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Characterization of Parameters Influencing Receptor-Mediated Endocytosis in Cultured Soybean Cells[1]

Mark A. Horn, Peter F. Heinstein, and Philip S. Low*

Departments of Chemistry (M.A.H., P.S.L.) and Medicinal Chemistry and Pharmacognosy (P.F.H.), Purdue University, West Lafayette, Indiana 47907

ABSTRACT

In a recent publication, we were able to demonstrate that biotin enters plant cells by receptor-mediated endocytosis and that impermeable macromolecules can be cotransported into cells by the same pathway if they are first covalently linked to biotin. In the present study, we have exploited the biotin endocytosis pathway to evaluate the variables in the cell wall and surrounding growth medium that influence the efficiency of endocytosis in plants. Under normal growth conditions, the major constraint limiting macromolecule endocytosis was found to be the size of the internalized macromolecule. Thus, a log-linear relationship with a negative slope exists between the molecular weight of the biotin-conjugated macromolecule and its rate of internalization by cultured soybean cells. This relationship, which extends from insulin (M_r approximately 5700) to immunoglobulin G (M_r approximately 160,000), is characterized by a slope of -1.04×10^5 molecules/cell/min per log M_r unit and an x intercept (no endocytosis detectable) of approximately log 160,000 daltons. Unfortunately, mild digestion with cell wall-degrading enzymes is unable to increase significantly the upper size limit of molecules that can be internalized, but uptake of lower molecular weight proteins can be enhanced by mild cell wall digestion. The optimal extracellular pH for endocytosis was found to be 4.6, *i.e.* near the normal pH of the cell culture medium. Furthermore, the osmotic strength at which endocytosis occurs most rapidly was observed to be isotonic to slightly hypotonic, suggesting that turgor pressure within the plant cell must not be a major determinant of endocytosis rates by cultured soybean (*Glycine max*) cells. Finally, cell age was found to impact significantly on the rate of macromolecule internalization, with maximal uptake rates occurring during early exponential growth and decreasing by a factor of 2 when the cells reach stationary growth phase.

Although once considered impossible because of high intracellular turgor pressure, endocytosis in plant cells has recently become a thoroughly documented process (for excellent reviews, see refs. 21 and 22). Fluid phase endocytosis has been well characterized in cultured plant cells and in protoplasts, and the morphological structures responsible for most endocytotic events in animals, termed coated pits, can be readily visualized in these plant systems (15–17, 21, 22, 24, 26, 28). Nonspecific uptake of electron-dense macromolecules from the medium has also been observed in protoplasts, and their pathways of intracellular transport have been monitored and characterized in some detail (7, 11, 14, 21, 22). Endocytosis has also been characterized in yeast (20).

Recently, evidence for receptor-mediated endocytosis involving several types of extracellular ligands has also been provided for cultured plant cells. In one case, macromolecular elicitors were found to bind to soybean cell surfaces and then enter the cells via receptor-mediated endocytosis, eventually passing into the cell's vacuole or tonoplast (9). Because uptake of the labeled elicitor was saturable (8) and could be competitively blocked by addition of unlabeled elicitor, and because the process was dependent on temperature in a manner characteristic of animal cell endocytosis, the elicitor endocytotic pathway was provisionally assumed to be similar to receptor-mediated endocytosis in animals. In a second series of experiments, the vitamin biotin was shown to enter plant cells via receptor-mediated endocytosis (10). In this case, however, the pathway was also exploited to cotransport otherwise impermeable macromolecules into the plant cell's cytoplasm. Thus, it was demonstrated that foreign proteins such as BSA, hemoglobin, ribonuclease, and insulin could be nondestructively delivered into cultured soybean cells via the biotin receptor endocytosis pathway if the proteins were first covalently attached to biotin. Because excess free biotin inhibited the cotransport of the foreign macromolecules, and because the foreign proteins were totally impermeable in the absence of an attached biotin, the receptor responsible for these endocytotic events was concluded to recognize only the attached biotin (10).

Although receptor-mediated endocytosis may now be established in both the animal and plant kingdoms, differences in cell structure, cell environment, and intracellular metabolism suggest that data concerning endocytosis in animals may not unequivocally extrapolate to endocytosis in plants. Thus, plant cells have cell walls with pores that are thought to exclude molecules larger than approximately 70,000 D (2, 27), suggesting that a size limit not observed in animals may exist for plant cell endocytosis. Still, whether the actual size limit for endocytosis in plants derives from cell wall porosity or some other variable remains to be determined. Likewise, most animal cells are bathed in an interstitial fluid of essentially constant osmolarity, whereas plant cells may be subjected to cycles of rain and drought during which highly variable osmotic pressures can be experienced. However, whether endocytosis will turn off and on during fluctuations in turgor pressure or whether compensatory mechanisms will exist to shield the cell from its environment are also questions

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that cannot be answered from the animal data. Other challenges faced by plants such as a change in extracellular pH, the senescence of certain cell types, or the loosening of the cell wall during growth are also likely to impact differently on endocytosis in the plant and animal kingdoms. Because of these major gaps in our understanding of receptor-mediated endocytosis in plants, we have undertaken a more thorough investigation of the process using the biotin endocytosis pathway as a simple, manipulatable model. We report here that endocytosis in plants is indeed sensitive to most of the variables mentioned above, but in many cases this sensitivity manifests itself in a manner not predicted from previous data.

MATERIALS AND METHODS

Plant Material

Soybean (*Glycine max* Merr cv Kent) cell suspension cultures were maintained in W-38 medium and subcultured every 7 to 10 d, as previously described (1, 13).

Preparation of Biotinylated Proteins

To 0.5 mL of a 1 mg/mL solution of the desired protein was added a 10-fold molar excess of *N*-hydroxysuccidimidyl biotin (Pierce Chemical Co.) dissolved in DMSO. The reaction was allowed to proceed for 1 h at room temperature, after which any unreacted *N*-hydroxysuccidimidyl biotin was "quenched" with 0.01 mL of ethanolamine. The resulting biotinylated protein was separated from free-labeling reagent by gel filtration chromatography on a disposable PD-10 column prepacked with Bio-Gel P-6 (Bio-Rad). Analysis of the biotin content of the labeled proteins by the method of Green (6) demonstrated that this procedure attached 1.5 biotins per insulin molecule, 2.5 biotins per RNase A molecule, 5 biotins per SBTI[2] molecule, 6 biotins per BSA molecule, and 10 biotins per IgG molecule.

Preparation of ^{125}I-Labeled Biotinylated Proteins

To 1 mL of a 300 mM phosphate buffer, pH 7.0, containing 3 iodobeads (Pierce Chemical Co.) was added 0.2 mCi [^{125}I] NaI (carrier free in 1 N NaOH; Amersham), and the mixture was allowed to incubate for 5 min at room temperature to liberate the active iodine species according to the supplier's instructions. After activation, 1 mg of desired biotinylated protein was added in 0.5 mL of iodination buffer. The iodination was allowed to proceed with stirring for 20 min, after which the product was isolated via gel filtration on a disposable PD-10 column (Bio-Rad). Typical iodinations yielded protein products emitting approximately 1×10^6 cpm/μg.

Preparation of FITC-Labeled Biotinylated Proteins

To 1 mL of a 1 mg/mL aqueous solution of the biotinylated protein was added 0.16 mL of a 1 mg/mL solution of FITC (Sigma Chemical Co.) dissolved in dimethylformamide, and the reaction was allowed to proceed for 4 h in the dark at room temperature. After this 4 h period, any unreacted FITC was quenched with 0.01 mL of ethanolamine, and the quenched reaction mixture was dialyzed against distilled water until the dialysate was free of fluorescence, as determined by fluorescence spectroscopy.

Assay for the Endocytotic Viability of the Cells

A 7-d-old suspension culture of soybean cells was gravity filtered using a fine nylon mesh, and 1 cm^3 of loosely packed cells was transferred to 20 mL of fresh W-38 medium and allowed to grow for 24 to 36 h at 23°C as described previously (9). This protocol yielded a cell population in the early exponential growth phase which was later found to be optimal for endocytosis. To these cells was added 20 μg of the desired biotinylated or nonbiotinylated fluorescent protein, and after 6 h of incubation, 1 mL of the cell suspension was removed, vacuum filtered on a glass fiber filter, and washed with 200 mL of fresh W-38 medium by vacuum filtration to remove unbound material. The washed cell pellet was then resuspended in 20 mL of fresh W-38 medium and examined at 40× magnification under a fluorescence microscope (Olympus BHT) equipped with a DM-500 (0–515) dichroic mirror and an EY-455 excitation filter and G-520 emission filter. Under these conditions, excitation wavelengths from A_{440} to A_{495} and emission wavelengths from A_{500} to A_{550} were allowed. The suspension was then evaluated for the fraction of cells with a fluorescent cytoplasm. Viable cells invariably took up the biotinylated fluorescent protein but were impermeable to the nonbiotinylated protein.

Assay for the Effect of Molecular Size on the Uptake of Biotinylated Proteins

Flasks containing 5 mL of a 36-h-old soybean suspension culture were treated with 25 μg of ^{125}I-labeled biotinylated bovine insulin (Sigma), RNase (Sigma), SBTI (Calbiochem), BSA (Sigma), or human IgG (ICN Biochemical Co.). The cells were then allowed to internalize the labeled protein for 2 h at 23°C, after which they were filtered on a glass fiber filter by vacuum filtration and washed on the filter with 50 mL of W-38 growth medium and placed in a gamma counter to determine internalized protein.

Determination of the Effect of Extracellular pH on the Uptake of Biotinylated Proteins

Approximately 5×10^6 36-h-old soybean cells were added to each of several 25 mL flasks containing 5 mL of W-38 growth medium initially adjusted to pH values 2.5, 3.5, 4.5, 5.5, 6.5, and 7.5. After 2 h of equilibration, the pH of the suspension was measured, 25 μg of ^{125}I-labeled biotinylated RNase or ^{125}I-labeled biotinylated IgG was added to the suspension, and the incubation was continued for an additional 2 h at 23°C. After the pH was measured again, the cells were filtered and washed with 50 mL of W-38 growth medium as described above and placed in a gamma counter to determine the amount of internalized protein.

[2] Abbreviations: SBTI, soybean trypsin inhibitor; FITC, fluorescein isothiocyanate; RNase, bovine RNase; IgG, human immunoglobin G; mOsM, milliosmolar; PGase, polygalacturonase.

Assay of the Effect of Osmotic Strength on the Uptake of Biotinylated Proteins

Approximately 5×10^6 36-h-old soybean cells were added to each of several 25 mL flasks containing 5 mL of W-38 growth medium that had been depleted or enriched in sucrose. This manipulation of sucrose concentration resulted in significant changes in the osmolarity of the growth medium, yielding deviations from normal osmolarity of −90, −60, −30, 0, +30, and +60 mOsM. After the cells were allowed to equilibrate for 2 h at 23°C, 25 μg of ^{125}I-labeled biotinylated RNase or ^{125}I-labeled biotinylated IgG was added to the suspension, and the cells were allowed to internalize the proteins for an additional 2 h. Following endocytosis, the cells were filtered and washed with 50 mL of W-38 growth medium as described above and placed in a gamma counter to determine the amount of internalized protein.

Examination of the Effect of Cell Age on the Uptake of Biotinylated Proteins

Either 25 μg of ^{125}I-labeled biotinylated RNase or ^{125}I-labeled biotinylated IgG was added to 5 mL of 0.5-, 1.5-, 2.5-, 3.5-, 4.5-, 5.5-, and 6.5-d-old (age after subculture into fresh growth medium) soybean cell suspension cultures, and the cells were allowed to internalize the protein for 2 h at 23°C. After this uptake period, the cells were filtered and washed with 50 mL of W-38 growth medium as described above and placed in a gamma counter to determine internalized protein.

Effect of Cell Wall Digestion on the Endocytosis of Biotinylated Proteins

In each of six flasks was placed 5 mL of a 36-h-old soybean cell suspension culture. To the individual flasks was added a variety of digestive enzymes of the types and quantities described in the figure legends. The digestion was then allowed to proceed for 2 h with gentle shaking at the normal growth temperature of the cells. After the digestion period, the cells were washed with 50 mL of W-38 growth medium to remove the digestive enzymes and resuspended in enzyme-free W-38 growth medium. At this point, the cells were checked to ensure viability. The cells were then treated with either 25 μg of ^{125}I-labeled biotinylated RNase or 25 μg of ^{125}I-labeled biotinylated IgG and allowed to internalize the proteins for 2 h. When the uptake period was complete, the cells were filtered and washed with 50 mL of W-38 growth medium and placed in a gamma counter to determine the amount of internalized protein.

Data Analysis

All data shown in "Results" represent the average of four determinations obtained by conducting each experiment in duplicate on two separate days. Because the variability among individual determinations never exceeded 20%, error bars are not shown.

RESULTS

Effect of Macromolecular Size on the Uptake of Biotinylated Proteins by Cultured Soybean Cells

To determine the effect of macromolecular size on the net rate of cell wall permeation and receptor-mediated endocytosis, cells were incubated for 2 h with ^{125}I-labeled biotinylated proteins of different mol wt and then examined for uptake of radioactivity. This 2-h incubation period was selected because by this time interval externally bound protein (assayed by incubation for 2 h at the nonpermissive temperature for endocytosis of 4°C) represented only 6% of the total cell-associated protein (*i.e.* externally bound plus internalized protein; assayed after 2 h at the permissive temperature for endocytosis of 23°C [9, 10]). A plot of the average velocity of internalization of the different biotinylated proteins *versus* the log of the protein mol wt is shown in Figure 1. Not surprisingly, this graph resembles the plot of retention time *versus* log mol wt of different proteins passing through a gel filtration matrix, suggesting that molecular sieving of some sort limits the rate of endocytosis. Because the size exclusion profile seen in our studies corresponds closely with that obtained in studies of cell wall permeability (2, 27), we assume the unidentified molecular sieve derives from the porosity of the plant cell wall.

Effect of Ionic Strength on the Endocytosis of Biotinylated Proteins

One of the classical arguments against endocytosis in intact plant cells has been that the high turgor pressure within the cell would prohibit the necessary invagination of the plasma membrane. Although endocytosis in plants is now well established, the question still arises as to whether turgor pressure might impact on the internalization process. To resolve this issue, cultured soybean cells were incubated in normal growth medium modified osmotically by varying the content of sucrose. After 2 h of incubation, the rate of endocytosis of biotinylated RNase was determined as described in "Materials and Methods." The rate of RNase·biotin uptake is indeed very sensitive to the osmolarity of the suspension medium

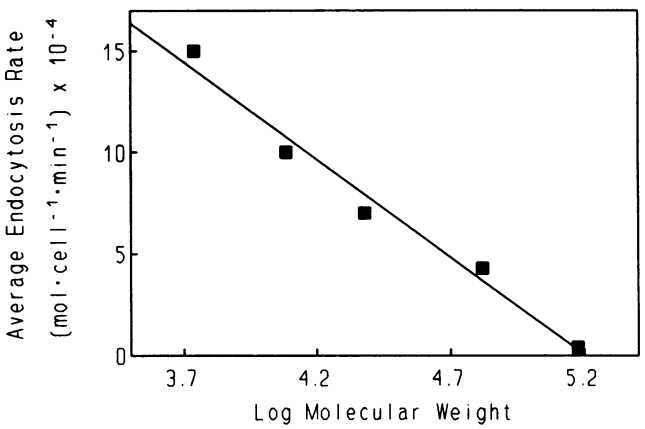

Figure 1. The effect of macromolecular size on the average rate of uptake of biotinylated proteins into soybean cells. Cells were incubated with 5 μg/mL of ^{125}I-labeled biotinylated proteins of different size for 2 h. After incubation was complete, the cells were washed, and the amount of internalized labeled protein was determined as described in "Materials and Methods." The mol wt assumed for the indicated proteins were: insulin, 5,770; RNase, 13,700; SBTI, 22,000; BSA, 68,000; IgG, 160,000.

(Fig. 2). However, instead of exhibiting the anticipated monotonic retardation with decreasing osmolarity, the rate of endocytosis actually displayed a biphasic response. Thus, endocytosis was maximal at or slightly below the osmotic strength of the normal growth medium and then diminished precipitously as sucrose content was further varied. This decline was shown not to be related to cell injury or death, because >95% of the cells remained capable of biotin receptor-mediated endocytosis at the end of the incubation period. It would, therefore, appear that the soybean cells have evolved a mechanism to cope with the usual turgor pressure in the cell. However, the same cells may be handicapped in conducting endocytosis as the turgor pressure deviates significantly from normal.

Effect of Extracellular pH on the Rate of Internalization of Biotin-Labeled Proteins

It has been hypothesized that changes in extracellular pH, namely, acidification, play an important role in triggering cell growth (18, 19). Because growth-related processes are commonly accompanied by increases in the rates of nutrient uptake, one might expect a concomitant enhancement of biotin and other nutrient endocytosis. This prediction is, in fact, incorrect (Fig. 3). Cells that were preincubated for 2 h at a pH different from their normal growth medium pH of 4.6 actually displayed decreased rates of endocytosis. Importantly, these reductions in endocytosis were again shown not to derive from massive cell death, because >95% of the cells remained viable and capable of biotin endocytosis at the end of the pH treatment. Thus, as previously hypothesized, these data suggest that the cells may be optimally adapted to conduct endocytosis under normal growth conditions. The data also imply that pH may be effectively used as a modulator of endocytosis, because the rates of internalization diminished precipitously as the pH was displaced from its normal value.

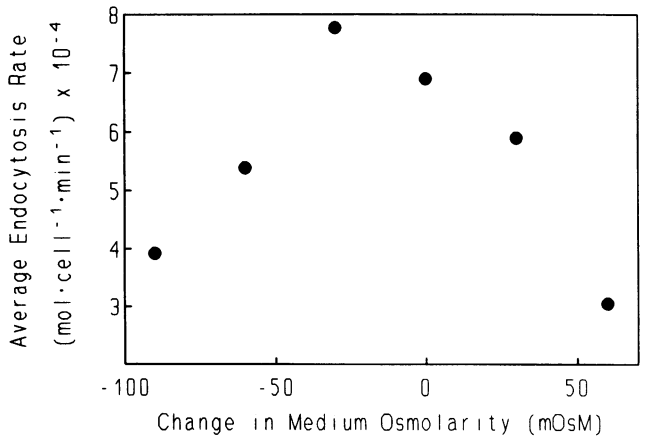

Figure 2. The effect of osmotic strength on the rate of uptake of biotinylated RNase into soybean cells. Cells were placed in W-38 growth medium of various osmolarities and allowed to equilibrate for 2 h at 23°C. After equilibration was complete, the cells were treated with 5 μg/mL of ^{125}I-labeled biotinylated RNase and allowed to incubate for 2 h. The cells were then washed and the amount of internalized labeled protein was determined. Identical results were obtained for the effect of growth medium osmolarity on the uptake of biotinylated IgG.

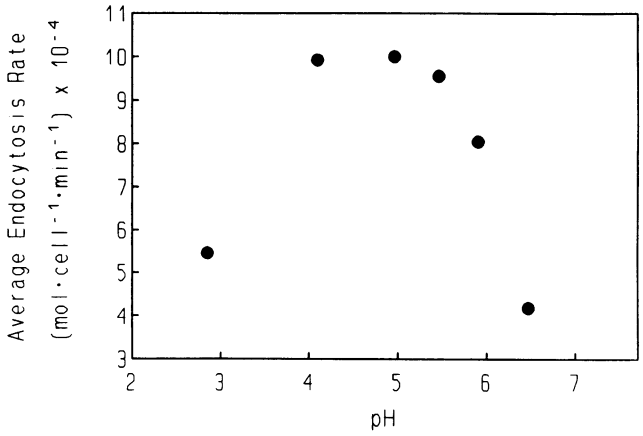

Figure 3. The effect of pH on the rate of uptake of biotinylated RNase into soybean cells. Cells were placed in W-38 growth medium of various pH values and allowed to equilibrate for 2 h at 23°C. After equilibration was complete, the medium pH was measured and recorded for use in the figure. The cells were then treated with 5 μg/mL of ^{125}I-labeled biotinylated RNase and allowed to incubate for an additional 2 h. The final pH was then measured, the cells were washed, and the amount of internalized labeled protein was determined. This internalized protein content is plotted against the measured pH at the beginning of the internalization period. The final pH values at the end of the second 2-h incubation were slightly different from those used in the figure and they are as follows: 3.35, 5.46, 5.70, 5.89, 6.20, and 6.61. Identical results were obtained for the pH dependence of the uptake of biotinylated IgG.

Effect of Cell Age on Endocytosis of Biotinylated Proteins

Because nutrient requirements are probably greater during periods of rapid cell growth, we undertook to evaluate the age dependence of biotin endocytosis by soybean cells. Again, this was most easily monitored using biotin-conjugated proteins, because the proteins could be easily radiolabeled and alternative routes of internalization could be largely eliminated due to the macromolecular size of the ligand. The rates of uptake of both RNase (M_r approximately 14,000) and IgG (M_r approximately 160,000) declined as the cells aged (Fig. 4). In fact, the average rate of endocytosis of RNase immediately following transfer of the cells into fresh growth medium was approximately twice its rate 6 d later when the cells had reached stationary growth phase. The relative decrease in the rate of IgG endocytosis was even more pronounced, suggesting that larger pores in the cell wall may disappear more rapidly than smaller ones. However, whether the observed diminution of RNase uptake actually reflects a change in cell wall porosity, a reduction in the number of biotin receptors, or a decrease in the rate of biotin endocytosis cannot be determined from the data.

Manipulation of Cell Wall Limitations on Endocytosis

The data in Figures 1 to 4 suggest that biotin receptor-mediated endocytosis in young cells under normal growth

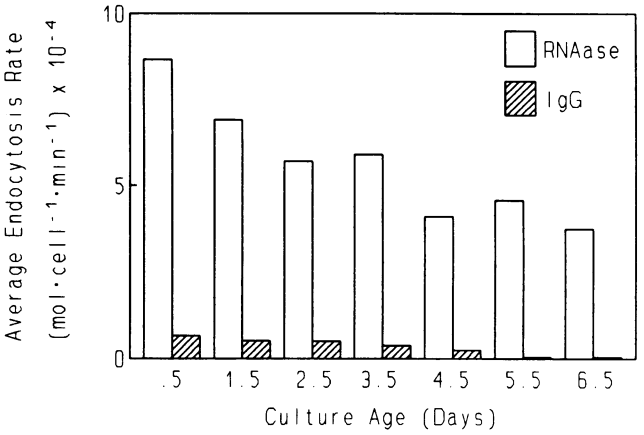

Figure 4. The effect of cell age on the rate of uptake of biotinylated RNase and biotinylated IgG into soybean cells. Soybean cells (1 cm^3) were placed into fresh W-38 growth medium and allowed to grow for the indicated times. After the desired growth period, the cells were treated with 5 μg/mL of either ^{125}I-labeled biotinylated RNase or ^{125}I-labeled IgG and allowed to internalize the biotin conjugates for 2 h. The cells were then washed, and the amount of internalized labeled protein was determined.

conditions is limited largely by cell wall porosity. Because it could prove useful to be able to reduce or remove the size limitation on macromolecules that can be delivered into cells by the biotin pathway, we elected to explore methods that might enlarge the channels through which the biotinylated proteins must pass. In each of the studies described below, care was taken to avoid extensive digestion of the cell wall, because as cultured cells are digested to the protoplast stage many of their properties are thought to change.

The effect of a 2 h digestion with different concentrations of pronase, a mixture of proteinases with broad specificity, on the average rates of endocytosis of RNase and IgG is shown in Figure 5. These two proteins were selected as the deliverable ligands because we believed their rates of uptake might provide somewhat different information concerning the cell wall modifications caused by digestion. Thus, RNase with a radius of only approximately 16Å may be limited in its penetration more by pore number than pore diameter. In contrast IgG, which apparently exceeds the size limit of most holes in the cell wall, would be expected to exhibit accelerated uptake only if existing channels could be enlarged or new larger pores could be generated. As shown in Figure 5, proteolysis of the cell exterior appears to enhance the uptake of both proteins, but mainly under the mildest of digestion conditions. As concentrations of pronase >5 units/mL were used, the accelerated uptake observed at low pronase concentration gradually diminished. Although the explanation of this behavior is not immediately apparent, it is conceivable that mild pronase digestion initially enlarges some cell wall cavities allowing accelerated protein uptake. However, more aggressive digestion must somehow lead to collapse of these pores or removal of biotin receptors, because a diminution of uptake then followed.

Several enzymes were also used to digest the polysaccharide components of the cell wall. In Figure 6 A and B, various PGases were used to selectively dismantle the polygalacturonic acid polymers of the cell wall. The PGase in Figure 6A is derived from a fungus and is presumably used by the pathogen to assist in the invasion of the host plant. The tomato PGase I in Figure 6B is thought to be involved in the breakdown of the tomato fruit cell wall during ripening. Importantly, neither treatment led to a major enhancement of IgG uptake, although a significant acceleration of RNase internalization was always observed under mild digestion conditions. Similar studies with tomato PGase II yielded results analogous to those obtained with PGase I (data not shown).

Finally, cellulase, an enzyme capable of degrading the cellulose fibers of the cell wall, was used by itself and in combination with the fungal PGase to learn whether it might promote enhanced endocytosis of larger macromolecules. As shown in Figure 6C, cellulase by itself either inhibited macromolecular uptake (for concentrations ≥20 units/mL) or exerted no effect on endocytosis (for concentrations <20 units/mL, data not shown). Thus, unlike the digestion protocols with the other enzymes, no acceleration of endocytosis was observed under even the mildest of digestion conditions. In combinations with PGase that have been used in some cells to prepare protoplasts, cellulase generally reduced the ability of PGase to accelerate endocytosis (Fig. 6D). Thus, cellulase treatment in general seems to serve only to retard endocytosis. Importantly, analysis of cell viability following all of the above cell wall digestion studies revealed >95% of the cells still participated to some extent in biotin receptor-mediated endocytosis. Thus, none of the treatments led to significant cell mortality.

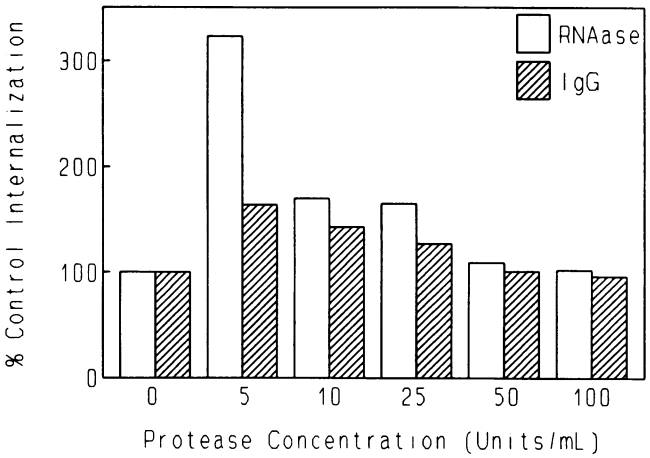

Figure 5. The effect of pronase pretreatment on the ability of soybean cells to internalize biotinylated RNase and biotinylated IgG. Cells were digested for 2 h with the concentrations of pronase (4 units/mg; Sigma) shown in the figure. After digestion was complete, the hydrolytic enzymes were removed via washing with 50 mL of W-38 growth medium. The washed cells were then resuspended in enzyme-free growth medium and treated with 5 μg/mL of either ^{125}I-labeled biotinylated RNase or ^{125}I-labeled biotinylated IgG. After an additional 2-h incubation, the cells were washed, and the amount of internalized labeled protein was determined.

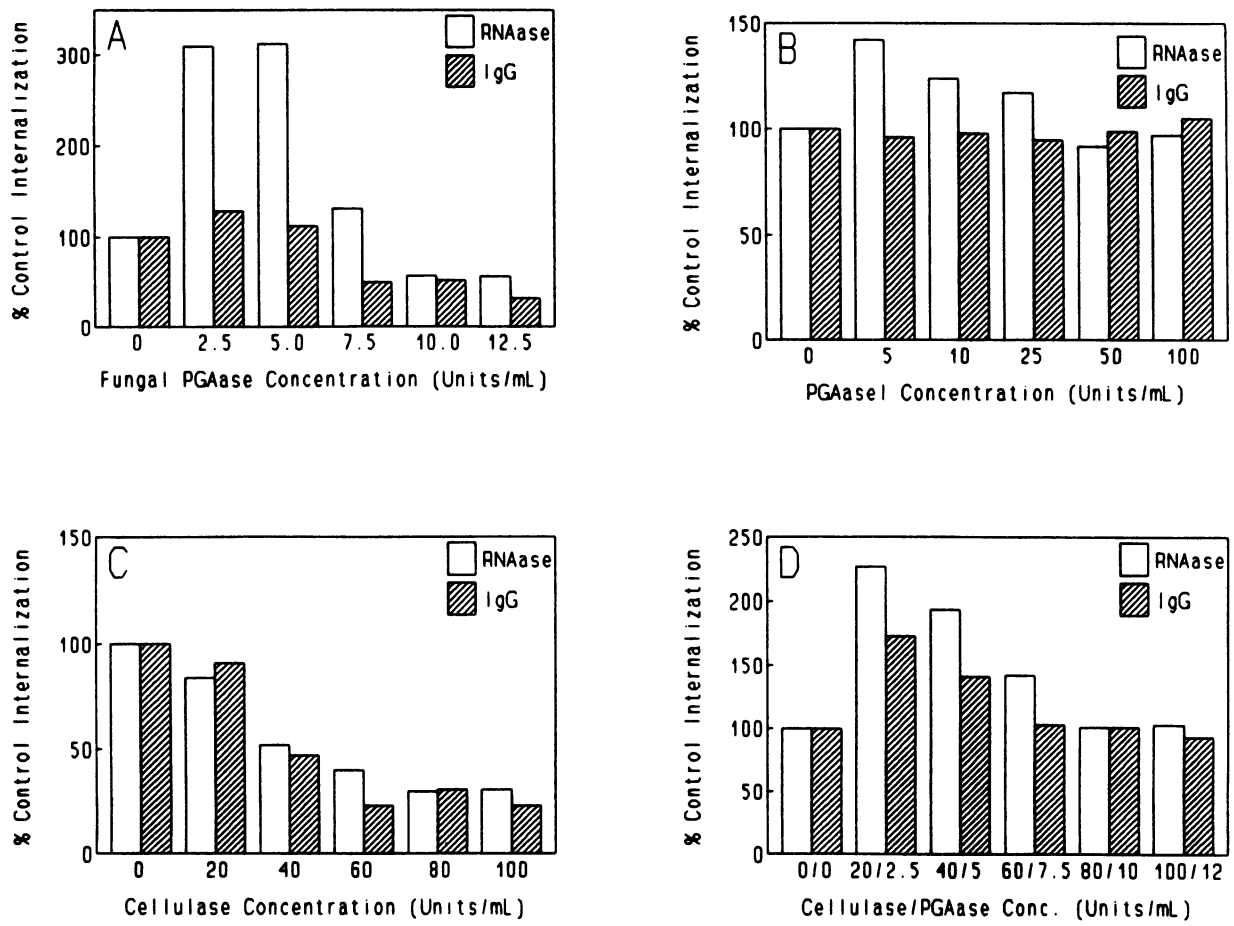

Figure 6. The effect of pretreatment with glycosidases on the ability of the soybean cells to internalize biotinylated RNase and biotinylated IgG. A, The effect of pretreatment with fungal PGase. Cells were digested for 2 h in different concentrations of fungal PGase (5.2 units/mg; Sigma catalog No. P5146). After the digestion period was complete, the hydrolytic enzyme was removed via washing the filtered cells with 50 mL of W-38 growth medium. The washed cells were then resuspended in enzyme-free growth medium and incubated for 2 h with 5 μg/mL of either ^{125}I-labeled biotinylated RNase or IgG. After uptake, the cells were washed, and the amount of internalized labeled protein was determined. B, The effect of pretreatment with tomato PGase I. Cells were digested for 2 h in different concentrations of tomato PGase I (300 units/mg; a kind gift of Dr. A. B. Bennett). After the digestion period was complete, the hydrolytic enzyme was removed via washing with 50 mL of W-38 growth medium and evaluated for endocytosis as described above. Identical results were also obtained after treatment with tomato PGase II (also a gift of Dr. A. B. Bennett). C, The effect of pretreatment with cellulase. Cells were digested for 2 h in different concentrations of cellulase (5.9 units/mg; Sigma catalog No. C0901). After the digestion period was complete, the hydrolytic enzymes were removed via washing with 50 mL of W-38 growth medium and evaluated for endocytosis as described above. D, The effect of simultaneous pretreatment with fungal PGase and cellulase. Cells were digested for 2 h in the concentrations of fungal PGase and cellulase shown in the figure. After digestion was complete, the hydrolytic enzymes were removed via washing with 50 mL of W-38 growth medium and evaluated for endocytosis as described above.

DISCUSSION

The use of a common ligand (biotin) linked to multiple proteins has allowed us to conduct a diversity of endocytosis studies not possible with a single, invariant ligand. For example, the effect of molecular size on the rate of endocytosis could be examined without significant concern regarding possible variations in the numbers or affinities of membrane receptors. Because all proteins were recognized by their linked biotins, the differences in uptake rates could be largely ascribed to the properties of the attached proteins. Although the proteins differed rather widely in isoelectric point, hydrophilicity, function, and size, only the latter variable appeared to significantly influence endocytosis. And even in this case, most of the data point to cell wall porosity as representing the major limiting element in the process as long as other growth medium conditions are normal. This absence of an intrinsic size limitation on the endocytotic process is, in fact, consistent with the observations that plant and animal endocytotic versicles are many times larger than any internalized ligand and that proteins as large as M_r 440,000 are readily endocytosed by protoplasts and animal cells which lack the restricting cell wall (7, 12, 21, 22, 25).

Unfortunately, attempts to enlarge the pores penetrating the cell wall were largely unsuccessful. Although enhanced rates of RNase uptake were commonly observed following mild cell wall digestion, endocytosis of the larger IgG molecules remained generally unaffected. We suspect this disparate response arises largely from an increase in the number of small diameter passages through the cell wall without a meas-

urable enlargement of existing pores. Only pronase treatment appeared to significantly facilitate IgG permeation. Perhaps, established channels through the cell wall are lined or stabilized with protein components that protect the channels from digestion by glycosidases.

Curiously, very extensive digestion of the cell wall with virtually any enzyme was found to inhibit the rate of endocytosis. Although larger gaps in the cell wall must certainly arise under these conditions, somehow they fail to facilitate receptor-mediated uptake. The fact that fluid phase endocytosis still proceeds in protoplasts (3, 12, 21, 22) suggests that the invagination process *per se* is not the defective step in digested cells. Furthermore, although polygalacturonic acid receptors are likely to be saturated under such digestion conditions, there is no reason to believe that biotin receptors will be similarly affected. Thus, the inability to enhance biotin conjugate uptake by gradually dismantling the cell wall remains a major enigma. Clearly, additional studies with alternative ligands will be necessary to resolve this issue.

Because virtually all recognition processes depend on charge interactions, we were not surprised by the strong pH dependence of endocytosis. However, in our minds, the biphasic effect of medium osmolarity on biotin endocytosis was totally unanticipated. If elevated turgor pressure impedes the invagination process as proposed by many (for example, see refs. 4 and 23), then why should a hyperosmotic medium with the associated reduced turgor pressure lead to reduced endocytosis? As shown by Gordon-Kamm and Steponkus (5), such elevated osmolarities directly lead to invagination and vesiculation of the plasma membrane as the cell tries to cope with the excess membrane surface area caused by cell shrinkage. Natural invaginations of this sort might at first be expected to simply accelerate any receptor-mediated invaginations occurring concurrently. However, exactly the opposite was observed. Although we were able to detect the osmotically induced plasmolysis, only inhibition of biotin receptor-mediated endocytosis was measured in the same cells. These data suggest that biotin receptor-mediated endocytosis is not a random process dependent largely on enhanced internalization of plasma membrane, but rather, a finely tuned pathway containing at least one event that is harmed more by reduced turgor pressure than it is helped.

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