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Biotin-Mediated Delivery of Exogenous Macromolecules into Soybean Cells[1]

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ABSTRACT

We have demonstrated that attachment of biotin to a variety of macromolecules allows the uptake of those macromolecules into cultured soybean cells (*Glycine max* Merr cv Kent). Macromolecules that were nondestructively delivered into intact cells in large numbers (>10^6/cell) by this technique include bovine insulin (M_r about 5,700), bovine ribonuclease (M_r about 14,000), human hemoglobin (M_r about 64,000), and bovine serum albumin (M_r about 68,000). It is hypothesized that this methodology may be useful for delivering antibodies, toxins, enzymes, and genetic material into living plant cells without requiring prior removal of the cell wall or infection with *Agrobacterium*.

The plant cell wall and plasma membrane are designed to serve as barriers to penetration by pathogens, extracellular macromolecules, toxins, and unwanted salts and solutes. Molecules required for normal cell growth such as nutrients and essential electrolytes are, therefore, commonly taken into the cell by specific membrane transport systems. Consequently, researchers who have needed to introduce exogenous macromolecules such as foreign genes or antibodies into plant cells have been forced to first dismantle the cell wall and then transiently puncture the plasma membrane to facilitate penetration of their foreign material. While such destructive techniques have been effective, producing many valuable results (3), they still suffer both from an inability to successfully impregnate all cells in a suspension and from a tendency to inflict damage on those cells which were successfully transformed (3).

Introduction of foreign macromolecules into animal cells has also met with similar obstacles and results. Unfortunately, cell disruption techniques such as electroporation, calcium phosphate or polyethylene glycol lysis, and detergent permeabilization still predominate as methods of choice for delivering macromolecules into animal cells (5, 16, 20, 21). Recently, however, a much gentler strategy has been developed, where one of the cell's endocytotic pathways is exploited to catalyze the internalization of foreign molecules. By first attaching the desired macromolecule to a protein ligand such as insulin or an asialoglycoprotein, the ligated macromolecule is then delivered into the target cell via the ligand's receptor-mediated endocytosis pathway (11, 17, 22). For example, a chloramphenicol acyl transferase gene has recently been introduced and expressed in rat liver *in vivo* by first linking the gene to an asialoglycoprotein that was targeted to the liver (22).

Unfortunately, examination of the endocytosis literature on plants immediately reveals that hormone and protein uptake systems analogous to those mentioned above are unknown in the plant kingdom. In fact, the only physiological endocytotic pathway described in plants to date is one which transfers elicitors from the cell surface to the vacuole (7). We reasoned, however, that endocytosis pathways must also exist for those essential nutrients that are too polar to passively diffuse across lipid bilayers and too large to penetrate proteinaceous channels. Large, water-soluble vitamins such as folate, vitamin B_{12}, and biotin are members of this category in animal cells (8, 15, 18), and could therefore be envisioned to penetrate plant cells by a similar method. Because covalent derivatization of macromolecules with biotin is relatively easy, and because the necessary biotinylation reagents are commercially available, we decided to explore the use of biotin to codeliver exogenous macromolecules nondestructively into living plant cells. We report here that an endocytosis pathway for biotin does exist in soybeans and that this system can be exploited to transfer large quantities of foreign proteins into intact cultured soybean cells.

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 described previously (1, 9).

Preparation of FITC[2]-Labeled Proteins

To 1 mL of a 1 mg/mL solution of the protein to be labeled was added 0.16 mL of a 1 mg/mL solution of FITC (Sigma Chemical Co.) in dimethylformamide. The reaction was allowed to proceed for 4 h in the dark at room temperature. After 4 h, any unreacted FITC was 'quenched' with 0.01 mL of ethanolamine, and the quenched reaction mixture was dialyzed against distilled H_2O until the dialysate was free of fluorescence.

Preparation of Biotinylated FITC-Labeled Proteins

To 0.5 mL of a 1 mg/mL solution of FITC-labeled protein was added a 10-fold molar excess of *N*-hydroxysuccidimidyl

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[2] Abbreviation: FITC, fluorescein isothiocyanate.

biotin (Pierce Chemical Co.) dissolved in dimethyl sulfoxide. 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 Pharmacia PD-10 column. Analysis of the biotin content of the labeled proteins by the method of (6) demonstrated that this procedure attaches 1 to 5 biotins per molecule, depending on the protein employed.

Assay for the Uptake of Fluorescein-Labeled Biotinylated Proteins

A 7-d-old suspension culture of *Glycine max* Merr cv Kent was gravity filtered using a fine nylon mesh. One cm^3 of loosely packed cells was then transferred to 20 mL fresh W-38 medium and allowed to grow for 24 to 36 h which yielded a cell population in the early exponential growth phase. To these cells was added 20 μg of the desired derivatized molecule, after which the suspension was incubated at the desired temperature. At different time intervals, 1 mL of the cell suspension was removed, vacuum filtered, and washed with 200 mL fresh W-38 medium. The washed cell pellet was then resuspended in 20 mL of fresh W-38 medium and examined under a fluorescence microscope (Olympus BHT). The washing procedure was required to remove nonendocytosed FITC derivatized proteins. Thus, only cells which had internalized the fluorescent proteins remained fluorescent.

Preparation of ^{125}I-Labeled Proteins

To 1 mL of a 300 mM phosphate buffer (pH 7.0), containing three 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 to liberate the active iodine species, according to the supplier's instructions. After activation, 1 mg of desired biotinylated or control protein was added in 0.5 mL of iodination buffer. The iodination was allowed to proceed with stirring for 20 min. After the iodination was complete, the product was isolated via gel filtration on a Pharmacia PD-10 column. Typical iodinations of ribonuclease A (Sigma Chemical Co.) yielded a product emitting 2×10^5 cpm/μg.

Assay of the Uptake of ^{125}I-Labeled Proteins

Soybean suspension culture cells in the early exponential growth phase were obtained as previously described. To each culture was added sufficient ^{125}I-labeled macromolecule to achieve a final concentration of 10 μg/mL, and the suspension was incubated at either 23 or 4°C for the desired time. After the desired incubation period, the cells were incubated for 5 min in growth media rebuffered to pH 8 with 15 mM glycylglycine to remove surface bound ligand. The cell suspension was then filtered, washed with 200 volumes growth media, and placed in counting vials.

Isolation of Internalized ^{125}I-Labeled Biotinylated Proteins

Soybean cells were allowed to internalize ^{125}I-labeled biotinylated serum albumin for 2 h, after which the cells were incubated for 5 min in growth medium rebuffered to pH 8 with 15 mM glycylglycine to remove surface bound ligand. The cell suspension (1 mL) was then filtered, washed with 200 volumes of growth medium, and ground by mortar and pestle with sand in 0.1 mL growth medium containing 1% sodium dodecylsulfate. The extract was boiled for 3 min, centrifuged at 3000*g* for 10 min., and 50 μL of supernatant were applied to a 1 × 16 cm Sephadex G-25 column. The column was developed with 300 mM Na_2HPO_4, 200 mM NaCl (pH 7), and the contents of 0.5 mL fractions were counted for radioactivity.

RESULTS

Uptake of Fluorescein-Labeled Biotinylated Insulin

To directly monitor the internalization of foreign macromolecules by cultured soybean cells, insulin, hemoglobin, and bovine serum albumin were derivatized with FITC and then either labeled with *N*-hydroxysuccinimidyl biotin or left unlabeled. Figure 1 shows the time course of uptake by intact

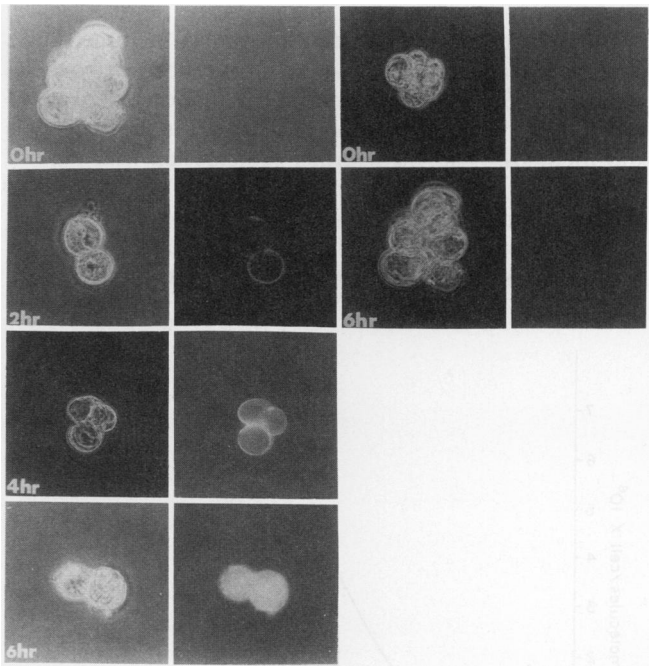

Figure 1. Comparison of the time course of internalization of biotinylated and nonbiotinylated insulin. Phase contrast micrographs (left image) are always paired with the corresponding fluorescence micrograph (right image) of the same field of cells. Cells incubated with biotinylated insulin are shown in the left two columns, while cells incubated with nonbiotinylated insulin are displayed in the right two columns. At the indicated times the cells were separated from their incubation medium, washed, and prepared for fluorescence microscopy as described in "Materials and Methods." Similar results were obtained with bovine serum albumin and human hemoglobin.

soybean cells of biotin-labeled (left set) and control (right set) insulin samples. At each time following addition of the appropriate protein solution, the soybean cells were filtered, washed with 200 volumes of growth medium, and then examined by either phase contrast (left image in each set of micrographs) or fluorescence (right image) microscopy to visualize the location of the fluorescent insulin in the field of cells. As can be seen from the micrographs, the biotinylated insulin first binds to the cell surface (2 h time point, left set) and is then gradually brought into the cell interior (4 and 6 h times, left set). That the labeled protein is indeed taken inside the cells and not simply superficially attached to the cell surface could be ascertained by changing the plane of focus and noting the presence of fluorescence at all depths within the cell. In contrast, the nonbiotinylated control insulin neither bound to the cells nor was internalized to any measurable extent (Fig. 1, right set). Importantly, similar results were also obtained with analogously treated hemoglobin and serum albumin samples (not shown), suggesting the uptake process is independent of the molecular properties of the biotinylated protein. Furthermore, about 95% of the cells displayed the ability to recognize and internalize the biotin-conjugated proteins, demonstrating that the pathway is a common property of most cells in the suspension.

Uptake of ^{125}I-Labeled Biotinylated RNAse A

To obtain a more quantitative measure of the time course of biotin-mediated macromolecule uptake, bovine ribonuclease, bovine serum albumin, and insulin were each derivatized with biotin or left underivatized as described in the "Materials and Methods," and then further radiolabeled with Na ^{125}I prior to introduction into the soybean cell suspension. As seen in Figure 2, after a brief lag period the rate of biotinylated ribonuclease uptake exceeded the rate of control ribonuclease uptake by more than 50-fold, confirming the strong preference

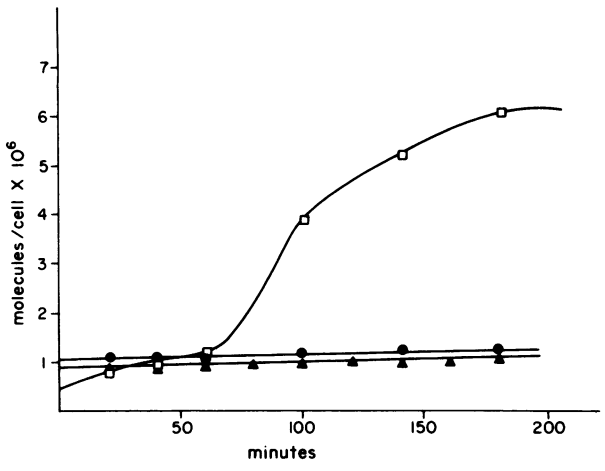

Figure 2. Kinetics of internalization of 1 μg/mL ^{125}I-labeled biotinylated (□) and nonbiotinylated (▲) ribonuclease in the absence (□, ▲) and presence (●) of 1 mM free biotin to compete for cell surface biotin receptors. All other conditions are as described in Figure 1. Similar results were obtained with insulin and bovine serum albumin.

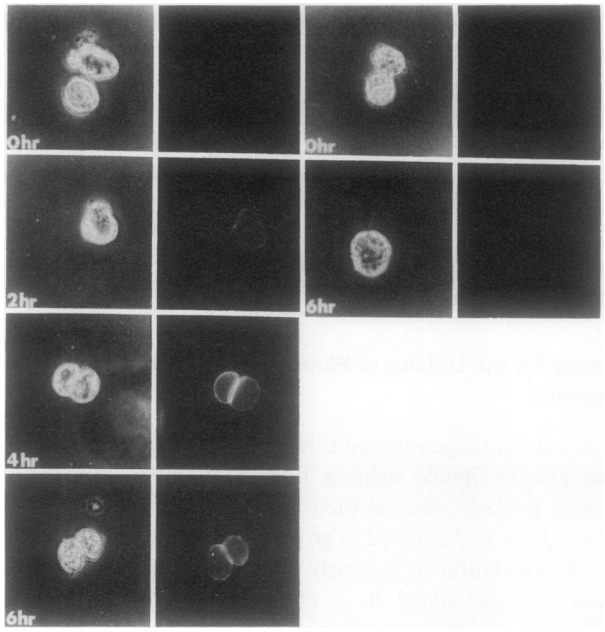

Figure 3. Inhibition of internalization of fluorescein-labeled biotinylated hemoglobin by chilling cells to 4°C. The micrographs are arranged as in Figure 1. The left pairs of micrographs show the phase contrast and fluorescence images of chilled cells treated with 1 μg/mL of fluorescein-labeled biotinylated hemoglobin, while the right set of micrographs shows the corresponding nonbiotinylated controls. Similar results were obtained with insulin and serum albumin. Note that biotinylated hemoglobin binds but is not internalized at 4°C.

of the soybean cells for the biotin-conjugated samples over the control samples. By 3 h of uninterrupted endocytosis, the cells were found to internalize more than 6×10^6 molecules of exogenous ribonuclease per cell. However, eventually even the biotin-mediated endocytotic process appeared to gradually level off, possibly due to internalization or down regulation of available biotin receptors. Again, similar results were also obtained with ^{125}I-labeled serum albumin and insulin.

To confirm that the above protein delivery process was specifically mediated by the attached biotin and not by some other modification incurred during the biotinylation process, the ability of free, unliganded biotin to compete with biotinylated ribonuclease for the uptake system was examined. In this case, the above [^{125}I]ribonuclease uptake study was repeated, only the cells were treated with 1 mM biotin directly prior to addition of the biotinylated ribonuclease. As revealed by the solid dots in Figure 2, no [^{125}I]ribonuclease uptake could be detected after saturating the system with biotin. Therefore, it can be inferred that the internalization process involves recognition of biotin by a limited number of receptors on the plant cell surface.

Inhibition of Internalization of Fluorescein-Labeled Biotinylated Hemoglobin

As mentioned previously, biotin could conceivably be internalized by either carrier-facilitated transport or receptor-mediated endocytosis (13, 18). Although the codelivery of

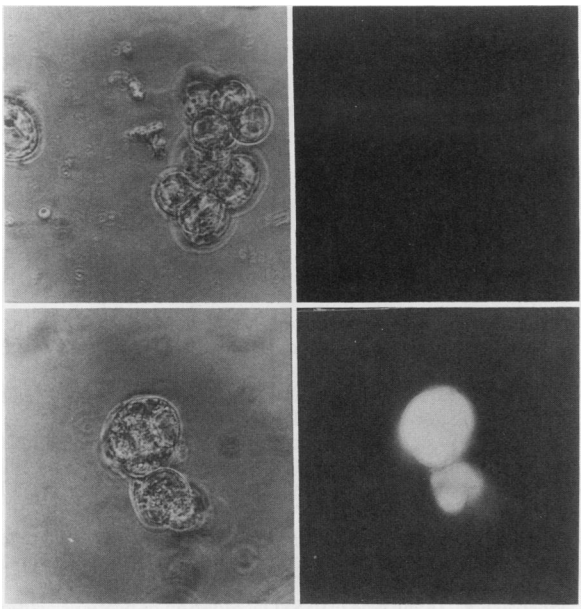

Figure 4. Resumption of internalization of fluorescein-labeled biotinylated hemoglobin after returning chilled cells to room temperature. The micrographs are arranged with the biotinylated test sample and nonbiotinylated control in the lower and upper micrographs, respectively. The micrographs were taken after allowing the cells in Figure 3 to incubate at room temperature for 3 h.

large proteins attached to biotin argues strongly for involvement of the latter mechanism in our studies, we decided to further test this hypothesis by examining the temperature sensitivity of the uptake events. Thus, endocytosis, but not ligand binding in both plants and animals, is characteristically halted by lowering the temperature to 4°C, and this interruption is quickly reversed upon return of the system to room temperature (7, 14, 19). As seen in Figures 3 and 4, these same characteristics were manifested by the biotin-mediated delivery system. Thus, biotinylated hemoglobin (left pairs of micrographs) bound normally to the soybean cells at 4°C, while nonbiotinylated hemoglobin exhibited no affinity for the same cell suspension (right pairs of micrographs). Still, no internalization of the biotinylated hemoglobin was observed even after 6 h of incubation at 4°C; *i.e.* all of the detectable fluorescence was localized at the cell periphery (*cf.* phase contrast and fluorescence image of 4 and 6 h time points). In contrast, upon warming the above cells to room temperature for 3 h, the fluorescent hemoglobin was observed to leave the cell surface and concentrate in the cell interior (Fig. 4, lower pair of micrographs). As usual, no nonbiotinylated hemoglobin either bound to or entered the soybean cells upon warming.

Molecular Nature of the Internalized ^{125}I-Labeled Bovine Serum Albumin

Although the cell surface associated fluorescence was seen to enter the cells in Figures 1 and 4, it was conceivable that the biotinylated proteins were first degraded by a cell surface proteinase and only thereafter internalized as amino acids or small mol wt fragments. To examine this possibility, radiolabeled, biotinylated bovine serum albumin was allowed to bind and enter the cultured soybean cells as above, after which the cells were thoroughly washed, homogenized, and extracted to remove the soluble proteins from the cell's cytoplasm. This cytoplasmic extract was then chromatographed on a Sephadex G-25 gel filtration column to determine whether any small mol wt fragments might be generated during the codelivery process. Figure 5 compares the elution profile of the ^{125}I-labeled material isolated with the cell extract (solid dots) with the profile of unmodified ^{125}I-serum albumin (open squares). While some of the extracted serum albumin migrated at lower mol wt (*i.e.* larger elution volume), clearly the majority of the internalized protein remained intact for at least the 2 h of the internalization study. This suggests that a major fraction of the albumin has entered the cell in its unmodified form. Some digestion of exogenous proteins was, in fact, anticipated, based on known half-lifes of injected proteins in cultured cells (10, 12).

DISCUSSION

In this paper we have described a nondestructive method for introducing exogenous proteins into plant cells. The method requires the prior attachment of the desired macromolecule to biotin, a vitamin which is brought into animal cells by receptor-mediated endocytosis (13, 18). Once the biotin is fixed to the macromolecule, the conjugate is internalized by the plant cell in a manner that is competitively blocked by free biotin. Based on quantitative measurements made with radiolabeled macromolecules, the capacity of the system appears to easily exceed 10^6 molecules/h.

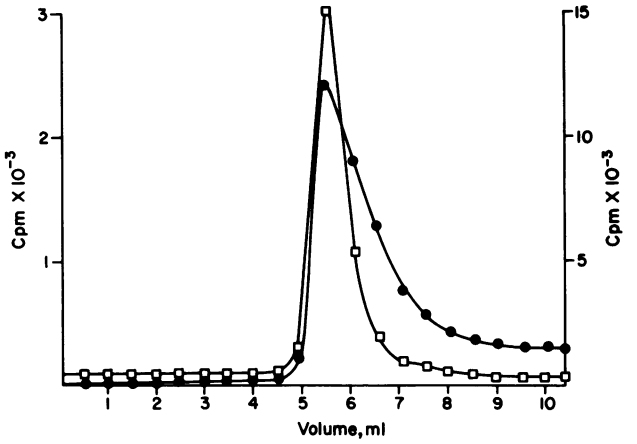

Figure 5. Comparison of the gel filtration elution profile of biotinylated ^{125}I-labeled bovine serum albumin before (□) and after (●) internalization by cultured soybean cells. The right ordinate scales the cpm of control serum albumin while the left ordinate corresponds to the cpm of the internalized sample. Similar results were obtained with ribonuclease and insulin. In each case, the cells were allowed to endocytose the labeled protein for 2 h before preparing the sample of G-25 Sephadex gel filtration chromatography, as described in "Materials and Methods."

Besides characterizing a possible tool for manipulating the intracellular contents of plant cells, the above experiments support our earlier conclusion that receptor-mediated endocytosis occurs in plants (7). In the earlier study, we provided evidence that elicitors which bind to receptors on plant cell membranes are subsequently removed via endocytosis of the receptors. The purpose of the internalization pathway was consequently presumed to be to assist in the clearance of the bound elicitor from the plasma membrane once its signal had been transduced. In the case of biotin uptake, where the ligand is specifically needed in the cell, we hypothesize that receptors exist expressly to bring the desired vitamin into the cytosol for use in metabolism. Thus, even though the capability is thought to exist for each plant cell to manufacture its own vitamins, it may also be possible for some cells to produce more than they require and thereby alleviate the need for other cells to engage in the biosynthetic pathway (2).

Although other macromolecular delivery systems have been developed for use in plants, our biotin-mediated protocol may find application in plant cell biology for the following reasons. First, it is nondestructive. Unlike cells infected with *Agrobacterium*, cells treated with biotin-conjugated proteins appear to show no ill effects of the internalization process. Second, the method can be carried out on intact cells. In contrast to current procedures which exploit the abilities of strong electric discharges, polyethylene glycol, or calcium phosphate to transiently break open protoplasts, our methodology requires no prior removal of the cell wall. This unique feature may eventually prove useful if the process can be adapted for gene delivery, since some plant species can be regenerated more readily from whole cells than from protoplasts (4). Third, the number of molecules internalized is large. Thus, where the capacity of uptake is important, such as might be the case in attempts to manipulate metabolite pools in the cytosol with exogenous enzymes, the vitamin-mediated delivery protocol may be preferred. Finally, the methodology is simple and could conceivably be adapted to numerous applications. While most scientific uses of the protocol remain to be explored, it is conceivable that anti-sense RNA, toxins, enzymes, F_{ab} fragments of antibodies, and individual genes might be delivered into living plant cells by this technique. Research is currently in progress to test these possibilities.

LITERATURE CITED

1. **Apostol I, Low PS, Heinstein PF, Stipanovic D, Altman DW** (1987) Inhibition of elicitor-induced phytoalexin formation in cotton and soybean cells by citrate. Plant Physiol **84:** 1276–1280
2. **Carlson PS** (1970) Induction and isolation of auxotrophic mutants in somatic cell cultures of *Nicotiana tabacum*. Science **163:** 487–489
3. **Draper J, Scott R, Kuman A, Dury G** (1988) Transformation of plant cells by DNA-mediated gene transfer. *In* J Draper, R Scott, P Armtage, R Walden, eds, Plant Genetic Transformation and Gene Expression: A Laboratory Manual. Blackwell, London, pp 161–198
4. **George EF, Sherrington PB** (1984) Plant Propagation by Tissue Culture: Handbook and Directory of Commerical Laboratories. Exegetics Ltd., Hants, England, pp 387–545
5. **Graham FL, van der Eb AJ** (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:** 456–467
6. **Green NM** (1965) A spectrophotometric assay for avidin and biotin based on binding of dyes by avidin. Biochem J **94:** 23c–24c
7. **Horn MA, Heinstein PF, Low PS** (1989) Receptor-mediated endocytosis in plant cells. The Plant Cell **1:** 1003–1009
8. **Kamen BA, Wang MT, Streckfuss AJ, Peryea X, Anderson RGW** (1988) Delivery of folates to the cytoplasm of MA104 cells is mediated by a surface membrane receptor that recycles. J Biol Chem **263:** 13602–13609
9. **Low PS, Heinstein PF** (1986) Elicitor stimulation of the defense response in cultured plant cells monitored by fluorescent dyes. Arch Biochem **249:** 472–479
10. **Moriyasu Y, Taxawa M** (1988) Degradation of proteins artifically introduced into vacuoles of *Chara austraolis*. Plant Physiol **88:** 1092–1096
11. **Poznansky MJ, Hutchison SJ, Davis PJ** (1989) Enzyme replacement therapy in fibroblasts from a patient with cholesteryl ester storage disease. FASEB J **3:** 152–156
12. **Rogers SW, Rehsteiner M** (1988) Degradation of structurally characterized proteins injected into Hela cells: basic measurements. J Biol Chem **263:** 19833–19842
13. **Said HM, Redha R** (1988) Biotin transport in rat intestinal brush-border membrane vesicles. Biochem Biophys Acta **945:** 195–201
14. **Schlessinger J** (1980) The mechanism and role of hormone-induced clustering of membrane receptors. Trends Biochem Sci **5:** 210–214
15. **Seetharam B, Alpers DH** (1985) Cellular uptake of Cobalamin. Nutr Rev **43:** 97–102
16. **Sen R, Baltimore D** (1987) In vitro transcription of immunoglobulin genes in a B-cell extract: effects of enhancer and promoter sequences. Mol Cell Biol **7:** 1989–1994
17. **Uchida T, Mekada E, Okada Y** (1980) Hybrid toxin of the A Chain of the ricin toxin and a subunit of *Wistaraia floribunds* lectin. J Biol Chem **255:** 6687–6693
18. **Vesely DL, Kemp SF, Elders MJ** (1987) Isolation of a biotin receptor from hepatic plasma membranes. Biochem Biophys Res Comm **143:** 913–916
19. **Wileman T, Harding C, Stahl P** (1985) Receptor-mediated endocytosis. Biochem J **262:** 1–14
20. **Willems PHGM, Van Den Broek BAM, Van Os CH, DePont JJHHM** (1989) Inhibition of inositol 1,4,5-triphosphate-induced Ca^{2+} release in permeabilized pancreatic acinar cells by hormone and phorbol ester pretreamtent. J Biol Chem **264:** 9762–9767
21. **Wong TK, Neumann E** (1982) Electric field mediated gene transfer. Biochem Biophys Res Commun **107:** 584–587
22. **Wu GY, Wu CH** (1988) Receptor-mediated gene delivery and expression in vivo. J Biol Chem **263:** 14621–14624