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The Resolution of Two Populations of Lysosomal Organelles Containing Endocytosed Wistaria floribunda Agglutinin From Murine Fibroblasts

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Subcellular fractionation of Balb/c 3T3 fibroblasts exposed to Wistaria floribunda agglutinin was performed to localize fractions containing internalized lectin. Employing two sequential self-generating silica sol density gradients, the postnuclear supernatant of cell homogenates was resolved into five distinct cellular components. Lysosome enzyme activities were displayed by two populations of vesicles, each separated from plasma membrane, golgi, and mitochondria markers. The more dense of these fractions exhibited morphological and biochemical properties ascribed to secondary lysosomes. The more buoyant population was similar to that reported by Rome et al [11] who noted that it may be a product of vesiculated golgi endoplasmic reticulum lysosome (GERL). Treatment with W floribunda agglutinin of cells, surface radioiodinated demonstrated that plasma membrane proteins were localized within both the buoyant and dense lysosome populations in as little as 10 min after exposure to lectin. Prolonged incubation of the cells with W floribunda agglutinin resulted in maintenance of this distribution. However, when nonradiactive cells were exposed to ^{125}I-labeled W floribunda agglutinin for 10 min, radioactivity was detected only in the buoyant population of lysosomes as well as the plasma membrane/golgi fraction. Treatment of cells with W floribunda agglutinin for 30 min resulted in appearance of lectin associated radioactivity in the dense lysosome fraction in addition to those populations containing radioactivity seen after a 10-min incubation. These data indicate that the endocytosis of W floribunda agglutinin differed substantially from the internalization of a portion of the plasma membrane proteins. Furthermore, we found radioactivity associated with both plasma membrane proteins W floribunda agglutinin in regions of the density gradient fractionation that virtually lacked golgi and lysosome markers. These fractions may have represented populations of nonlysosome vesicles formed during the process of endocytosis.

Key words: endocytosis, GERL, lectin, lysosome, subcellular fractionation, Wistaria floribunda, agglutinin

In recent years a number of laboratories have demonstrated that the action of certain polypeptide hormones and light density lipoprotein (LDL) on target cells is mediated through their interaction with plasma membrane bound receptors. These

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ligands bind to the plasma membrane of their target cells and, at permissive temperatures, they (and their receptors) are internalized at clathrin associated regions of the plasmalemma. It has been suggested that in the case of insulin [1] and epidermal growth factor (EGF) [2,3] the internalization of the hormone receptor represents a down regulation process of the target cell. However, reports in the literature differ as to the intracellular fate of the ligand and/or receptor. It has been suggested that endocytic vesicles containing LDL receptors fuse directly with lysosomes resulting in a shuttle-like reappearance of the receptors on the plasmalemma [4]. In contrast, it has been indicated that endocytic vesicles containing EGF receptors interact initially with a golgi associated region prior to their localization with lysosomes [5]. Such observations have been made in different experimental systems, and the nature of the ligand as well as the receptor may influence the ultimate fate of the receptor.

In order to study the intracellular fate of different plasmalemmal glycoproteins associated with the adsorptive endocytosis of a single ligand, we have employed Wistaria floribunda agglutinin (WFA). This plant lectin is a tetrameric protein of 116,000 daltons possessing a carbohydrate binding specificity toward α-and β-linked terminal D-galactosyl and N-acetyl -D-galactosaminyl residues [6,7]. This protein is capable of specifically interacting with five surface proteins, ranging in molecular weights from 50 to 250 Kd, isolated from the plasma membrane of Balb/c 3T3 fibroblasts [8]. Upon interaction with the plasma membrane of in vitro cultured fibroblasts, WFA is internalized by adsorptive endocytosis mediated through the carbohydrate binding site of the lectin [9]. Ferritin-conjugated WFA binds to both smooth and clathrin associated regions of the plasma membrane and is internalized with clathrin coated vesicles as well as micropinosomes [8]. Prolonged exposure of the cells to ferritin-WFA results in the localization of the conjugate within secondary lysosomes [9,10].

In this paper we demonstrate that subcellular fractionation of Balb/c 3T3 fibroblasts exposed to WFA resulted in the localization of the lectin within two discrete populations of vesicles, each separated from plasma membrane and golgi markers, which displayed characteristic lysosomal enzyme activities. The heavier fraction exhibited morphological and biochemical properties ascribed to secondary lysosomes. A more buoyant fraction appears to be similar to that reported by Rome et al [11], who have suggested that it is a product of vesiculated golgi endoplasmic reticulum lysosome (GERL). The temporal relationship of the adsorptive endocytosis of WFA to the internalization of radiolabeled plasma membrane proteins indicates that the lectin was internalized by an intracellular pathway distinct from that of the bulk of plasma membrane proteins.

MATERIALS AND METHODS

Cell Culture

Balb/c 3T3 fibroblasts were grown as monolayer cultures in Dulbecco's minimum essential medium (Gibco, Grand Island, New York) containing 10% calf serum (Gibco, Grand Island, New York).

Cell Fractionation

Homogenization medium (HM) consisted of 0.25 M sucrose containing 1 mM disodium ethylenediaminetetraacetate (EDTA) and adjusted to pH 6.8 with KOH.

Cells at 75% confluency were harvested in HM by scraping with a rubber policeman. The cells were washed several times by centrifugation and resuspension in HM. Cells, as a 20% v/v suspension in HM were homogenized with a model 45 Virtis homogenizer (Research Equipment, Gardner, New York) until approximately 50% cell breakage occurred as judged by phase contrast microscopy. The resulting homogenate was centrifuged at 800g for 10 min at 4°C. The supernatant was reserved and the pellet was washed by resuspension in HM and centrifugation as before. The supernatants were combined and designated postnuclear supernatant (PNS).

A "Percoll Stock" was prepared by adding 9 parts Percoll (Pharmacia Fine Chemicals, Piscataway, New Jersey) to 1 part of 10 times concentrated HM and the mixture was adjusted to pH 6.8 with HCl. A Percoll suspension with a density of 1.070 gm/ml was prepared by mixing 3 parts of "Percoll Stock" with 7 parts of HM. Centrifugation of PNS through a self-forming Percoll density gradient was performed in nitrocellulose tubes (1 × 3-1/2 inches) by the sequential layering of 22 ml of Percoll (density of 1.070 gm/ml) onto 3 ml of 60% w/v aqueous sucrose (pH 6.8 adjusted with KOH) followed by the addition of PNS onto the Percoll layer. Following centrifugation in a model 60Ti rotor (Beckman Instruments, Palo Alto, California) at 20,000g for 2 h at 4°C, the contents of the tube were collected as 1-ml fractions by upward flow displacement [12].

Further fractionation of the unresolved material found in fractions 7–9 of the separation by the above density gradient was performed by use of a second density gradient procedure. A suspension of Percoll in HM with a density of 1.045 gm/ml was prepared by adding 1 part of "Percoll Stock" to 12.33 parts of HM. A mixture of this Percoll suspension (20 ml) and fractions 7–9 of the previous density gradient separation was layered onto 3 ml of a 60% aqueous sucrose solution. Following centrifugation at 20,000g for 40 min at 4°C, the contents of the tube were collected as described previously.

Wistaria floribunda Agglutinin

WFA was purified as a 116,000-dalton protein as previously described [6].

Iodination of WFA

The lectin was radiolabeled with ^{125}I by the method of McConahey and Dixon [13] using 0.5 mCi ^{125}I/mg protein.

Iodination of Cell Monolayers

Cell surface lactoperoxidase catalyzed iodination was accomplished by a procedure similar to that reported by Schenkein et al [14]. Cells in Hanks' Balanced Salt Solution (HBSS) (Gibco, Grand Island, New York) containing 50 mM D-glucose and enzymes in HBSS were maintained at 4°C. The reaction was performed by the sequential addition of: carrier free ^{125}I (50 μCi/ml); lactoperoxidase (Sigma Chemical Company, St. Louis, Missouri) 50 μg/ml; and glucose oxidase (Sigma Chemical Company, St. Louis, Missouri) 20 μg/ml; followed by incubation for 30 min. After washing the cell monolayer with HM, cells were treated as described and prepared for fractionation.

Measurement of Cell-Associated Radioactivity

The radioactivity of ^{125}I-labeled plasma membrane proteins precipitated with

5% trichloroacetic acid (TCA) was determined in aliquots of individual fractions. ^{125}I-WFA within subcellular fractions was determined by measuring the total radioactivity of the individual fractions.

Enzyme Assays

N-acetyl-β-D-glucosaminidase was assayed according to the method of Bosmann [15].

Aryl sulfatase A and B were quantified according to the procedure of Roy [16].

β-D-Galactosidase was analyzed as described by Rome et al [11] with the use of p-nitrophenyl β-D-galactoside as substrate.

Cytochrome c oxidase was determined according to the method of Appelmans et al [17].

Galactosyl transferase was detected by the procedure of Rome et al [11].

Lactate dehydrogenase was assayed according to the method of Wroblewski and LaDue [18].

Electron Microscopy of Subcellular Fractions

Fractions were combined and made to 2% glutaraldehyde and centrifuged at 150,000g for 40 min at 4°C. Organelles were removed by suction and mixed with an equal volume of 2% agarose (45°C) and immediately placed on ice. The solidified agarose was minced and washed three times for 5 min each with 0.01 M sodium phosphate buffer, pH 6.8, containing 0.12 M sodium chloride (PBS). The sample was postfixed in 2% OsO_4 in PBS for 1 hr, dehydrated with a graded alcohol series, followed by acetone, and embedded in Epon 812. Blocks were sectioned on a Sorvall ultramicrotome (model MT2-B) and poststained with 2% uranyl acetate and 3% lead citrate. Sections were visualized on a Siemans Elmiskop IA microscope at 80 keV.

RESULTS

Figure 1A shows the enzyme profiles of the density gradient fractionation in 30% "Percoll stock" of PNS from homogenates of Balb/c 3T3 fibroblasts. Prior to homogenization, plasma membrane proteins of monolayer cells were iodinated with ^{125}I. The enzyme markers investigated were: golgi (galactosyl tranferase); lysosomes (N-acetyl-β-D-glucosaminadase, aryl sulfatase A and B, β-D-galactosidase); mitochondria (cytochrome c oxidase); and for soluble enzymes (lactate dehydrogenase). The linear range of density for the gradient was between 1.040 and 1.080 gm/ml. Such a gradient resulted in soluble enzyme localizing in a single band centering at a density of 1.025 gm/ml. It was also evident that lysosomal enzyme markers were detected at two distinct densities. The more buoyant region (fractions 7–9) at a density of 1.040 gm/ml was coincident with galactosyl transferase activity indicative of golgi and plasma membrane as shown by ^{125}I-labeled protein from cells iodinated at 0°C and homogenized at zero time. The second area of lysosomal enzyme activities centered at a density of 1.080 gm/ml. The mitochondrial enzyme marker, cytochrome c oxidase, is resolved from all other enzyme studies and occurred as a broad unimodal distribution about a density of 1.050 gm/ml.

In order to separate the more buoyant particles displaying lysosomal enzyme activities from the golgi associated marker and plasma membrane proteins, the con-

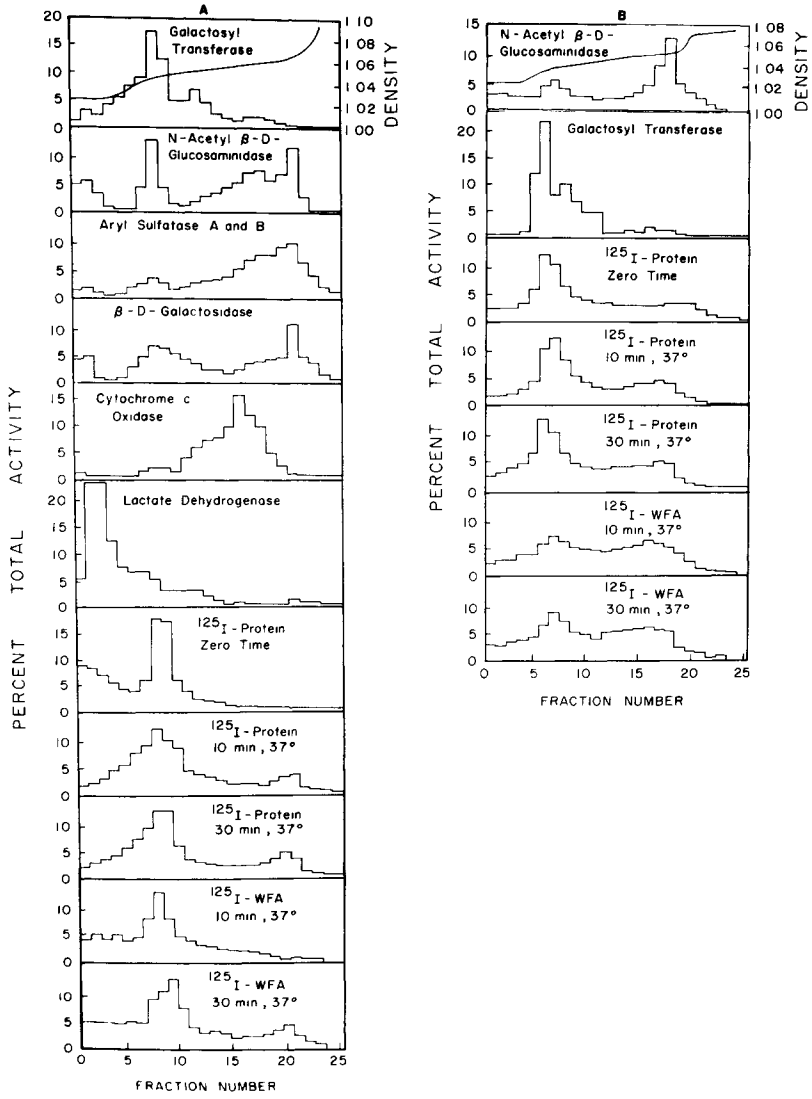

Fig. 1. Subcellular fractionation of the postnuclear supernatant of Balb/c 3T3 fibroblasts. Prior to homogenization of the plasma membrane proteins of monolayer cells were iodinated with ^{125}I using lactoperoxidase and incubated with WFA (100 μg/ml) for the indicated time. Alternatively, cells not labeled with ^{125}I were treated with ^{125}I-WFA (100 μg/ml, 20,000 cpm/μg) for the times indicated. (A) This describes the activity profiles of the density gradient fractionation in 30% "Percoll stock" (20,000g for 2 hr); (B) this describes the activity profiles of the density gradient fractionation in 7.5% "Percoll stock" (20,000g for 45 min) of combined fractions 7–9 from the 30% "Percoll stock" gradient.

tents of fractions 7–9 of the previous fractionation were incorporated into a second Percoll density gradient. The latter density gradient possessed a less dense midpoint and a more shallow rise in density than the former density gradient. It is evident from Figure 1B that the iodinated plasma membrane proteins and galactosyl transferase activity still comigrated at a density of 1.037 gm/ml. However, 80% of the

N-acetyl-β-D-glucosaminidase activity was resolved from the plasma membrane and golgi markers and banded at a density of 1.045 gm/ml. The remaining 20% of the enzymatic activity comigrated with the plasma membrane/golgi fraction and may have represented adsorbed enzyme as indicated by the lack of latency displayed by this enzyme activity (see below).

In experiments in which the material in fractions 7–9 of the first density gradient were rerun on an identical gradient, only 10% of the N-acetyl-β-D-glucosaminidase activity sedimented as would be expected for dense lysosomes (density, 1.080 gm/ml). The remaining 90% banded in the same position as in the initial fractionation.

The coordinated use of two Percol density gradients allows the resolution of the cellular components of Balb/c 3T3 fibroblasts into five pools: (a) soluble enzyme; (b) plasma membrane and golgi; (c) buoyant lysosomes; (d) mitochondria; and (e) dense lysosomes.

In order to determine the accessibility to substrate of N-acetyl-β-D-glucosaminidase within lysosomal pools, studies were conducted with Triton X-100. Enzyme assays were performed with aliquots of each lysosomal pool as described under the Methods section, or in the absence of Triton X-100 with the assay solution adjusted to 0.25 M sucrose. A latency factor could be taken as a measure of enzyme sequestration [19] and is calculated as:

$$\frac{\text{Enzymatic activity with Triton X-100}}{\text{Enzymatic activity without Triton X-100}}.$$

A value of 1.00 indicates total accessibility of the substrate to the enzyme and values greater than 1.00 indicate sequestration of the enzyme, presumably within Triton X-100 soluble vesicles. Such studies resulted in latency factors of 3.00 ± 0.50 for the buoyant lysosomal fractions, 7.00 ± 1.00 for the dense lysosomal population, and 1.05 ± 0.05 for the small amount of activity remaining with the plasma membrane/golgi pool in the second density gradient fractionation.

Electron microscopic examination performed on the two particulate fractions isolated from the second density gradient as well as the dense lysosomal fraction obtained from the first density gradient demonstrated significant morphological differences. Figure 2A shows that the plasma membrane/golgi fraction consisted largely of membranous sheets as well as a few small vesicles of 0.1 μm in diameter. However, Figure 2B shows that the buoyant lysosomal fraction contained primarily small, irregularly shaped, apparently empty, vesicles ranging in size from 0.1 to 0.2 μm. In contrast to the buoyant lysosome fraction, electron micrographs of the dense lysosome fraction (Fig. 2C) demonstrated that this material was primarily single membrane bound vesicles ranging in size from 0.1 to 0.3 μm. These vesicles contained a host of membranous structures and appeared similar to cellular organelles considered to be secondary lysosomes [11].

Experiments were conducted to ascertain the intracellular fate of WFA and protein components of the plasma membrane of cells treated with the lectin. Subcellular fractionation was conducted on cells, surface radioiodinated at 4°C and incubated in the presence of nonradioactive WFA (100 μg/ml) at 37°C for either 10 min or 30 min. The results of such experiments are shown in Figure 1. It is evident that radioactivity was detected as early as 10 min within both the buoyant and dense ly-

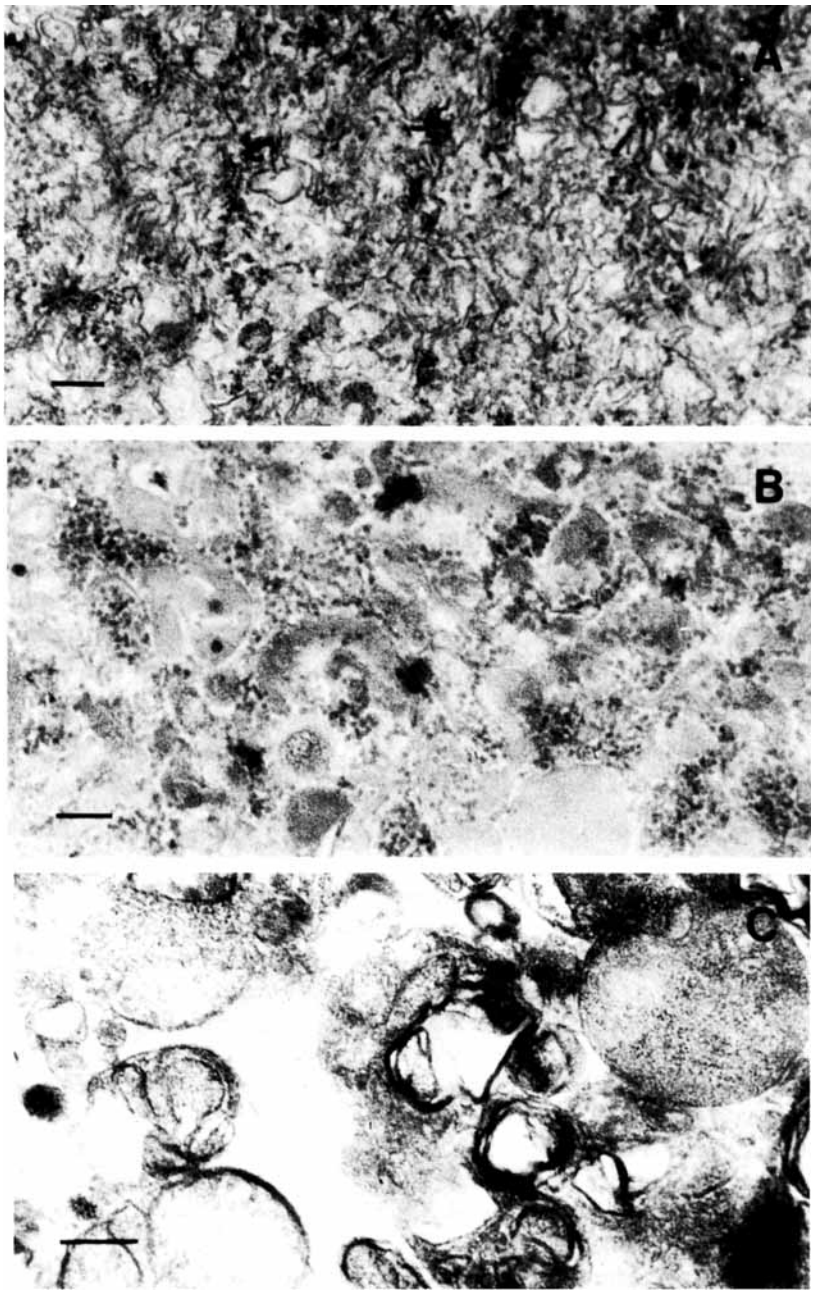

Fig 2 Electron micrographs of subcellular components from 30 and 7 5% "Percoll stock" density gradient fractionation (bar, 0 5 μm) (A) Plasma membrane/golgi fraction from a 7 5% "Percoll stock" gradient (fractions 5–8), (B) buoyant lysosome fraction from a 7 5% "Percoll stock" gradient (fractions 15–20), (C) dense lysosome fraction from a 30% "Percoll stock" gradient (fractions 19–23)

sosome fractions, as well as remaining in the membrane/golgi fraction. Continued exposure of the cells to WFA for 30 min resulted in a 15–25% increase in the amount of radioactivity associated with the dense lysosome pool. The level of radioactivity in the buoyant lysosome fractions remained similar to that seen for a 10-min exposure. In contrast to the localization of the internalized plasma membrane proteins, when nonradiolabeled cells were incubated with ^{125}I-WFA (100 μg/ml; 20,000 cpm/μg) for 10 min the radioactivity associated with the lectin was detected only in the buoyant lysosome fraction as well as the plasma membrane/golgi fraction. Only upon continued incubation of the cells in the presence of ^{125}I-WFA for 30 min did radioactivity appear in the dense lysosome fraction. However, both significant TCA precipitable radioactivity derived from plasma membrane protein and total radioactivity from WFA were also detected in a region between the plasma membrane/golgi pool and light lysosomes which contained virtually no galactosyl transferase or N-acetyl-β-D-glucosaminidase activity. It is noteworthy that the radioactivity associated with either plasma membrane or WFA that migrates in the region of the buoyant lysosomes (1.045 g/ml) consistently peaked one fraction less dense than the enzymatic activity. This difference in the profiles may be due in part to the presence of contaminating dense lysosomes, representing 10–15% of the total enzyme activity and little or no radioactivity, which localized toward the dense side of the vesicles showing N-acetyl-β-D-glucosaminidase activity. The influence of such a contaminant may be the cause of an apparent shift in the enzymatic activity profile. Alternatively, the differences seen may indicate the partial resolution of two or more populations of vesicles containing different proportions of radioactivity and enzyme activity.

DISCUSSION

We are studying the internalization into fibroblasts of WFA to explore the characteristics of adsorptive endocytosis of a single ligand capable of binding to a number of cell surface glycoproteins. In order to localize the ligand and specific WFA reactive plasmalemma glycoproteins within intracellular pools biochemically, we have undertaken a series of experiments centered about the subcellular fractionation of cells exposed to WFA. Application of two sequential self-generating silica sol density gradients to the PNS of cellular homogenates of Balb/c 3T3 fibroblasts has allowed us to resolve completely two discrete vesicular fractions displaying lysosome enzyme activities from the plasma membrane/golgi fraction as well as from a mitochondrial fraction.

Recently, Rome et al [11] obtained a bimodal distribution of vesicles displaying lysosome enzyme activities from human fibroblasts fractionated on a single silica sol density gradient. Although these researchers were unable to resolve the buoyant region of lysosomal enzymes from galactosyl transferase (a golgi marker) and 5′-nucleotidase (a plasma membrane marker), electron microscopic examination in conjunction with cytochemistry and the biochemical localization of an endogenous biosynthetic product led the authors to suggest that the buoyant fraction of lysosome enzyme activities may be derived from golgi endoplasmic reticulum lysosome. GERL was first introduced by Novikoff [20] to describe structures morphologically distinct from lysosomes which stain positive for the cytochemical localization of acid phosphatase and which appear to be associated with the golgi apparatus and the endoplasmic reticulum.

Electron micrographs of the two lysosome populations we have isolated, each lacking detectable galactosyl transferase activity, indicate that the buoyant population of vesicles are similar to that pool described by Rome et al [11] as having the characteristics expected for GERL, and the more dense fraction of vesicles are isolated secondary lysosomes.

Our studies utilizing ^{125}I-WFA and surface iodinated cell monolayers clearly demonstrate that internalized lectin localized initially (within 10 min) in intracellular elements which contained a portion of the internalized plasma membrane proteins. This pool was totally resolved during subcellular fractionation from dense lysosomes which also contained internalized plasma membrane proteins but had no detectable lectin. Only upon prolonged exposure of the cells to WFA was the lectin detected within all subcellular fractions containing internalized plasma membrane proteins. Such data strongly indicate that the endocytosis of WFA differs substantially from the internalization of a portion of, if not all, plasma membrane proteins. Furthermore, the localization of WFA and plasma membrane derived proteins in fractions possessing little, if any, N-acetyl-β-D-glucosaminidase and galactosyl transferase activities indicates that the internalized WFA and some plasma membrane proteins may enter a population of nonlysosomal vesicles.

Recently, Willingham and Pastan [5] and Wall et al [21] have demonstrated that ligands internalized following interaction with receptors associated with clathrin coated regions of the plasma membrane may be localized in single membrane bound vesicles concentrated near the golgi and which appear distinct from lysosomes by phase contrast microscopy and lack of cytochemically detectable aryl sulfatase activity. From video intensified microscopy [5] and ultrastructural examination of hepatocytes exposed to lactosaminated ferritin [21], these researchers have noted that appropriately labeled ligand is localized first in the nonlysosomal vesicles followed by the appearance of ligand within organelles with characteristics of lysosomes.

Similarly, that portion of WFA which binds to glycoproteins associated with clathrin coated regions of the plasma membrane may, upon endocytosis, enter a nonlysosomal population of vesicles. However, improved resolution of presumed populations of vesicles that concentrate in the region of the buoyant lysosomes during density gradient centrifugation, as well as cytochemical localization of WFA within individual fractionated vesicles, is required to clarify this aspect of the endocytosis of the lectin.

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