells are mediated by lectins, a class of nonimmune carbohydrate binding proteins that have specificity for particular sugar residues (Lis & Sharon, 1985). Some lectins are involved in the cellular translocation of glycoproteins. Among these are the hepatic lectin or asialoglycoprotein receptor, which is believed to function as a cell surface receptor for circulating asialoglycoproteins (Ashwell & Harford, 1982), and the mannose 6-phosphate receptors, which are involved in transport of lysosomal enzymes (Neufeld & Ashwell, 1980; Sly & Fischer, 1982; Hoflack & Kornfeld, 1985a). Others are believed to mediate cell–cell and cell–substratum adhesion. These include the lymphocyte homing receptor, which mediates attachment of lymphocytes to high endothelial venules of target organs (Gallatin et al., 1986), and discoidin 1, a lectin from the slime mold *Dictyostelium discoideum*, which promotes cell–substratum adhesion and ordered cell migration during morphogenesis (Springer et al., 1984). A number of host–parasite interactions, including those between *Plasmodium falciparum* merozoites and erythrocytes (Jungery et al., 1983), *Leishmania donovani* promastigotes and macrophages (Chang, 1981), and *Trypanosoma cruzi* trypomastigotes and myoblasts (Villalta & Kierzenbaum, 1983), are also postulated to involve cell surface lectins. We recently described (Lev et al., 1986) the presence of a mannose 6-phosphate binding, membrane-bound lectin in *Giardia lamblia*, a widely distributed protozoan parasite that causes diarrheal disease. *Giardia* trophozoites exert their pathogenic effect after selectively adhering to the surface of proximal small intestinal enterocytes. The lectin is novel in that it is specifically activated by exposure to trypsin, an enzyme that is present in abundance at the site of infection. When activated, the lectin agglutinates enterocytes to which the parasite adheres in vivo. In addition, the activated lectin binds specifically to isolated surface brush border membranes of these cells (Ward et al., 1987). In this paper we report the identification of this lectin, which we have named taglin, and describe some of its properties.

Experimental Procedures

Materials. Sugars, trypsin (from bovine pancreas, type III), subtilisin (from *Bacillus subtilis*, type VIII), alkaline phosphatase (from bovine intestinal mucosa, type XX), protein A–Sepharose, lectins, and salts were obtained from Sigma Chemical Co., St. Louis, MO. CM Affigel Blue Sepharose was from Bio-Rad, Richmond, CA. Protoblot immunoblotting system was obtained from Promega Biotec, Madison, WI. Rabbit anti-mouse IgG[1] was obtained from Cooper Biomedical, Malvern, PA. Prestained molecular weight standards were from Bethesda Research Laboratories, Gaithersburg,

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[1] Abbreviations: PBS, 20 mM phosphate, pH 7.2, containing 50 mM sodium chloride; TBS, 20 mM Tris-HCl, pH 8.0, containing 150 mM sodium chloride; TTBS, 20 mM Tris-HCl, pH 8.0, containing 150 mM sodium chloride and 0.05% Tween 20; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; IgG, immunoglobin G; EDTA, ethylenediaminetetraacetic acid.

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MD. Phosphomannans were a gift from Dr. M. E. Slodki, Northern Regional Research Laboratory, Peoria, IL. Control monoclonal antibody 17 D6 against a human B cell class 1 marker was a gift from Dr. D. Thorley-Lawson, Tufts University, Boston, MA. Bovine testicular β-galactosidase was a gift from Dr. G. S. Sahagian, Tufts University, Boston, MA.

Parasites. Trophozoites of the Portland 1 strain of *Giardia lamblia* (a gift from Dr. L. S. Diamond, National Institutes of Health, Bethesda, MD) were axenically cultivated in TYI-S-33 medium supplemented with bile (Keister, 1983) for 72 h at 37 °C in 15 × 125 mm screw-cap borosilicate glass tubes or in roller bottles (Farthing et al., 1982). Parasites in late-log phase were harvested by chilling the tubes or bottles on ice at 4 °C for 15 min, pelleted at 500*g* for 10 min, and washed 3 times in PBS.

Trypsin Activation. Washed trophozoites were lysed by sonication at 4 °C for three pulses of 30 s each at an output of 30 W with a Branson sonifier and filtered through a 0.22-μm Millipore filter. Lectin activity was induced by incubating the lysates with 40 μg of trypsin per 10^7 trophozoites in 1 mL of PBS at room temperature for 10 min. Proteolysis was stopped with 2 mM phenylmethanesulfonyl fluoride.

Hemagglutination Assay. Lectin activity was determined by a hemagglutination assay as described earlier (Lev et al., 1986). Briefly, 2% rabbit erythrocytes in PBS were added to serial doubling dilutions of activated lysates in PBS containing 2 mg/mL BSA and 1 mM $CaCl_2$ and incubated at 4 °C for 60 min. Lectin titer was defined as the reciprocal of the highest agglutinating dilution, as determined by direct observation. This concentration was considered to equal 1 hemagglutination unit.

Identification of Taglin Using Monoclonal Antibodies. The production of monoclonal antibodies to the *Giardia* lectin has been described earlier (Ward et al., 1987). Monoclonal antibody α-L2 and isotype-matched control antibody 17 D6 were purified from ascites fluid by chromatography on CM Affigel Blue Sepharose followed by fractionation with 45% ammonium sulfate as described (Izant et al., 1982). After extensive dialysis against PBS, the IgG purified in this way was adjusted to a concentration of 1 mg/mL in PBS. Aliquots of purified monoclonal antibody or of PBS alone were incubated with an equal volume of trypsin-activated lysate for 18 h at 4 °C, followed by incubation for 2 h at 4 °C with protein A–Sepharose, which had been adsorbed with rabbit anti-mouse IgG. This mixture was then centrifuged at 7000*g* for 10 min, and the supernatants were assayed for lectin activity.

Lysates of *Giardia* trophozoites (10^7/mL) were treated with trypsin or subtilisin (40 μg/mL for 10 min at room temperature). Aliquots of untreated lysate or of lysates treated with trypsin or subtilisin were boiled for 5 min under reducing conditions and electrophoresed on 10% SDS–polyacrylamide gels (Laemmli, 1970) and then electroblotted to nitrocellulose (Towbin et al., 1979). Blots were blocked with 3% BSA in TTBS at 37 °C for 1 h and then probed with purified α-L2 or 17 D6 diluted to a concentration of 2.5 μg/mL in 3% BSA in TTBS, at 4 °C overnight. Blots were washed with TTBS for 30 min with three changes at 23 °C and then incubated with goat anti-mouse IgG conjugated to alkaline phosphatase at a dilution of 1:7500 in TTBS for 1 h at 23 °C. Proteins recognized by the antibody were visualized by incubating the filters in a substrate solution containing 330 μg/mL nitro blue tetrazolium, 165 μg/mL 5-bromo-4-chloro-3-indolyl phosphate, 100 mM sodium chloride, 5 mM magnesium chloride, and 100 mM Tris-HCl, pH 9.5. Once the bands were seen, the reaction was stopped by transferring the blots to 20 mM Tris-HCl, pH 8.0, containing 5 mM EDTA.

Identification of Taglin by Direct Erythrocyte Binding to Nitrocellulose Blots. Untrypsinized as well as trypsinized lysates were electrophoresed on 10% SDS–polyacrylamide gels as described above except that reducing agents were not used and samples were not boiled. Proteins thus separated were then electroblotted to nitrocellulose as described (Towbin et al., 1979), except that methanol was omitted. The filter was then blocked with 5% (w/v) nonfat dry milk (Carnation) in 30 mM Hepes, pH 7.5. Proteins bound to the filter were denatured and renatured as described (Celenza & Carlson, 1986). Briefly, filters were denatured with 7 M guanidine hydrochloride in 50 mM Tris-HCl, pH 8.3, and 0.25% nonfat dry milk, for 1 h at 25 °C. They were then allowed to renature in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Nonidet P-40, and 0.25% nonfat dry milk, for 16 h at 4 °C. Filters were washed 3 times in PBS containing 5 mg/mL BSA for 20 min each time and then attached to the bottom of a Petri dish with double-sided Scotch tape. The Petri dish was filled with a 0.25% suspension of erythrocytes in PBS and incubated at 4 °C for 1 h. A glass slide was placed over the dish, which was then inverted and placed in a large vessel containing 150 mM NaCl in water to enable the red cells that were nonspecifically bound to detach and sediment on the glass. The dish was then placed right side up, and protein bands with lectin activity were identified by direct visual observation of bound erythrocytes. This method is a modification of that described to screen clones for monoclonal antibody production (Sharon et al., 1979).

Sugar Specificity. Sugar specificity of taglin was determined with a standard hemagglutination inhibition assay; 2% rabbit erythrocytes were added to serial doubling dilutions of the test compound in PBS containing 2 mg/mL BSA and 1 mM $CaCl_2$, to which was added 4 hemagglutination units of lectin, followed by incubation at 4 °C for 60 min. The minimum inhibitory concentration of a compound was defined as the minimum concentration that inhibited 4 hemagglutination units of lectin.

Alkaline Phosphatase Treatment of Erythrocytes. Rabbit erythrocytes were washed 3 times in TBS and adjusted to a concentration of 50% v/v in the same buffer. Aliquots (500 μL) of this suspension were incubated with varying concentrations of alkaline phosphatase in TBS at 37 °C for 2 h. The cells were then washed 3 times in TBS, and an aliquot of cells was removed for phosphate assay. The remaining cells were then washed 3 times in PBS, adjusted to a 2% suspension, and used to assay for lectin activity in a trypsinized lysate as described above. As a control, 500 μL of erythrocytes was treated with an equal volume of TBS under the same conditions. Aliquots of control and alkaline phosphatase treated erythrocytes were lysed to yield ghosts (Hanahan & Ekholm, 1974), which were then ashed and assayed for organic phosphate content as described (Ames & Dubin, 1960). In order to ascertain that the effect of the alkaline phosphatase was due to the removal of phosphate groups and not due to the effect of possible contaminants, inhibitors of alkaline phosphatase such as 100 mM sodium phosphate or 1 mM sodium metavanadate were included in the reaction mixture of control experiments.

Control and alkaline phosphatase treated erythrocytes were also used to determine hemagglutinating activity of the following lectins: concanavalin A, wheat germ agglutinin, peanut agglutinin, *Helix pomatia* agglutinin, and soy bean agglutinin. All these lectins were dissolved in PBS at a concentration of 1 mg/mL.

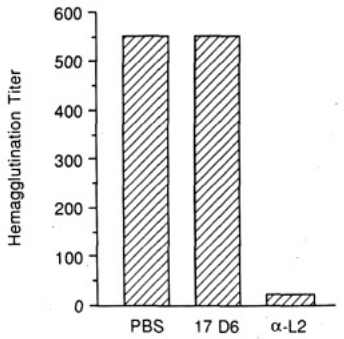

FIGURE 1: Depletion of taglin activity from *Giardia* lysates by monoclonal antibody α-L2. Aliquots of a trypsin-activated *Giardia* lysate were incubated with PBS and monoclonal antibody α-L2 or isotype-matched control antibody 17 D6, followed by incubation with protein A–Sepharose that had been adsorbed with rabbit anti-mouse IgG, as described under Experimental Procedures. The mixture was then centrifuged and the supernatant assayed for lectin activity.

Alkaline Phosphatase Digestion of Phosphomonoester Core of Hansenula holstii Y2448. A solution of *H. holstii* phosphomonoester core (100 μL, 25 mg/mL) in TBS was incubated with alkaline phosphatase (4 units/mg) at 37 °C for 24 h, dialyzed against PBS, and then used to inhibit lectin activity as described above. As a control, 100 μL of the same solution was treated with TBS alone under the same conditions.

Determination of pH Optimum for Lectin Activity. The optimum pH for lectin activity was determined as follows. Aliquots of a trypsin-activated lysate were dialyzed against buffers containing 160 mM sodium barbital, 143 mM sodium acetate, and 150 mM NaCl, adjusted to various pHs ranging from 5.5 to 8.5, and then assayed for lectin activity in the respective buffer containing 2 mg/mL BSA. Erythrocytes used in the assay were washed 3 times in the same buffer.

Requirement of Divalent Cations for Lectin Activity. A trypsin-activated lysate (lectin titer 128) was treated with 10 mM EGTA and then dialyzed extensively against PBS to remove divalent cations. The lysate thus treated was devoid of lectin activity. Increasing concentrations of $CaCl_2$, $MnCl_2$, $MgCl_2$, $ZnCl_2$, $CuCl_2$, and $FeCl_2$ in water were added to aliquots of this lysate, which were then assayed for lectin activity as described above.

RESULTS

A Monoclonal Antibody to Taglin Recognizes a 28/30-kDa Protein in Giardia Lysates. We had earlier described the production of monoclonal antibodies to the trypsin-activated *Giardia* lectin (Ward et al., 1987). These antibodies were raised by immunizing mice with a trypsin-activated *Giardia* lysate and screening clones for anti-lectin antibodies by hemagglutination inhibition. One of the positive clones obtained, α-L2, was of the IgG 2b subclass and reacted with the surface of live trophozoites in an immunofluorescence assay, confirming a plasma membrane location for the lectin. In order to be certain that this antibody was indeed recognizing taglin, experiments were performed to determine whether immobilized antibody could deplete lectin activity from an activated lysate. As shown in Figure 1, the monoclonal antibody immobilized on protein A–Sepharose (via a rabbit anti-mouse IgG bridge) did indeed deplete lectin activity from an activated lysate whereas an isotype-matched control 17 D6, immobilized in a similar fashion, had no such effect.

Identification of the protein determinant recognized by the anti-taglin antibody was ascertained by Western blotting. As shown in Figure 2, lane A, the antibody predominantly recognized a doublet of 28/30 kDa in an untrypsinized aliquot

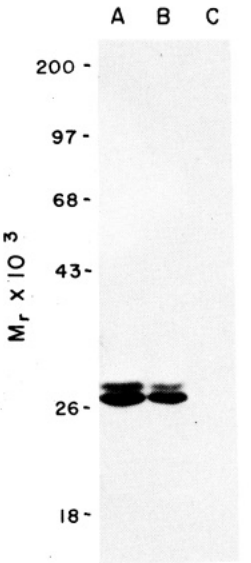

FIGURE 2: Identification of taglin by Western blotting with monoclonal antibody α-L2. Aliquots of a *Giardia* lysate, either untreated (lane A) or treated with trypsin (lane B) or subtilisin (lane C), were electrophoresed on SDS–polyacrylamide gels, electroblotted to nitrocellulose, and probed with monoclonal antibody α-L2 as described under Experimental Procedures. Prestained molecular mass standards used were myosin (200 000 kDa), phosphorylase B (97 000 kDa), bovine serum albumin (68 000 kDa), ovalbumin (43 000 kDa), α-chymotrypsinogen (25 000 kDa), and β-lactoglobulin (18 000 kDa).

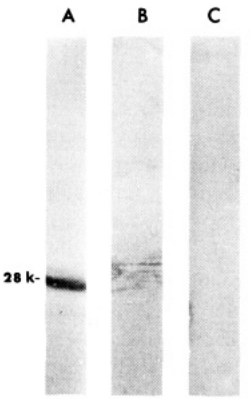

FIGURE 3: Identification of taglin by direct erythrocyte binding to nitrocellulose blots. Trypsinized (lanes A and B) as well as untrypsinized (lane C) aliquots of a *Giardia* lysate were electrophoresed on 10% polyacrylamide gels and transferred to nitrocellulose filters. Proteins bound to the filter were denatured and renatured as described under Experimental Procedures. Filters (lanes B and C) were incubated with 0.25% rabbit erythrocytes, and protein bands with lectin activity were identified by direct visual observation of bound erythrocytes. Lane A was probed with monoclonal antibody α-L2 as described under Experimental Procedures.

of a *Giardia* lysate that was devoid of lectin activity. In lane B, which represents an aliquot of the same lysate with a lectin titer of 2048 after trypsin activation, the antibody recognized the same doublet. When the lysate was treated with subtilisin (lane C), a condition that destroys lectin activity (Lev et al., 1986), the monoclonal antibody no longer recognized the protein doublet.

Identification of Taglin by Direct Erythrocyte Binding to Nitrocellulose Blots. In order to confirm that the 28/30-kDa bands recognized by the monoclonal antibody did indeed represent taglin lectin activity, we employed the technique of direct erythrocyte binding to proteins electrophoresed on SDS–polyacrylamide gels and transferred to nitrocellulose. As seen in Figure 3, erythrocytes bound to an area of the nitrocellulose blots, corresponding in molecular weight to those

Table I: Sugar Specificity of Taglin[a]

sugar	minimal inhibitory concn (mM)	relative inhibitory power compared to D-mannose
D-mannose	150	1.0
D-mannose 6-phosphate	20	7.5
D-mannosamine	65	2.3
N-acetyl-D-galactosamine	100	1.5
N-acetyl-D-mannosamine	100	1.5
D-arabinose	150	1.0
D-fucose	150	1.0
D-galactose 6-phosphate	>100	<1.5
D-fructose 1-phosphate	>100	<1.5
D-mannose 1-phosphate	>100	<1.5
D-glucose 1-phosphate	>100	<1.5
D-fructose 1,6-diphosphate	>100	<1.5
D-galactose 1-phosphate	>100	<1.3
D-glucosamine 6-phosphate	>100	<1.5
D-fructose 6-phosphate	>100	<1.5
D-galactose	>200	<1.3
D-glucose	>200	<1.3
N-acetyl-D-glucosamine	>200	<1.3
D-glucose 6-phosphate	>200	<1.3

[a] Sugar specificity of taglin was determined by a hemagglutination inhibition assay as described under Experimental Procedures. The minimal inhibitory concentration is defined as the minimal concentration of a sugar required to inhibit 4 hemagglutination units of lectin activity.

recognized by the monoclonal antibody. Further evidence that the erythrocytes were bound by taglin is provided by the finding that erythrocyte binding occurred to trypsin-activated lysates but not to untrypsinized lysates that were devoid of lectin activity.

Taglin Is Most Specific for Terminal Mannose 6-Phosphate. In earlier studies, using a variety of monosaccharides, polysaccharides and glycoconjugates, taglin was found to be most specific for the monosaccharide mannose 6-phosphate, which inhibited lectin activity at a minimum concentration of 20 mM (Lev et al., 1986). In the present study we confirmed this finding using a wider range of sugars, including a number of phosphorylated and acetylated derivatives (Table I). Mannose 6-phosphate was 7 times as potent as the unphosphorylated hexose in inhibiting lectin activity. Mannose 1-phosphate, however, did not inhibit lectin activity, at a concentration as high as 100 mM, indicating the importance of phosphorylation at C-6 of mannose. Furthermore, other phosphorylated sugars such as glucose 6-phosphate, fructose 1-phosphate, and arabinose 5-phosphate had no inhibitory activity, suggesting that phosphorylation per se was not required for lectin binding to sugars.

In order to confirm the specificity of taglin for phosphomannosyl residues, a number of phosphomannose-containing compounds including yeast phosphomannans and their derivatives were tested for inhibitory activity. Phosphomannans are polymers that contain phosphate in a phosphodiester structure wherein it links the anomeric hydroxyl of a hexose unit (either mannose or glucose) to the primary hydroxyl of a mannosyl unit (Slodki et al., (1972). Mild acid hydrolysis yields a phosphomonoester core with exposed mannose 6-phosphate. As shown in Table II, the α-D-mannan of *Torulopsis pinus Y2023* and the α-D-glucomannan of *Pichia pinus Y2579* did not inhibit lectin activity, whereas the *O*-phospho-α-D-mannans of *T. pinus Y2023* and *H. holstii Y2448* did inhibit, albeit at a relatively high concentration. The best inhibition was obtained with the phosphomonoester core of all three phosphomannans, indicating the specificity of taglin for terminal mannose 6-phosphate residues. Digestion of the phosphomonoester core of *H. holstii* with alkaline phosphatase

Table II: Inhibition of Taglin by Phosphomannose-Containing Compounds[a]

phosphomannose-containing compound	minimal inhibitory concn (mg/mL)
(A) mannose 6-phosphate	3.6
(B) yeast phosphomannans	
T. pinus Y2023	
α-D-mannan	>25.0
O-phospho-α-D-mannan	6.2
phosphomonoester	3.1
P. pinus Y2579	
α-D-glucomannan	>25.0
O-phospho-α-D-glucomannan	>25.0
phosphomonoester	3.1
H. holstii Y2448	
O-phospho-α-D-mannan	6.2
phosphomonoester	3.1
alkaline phosphatase treated phosphomonoester	12.5
(C) bovine testicular β-galactosidase	1.0

[a] Inhibition of hemagglutinating activity of taglin by phosphomannose-containing compounds was determined by a hemagglutination inhibition assay as described under Experimental Procedures. Minimal inhibitory concentration is defined as the minimal concentration of a compound or its derivative required to inhibit 4 hemagglutination units of lectin activity.

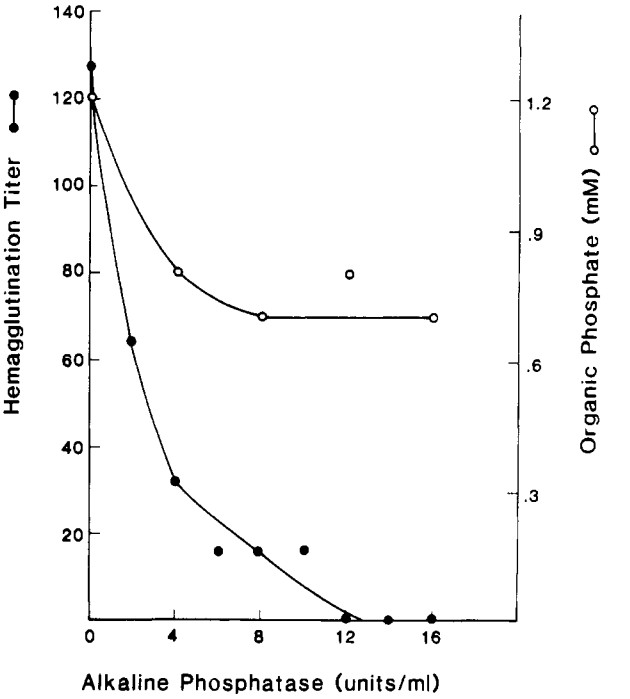

FIGURE 4: Cell surface phosphate is required for taglin binding to erythrocytes. Rabbit erythrocytes were treated with alkaline phosphatase or with buffer alone as a control, as described under Experimental Procedures. Aliquots of control and alkaline phosphatase treated cells were assayed for organic phosphate content. Alkaline phosphatase treated and control erythrocytes were used to assay for lectin activity in a trypsin-activated *Giardia* lysate.

reduced its inhibitory capacity by 75%, emphasizing the importance of exposed phosphate residues esterified to C-6 of mannose. In addition to phosphomonoesters of various yeast mannans, other phosphomannose-containing macromolecules such as bovine testicular β-galactosidase were potent inhibitors of taglin-induced hemagglutination.

Cell Surface Phosphate Is Required for Binding of Taglin to Erythrocytes. In order to study the role of phosphate residues in lectin binding, rabbit erythrocytes were treated with alkaline phosphatase to remove cell surface phosphate and then used to assay for lectin activity in a trypsin-activated lysate.

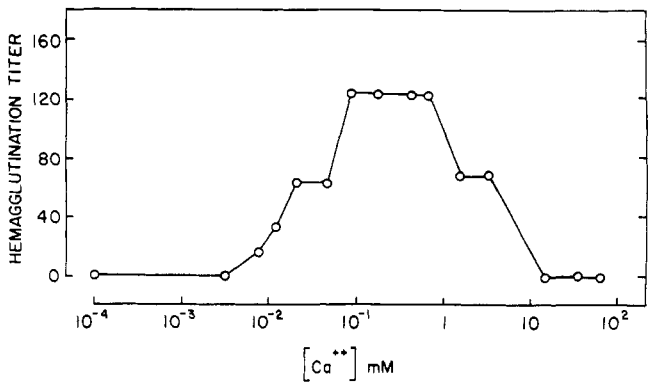

FIGURE 5: Requirement of divalent cations for lectin activity. Increasing concentrations of $CaCl_2$ were added to aliquots of a trypsin-activated *Giardia* lysate that had been depleted of Ca^{2+} as described under Experimental Procedures, and the lysates were then assayed for lectin activity.

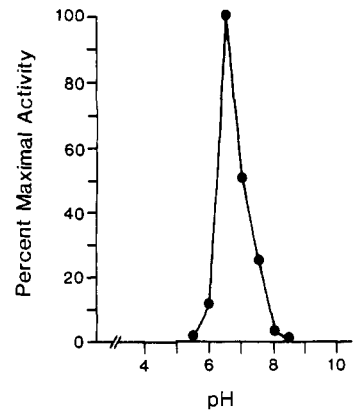

FIGURE 6: Determination of pH optimum for lectin activity. Trypsin-activated lysates of *Giardia* were assayed for lectin activity at various pHs ranging from 5.5 to 8.5 as described under Experimental Procedures. Results are expressed as a percent of maximal lectin activity, which was at pH 6.5.

As shown in Figure 4, digestion of erythrocytes with increasing concentrations of alkaline phosphatase resulted in decreasing lectin titers. This decrease in lectin titer was paralleled by a corresponding reduction in the amount of phosphate that remained bound to the erythrocytes, indicating that the fall in lectin titers was due to removal of phosphate by the enzyme. Further evidence that the effect of alkaline phosphatase was due to the removal of phosphate residues is provided by the finding that the effect of the enzyme could be inhibited by inhibitors of alkaline phosphatase such as sodium phosphate and sodium metavanadate (data not shown). These results indicate that cell surface phosphate is involved in the binding of taglin to erythrocytes. Whether the active phosphate residues were linked to mannose however, remains to be determined. Nevertheless, this finding is unique to taglin since a panel of conventional lectins including concanavalin A, wheat germ agglutinin, and peanut agglutinin agglutinated alkaline phosphatase treated red cells to the same extent as untreated erythrocytes.

Divalent Cations Are Required for Lectin Activity. The hemagglutinating activity of taglin was dependent on the presence of divalent cations. EGTA inhibited lectin activity at a minimum concentration of 3 mM (data not shown), suggesting that Ca^{2+} was required for lectin binding to the erythrocytes. As shown in Figure 5, by adding increasing concentrations of $CaCl_2$ to a lysate that had been depleted of hemagglutinating activity by treatment with EGTA and extensive dialysis against PBS, a minimum concentration of 0.1 mM was required to recover the original lectin activity, whereas concentrations higher than 10 mM inhibited hemagglutinating activity. In addition to Ca^{2+}, Mn^{2+} also restored lectin activity of trypsinized *Giardia* lysates that had been treated with EGTA; the dose–response curve of Mn^{2+} was similar to that of Ca^{2+} (data not shown).

Effect of pH on Hemagglutinating Activity of Taglin. Taglin was maximally active within a narrow pH range. The optimum hemagglutinating activity was at pH 6.5 (Figure 6), and a reduction of 1 pH unit resulted in abolition of lectin activity.

DISCUSSION

In the present study we have identified taglin, a trypsin-activated lectin from *Giardia lamblia*, by Western blotting, using a mouse monoclonal antibody specific for the lectin. By this method, a 28/30-kDa protein doublet was identified as being taglin. Support for this conclusion was provided by the finding that trypsinization of the *Giardia* lysate, with resultant induction of high titers of lectin activity, had no apparent effect on the molecular weight of the doublet whereas treatment with subtilisin, which destroys lectin activity, resulted in complete abolition of antibody recognition of the bands. Taglin was also directly identified in *Giardia* lysates that had been separated by SDS–PAGE, transferred to nitrocellulose, denatured, and then renatured. Lectin activity was demonstrated by direct binding of rabbit erythrocytes to proteins in the same molecular weight range as those recognized by the antibody. Again, binding occurred only to lysates that had been trypsinized. We had earlier reported a preliminary finding that partial purification of the lectin by chromatography of [^{35}S]methionine-labeled *Giardia* lysates on Blue Sepharose yielded a major band of 56 kDa (Lev et al., 1986). We have subsequently not been able to detect the 56-kDa band either by chromatography on Blue sepharose or by Western blotting using the taglin-specific monoclonal antibody. However, the monoclonal antibody does recognize the 28/30-kDa band both in total lysates and in preparations of the lectin that have been purified by chromatography on Blue Sepharose (data not shown). Furthermore, direct erythrocyte binding to nitrocellulose blots revealed bands in the same molecular weight range as those recognized by the monoclonal antibody. These results indicate that the molecular mass of taglin is a doublet of 28/30 kDa. The most likely explanation for the 56-kDa band obtained earlier is that it may represent a dimer of the 28-kDa band that was formed during the purification of the metabolically labeled material. This possibility is supported by the fact that both the 56-kDa band reported earlier (Lev et al., 1986) and the 28/30-kDa doublet recognized by the monoclonal antibody are resistant to trypsinization but are completely destroyed by subtilisin treatment, in accordance with lectin activation by trypsin and abolition of lectin activity by subtilisin.

We had earlier proposed that activation of the lectin by limited proteolysis could be explained by preferential trypsin digestion of an endogenous ligand that might be complexed to the lectin in vivo or by activation of a lectin precursor or prolectin by limited proteolysis with trypsin (Lev et al., 1986). The observation that the molecular weight of taglin did not change upon trypsinization does not discriminate between these two possibilities. If the first supposition was correct, then the molecular weight of the protein should not change upon trypsinization. If the second hypothesis is correct, the lack of a detectable change in molecular weight upon proteolysis could also be explained if trypsinization results in cleavage of a very small peptide from the inactive lectin that would not be detectable by SDS–PAGE. This possibility may be resolved by comparing the N-terminal amino acid sequence of the

inactive protein to that of the activated lectin.

A lectin (or antibody) site is considered to be most specific for the hapten or macromolecule that inhibits at the lowest concentration (Kabat, 1976). Taglin was found to be most specific for terminal mannose 6-phosphate. This specificity is unusual since there are very few proteins known to specifically recognize phosphomannosyl residues. These include the mannose 6-phosphate receptors, ligatin, and the lymphocyte homing receptor. Mannose 6-phosphate receptors are involved in the transport of lysosomal hydrolases from the Golgi complex to lysosomes (Sly & Fischer, 1982). Two immunologically distinct receptors have been described, a 215-kDa protein (Sahagian et al., 1981) that is cation independent and a 46-kDa receptor that is dependent on divalent cations for binding activity (Hoflack & Kornfeld, 1985b). Although the enzymes required for phosphorylation of high-mannose oligosaccharides of lysosomal hydrolases have been described in lower eukaryotes (Lang et al., 1986), it is not known whether protozoa such as *Giardia* employ the phosphomannosyl recognition pathway for transport of lysosomal enzymes, particularly since lysosomes have not been identified as such in these organisms (Friend, 1966). Taglin is similar to the 46-kDa receptor in its requirement for divalent cations as well as in its optimum pH for binding. However, both the mannose 6-phosphate receptors appear to differ from taglin in many ways. Apart from the obvious differences in molecular weight, neither of these receptors require trypsinization for binding activity. Furthermore, both the mannose 6-phosphate receptors differ from taglin in their sugar specificity; the 215-kDa receptor binds to fructose 1-phosphate in addition to mannose 6-phosphate (Neufeld & Ashwell, 1980), whereas taglin hemmaglutinating activity was not inhibited even by high concentrations of fructose 1-phosphate. The 46-kDa receptor binds (methylphospho)mannosyl diesters in addition to mannose 6-phosphate monoesters (Hoflack & Kornfeld, 1985a). Finally, neither of these receptors shows immunological cross-reactivity with taglin since antibodies to the 215-kDa receptor (provided by Dr. G. Sahagian, Tufts University, Boston) as well as antibodies to the 46-kDa receptor (obtained from Dr. S. Kornfeld, Washington University, St. Louis) do not react with taglin by Western blotting or by immunoprecipitation.[2]

Ligatin, a plasma membrane protein that functions as a base plate for the attachment of peripheral glycoproteins to the external cell surface also binds phosphomannosyl residues (Jakoi et al., 1981). However ligatin only recognizes oligosaccharides in which the phosphate is present in phosphodiester bonds linking penultimate mannose residues to terminal glucose residues (Marchase et al., 1982). The third lectin with specificity for phosphomannosyl residues is the lymphocyte homing receptor, which is involved in binding of lymphocytes to high endothelial venules of lymphoid organs (Stoolman et al., 1984). The relationship, if any, between the homing receptor and taglin is unknown.

In addition to the specificity for terminal phosphomannosyl residues as determined by hemagglutination inhibition assays, we found that cell surface phosphate was critical for taglin binding to erythrocytes. Removal of cell surface phosphate by treatment with alkaline phosphatase resulted in loss of taglin-induced hemagglutination. This is an unusual property of the *Giardia* lectin since lectins are not known to require phosphate for hemagglutinating activity. In contrast to most known lectins (Goldstein & Hayes, 1978), taglin was active within a very narrow pH range; the optimum pH was 6.5, which is the average pH of the small intestine where *Giardia* thrives (Rhodes & Prestwich, 1966). The optimal pH for taglin activity could therefore be a clue to its possible role in parasite attachment to enterocytes. On the basis of our earlier findings (Lev et al., 1986) that the *Giardia* lectin was specifically activated by trypsin, an enzyme present in abundance at the site of infection, and that the activated lectin agglutinated the intestinal cells to which the parasite adheres in vivo, we proposed that taglin may be of importance in attachment of the trophozoite to the host cells. Recently Wilson and Pearson have reported that attachment of another protozoan parasite, *L. donovani*, to macrophages is also inhibited by mannose 6-phosphate (Wilson & Pearson, 1985). Whether taglin or a similar lectin is also involved in other host–parasite interactions remains to be determined. The identification of taglin and elucidation of some of its properties should therefore assist in ascertaining its possible function in the *Giardia*–enterocyte interaction.

[2] H. D. Ward, B. I. Lev, G. T. Keusch, and M. E. A. Pereira, unpublished observations.

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Registry No. Ca, 7440-70-2; Mn, 7439-96-5; mannose 6-phosphate, 3672-15-9.

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Saturation-Transfer Electron Spin Resonance Studies on the Mobility of Spin-Labeled Sodium and Potassium Ion Activated Adenosinetriphosphatase in Membranes from *Squalus acanthias*†

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ABSTRACT: The sodium and potassium ion activated adenosinetriphosphatase [(Na^+,K^+)-ATPase] in membranous preparations from *Squalus acanthias* has been spin-labeled on sulfhydryl groups after prelabeling with *N*-ethylmaleimide. Saturation-transfer electron spin resonance spectroscopy has been used to study the rotational motions of the labeled protein on the microsecond time scale. Effective rotational correlation times deduced from the diagnostic line-height ratios in the second-harmonic, 90° out-of-phase (V_2') spectra are much larger than those deduced from the spectral integrals, indicating the presence of large-scale segmental motions, in addition to rotation of the protein as a whole. Experiments involving controlled cross-linking of the protein by glutaraldehyde, as well as measurements of the line broadening of the conventional electron spin resonance spectra, support this interpretation. Both the spectral integrals and diagnostic line-height ratios are found to increase irreversibly with time on incubation at temperatures greater than 20 °C, corresponding to a decrease in the segmental motion of the protein and probably also in the overall protein rotation. The native enzyme displays a marked nonlinearity in the Arrhenius temperature dependence of the activity at temperatures above 20 °C, and the activity decreases with a half-life of ca. 70 min on incubation at 37 °C (but not on incubation at low temperature), paralleling the time- and temperature-dependent changes in the saturation-transfer spectra of the labeled protein. Both of these observations suggest that the changes observed in the molecular dynamics could correspond to functional properties of the protein. The effective rotational correlation time of the membranous enzyme, deduced from the low-field and high-field spectral line-height ratios using calibrations from isotropically rotating spin-labeled hemoglobin, lies in the region of 50 μs, implying an upper limit of $\tau_{R_\parallel} = 25$ μs for the true rotational correlation time of the protein.

The sodium and potassium ion activated adenosinetriphosphatase [(Na^+,K^+)-ATPase][1] (EC 3.6.1.8) is a membranous active-transport enzyme involved in maintaining ion levels and osmotic balance in the cell [for reviews, see, e.g., Skou (1965) and Glynn (1985)]. Functionally, the enzyme mediates the extrusion of three Na^+ ions and the uptake of two K^+ ions, coupled to the hydrolysis of one molecule of ATP. The catalytic transport cycle involves the formation of a phosphorylated enzyme intermediate and a conformational transition between the E_1 and E_2 forms of the enzyme, which display different affinities for Na^+ and K^+. Structurally, the

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[1] Abbreviations: (Na^+,K^+)-ATPase, sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.8); NEM, *N*-ethylmaleimide; MSH, 2-mercaptoethanol; GA, glutaraldehyde; EDTA, ethylenediaminetetraacetic acid; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; SDS, sodium dodecyl sulfate; 5-MSL, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy; ESR, electron spin resonance; STESR, saturation-transfer ESR; V_1, first harmonic ESR absorption signal detected in-phase with respect to the field modulation; V_2', second harmonic ESR absorption signal detected 90° out-of-phase with respect to the field modulation; kDa, kilodalton(s).

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