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Ricin linked to monophosphopentamannose binds to fibroblast lysosomal hydrolase receptors, resulting in a cell-type-specific toxin

(plasma membranes/protein transport/hybrid proteins/glycoproteins/cellular compartmentalization)

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ABSTRACT **The receptor specificity of the plant seed toxin ricin, which ordinarily binds to galactose-containing receptors, has been altered by coupling monophosphopentamannose residues to ricin by reductive amination and by reversibly binding lactose to the modified ricin. The added monophosphopentamannose residues provide ricin with the recognition factor common to fibroblast lysosomal hydrolases and enable the modified ricin (Man6*P*-ricin) to bind to the fibroblast Man6*P* receptor and inhibit protein synthesis in the cells via this receptor. The addition of lactose to Man6*P*-ricin saturates the galactose site on Man6*P*-ricin and prevents the binding of Man6*P*-ricin to cells via galactose-containing ricin receptors. The Man6*P* receptor-mediated toxicity of Man6*P*-ricin, identified in human fibroblasts by competition by Man6*P* and blockade by alkaline phosphatase treatment, was not detected in HeLa cells or human amnion cells. Consequently, in the presence of lactose, the fibroblasts were 8 and 13 times more sensitive than amnion and HeLa cells, respectively. These results show that highly toxic cell-type-specific reagents can be made by the proper alteration of toxin receptor specificities. An attempt to construct a highly toxic altered toxin by adding Man6*P* residues to diphtheria toxin fragment A was unsuccessful. A possible explanation is that in Man6*P*-ricin the ricin B chain performs some entry function, even though the initial binding step occurs at the Man6*P* receptor.**

A wide variety of proteins are capable of entering cells by receptor-mediated transport processes. Having gained entry these proteins are directed to specific cellular compartments where they exert either a physiological or pathological function (1).

In general it appears that only a discrete portion of these proteins contains the receptor binding activity that is involved in the entry process, whereas another portion of the protein performs the intracellular function. Therefore, it is possible to split and reassemble two such proteins with a new combination of receptor entry specificity and intracellular function. Such proteins we call artificial hybrid proteins, and previous reports from this laboratory (2–4) have suggested that such hybrids should have utility both as probes of entry processes and as a new class of pharmacologic reagents with custom-made receptor and therefore cell-type specificity.

The functional activity of artificial hybrid proteins has already been demonstrated by this laboratory and others. Thus, the hybrids of *Wistaria* lectin-diphtheria toxin fragment A (5), concanavalin A-diphtheria toxin fragment A (6), and ricin-diphtheria toxin fragment A (4) have all been shown to mediate the entry of the enzymatically active toxin A chain into the cytosol via the lectin receptor with a resulting cessation of protein synthesis or loss of functional elongation factor 2. Hybrid concentrations of 2 nM were sufficient to elicit cytotoxicity. Two attempts have been reported to prepare cell-type specific toxic hybrids by utilizing protein hormones as the receptor-binding moiety. The hybrid human placental lactogen-diphtheria toxin fragment A lacked cytotoxicity at 100 nM when assayed with cells carrying lactogenic receptors, even though the *in vitro* binding and enzymatic functions were largely intact (2, 3). However, the hybrid human chorionic gonadotropin-ricin fragment A prepared by similar methodology showed definite cell-type specific toxicity towards a Leydig cell-derived tumor when assayed at 800 nM (7, 8).

The present work is a further attempt to construct a cell-type-specific cytotoxic reagent utilizing the concepts previously developed for artificial hybrid proteins. This work utilizes as a binding moiety an analog of the receptor recognition factor for fibroblast lysosomal hydrolase binding and uptake. This analog was developed in a previous study of low-density lipoprotein (LDL) uptake by an alternate receptor induced by coupling a foreign recognition factor to LDL (unpublished data). Earlier work by Rogers and Kornfeld (9) and later by Lee and coworkers (10, 11) had shown that carbohydrate recognition factors when attached to various proteins rendered them accessible to the particular receptor-mediated uptake processes. Mannose 6-phosphate had been identified as the recognition factor for fibroblast lysosomal hydrolases both by inhibition of uptake (12, 13) and by isolation of the active phosphorylated oligosaccharide from hydrolases (14). Therefore, a search was made for a similar compound with the same binding specificity which could be easily coupled to LDL. Monophosphopentamannose obtained from *Hansenula holstii* (15) was assayed and found to be a competitive inhibitor of iduronidase uptake by fibroblasts with a K_i of 20–30 μM (E. M. Karson, G. N. Sando, and E. F. Neufeld, personal communication). This compound was then coupled to LDL by reductive amination.

Monophosphotetramannosyldeoxymannitolyl-LDL (Man6*P*-LDL) was found to gain entry to human fibroblasts through the Man6*P* receptor and to effect down regulation of 3-hydroxy-3-methylglutaryl CoA reductase. In addition, Man6*P*-LDL that entered through the Man6*P* receptor was degraded like native LDL. This suggested that the Man6*P*-LDL, like LDL and like the lysosomal hydrolases, is routed to the lysosomal compartment.

Therefore, to extend our work on modifying toxin receptor-binding specificities, monophosphopentamannose residues were linked to the toxin ricin. The monophosphotetramannosyldeoxymannitolyl-ricin (Man6*P*-ricin) was found to bind Man6*P* receptors on cell membranes and subsequently inhibit

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Abbreviations: LDL, low-density lipoprotein; Man6*P*-ricin, monophosphotetramannosyl-1-deoxymannitolyl-ricin; Glc-ricin, maltosyl-1-deoxysorbitolyl-ricin; Man6*P*-diphtheria toxin fragment A, monophosphotetramannosyl-1-deoxymannitolyldiphtheria toxin fragment A.

protein synthesis in these cells. The Man6*P* receptor-mediated toxicity of Man6*P*-ricin was found to be cell-type specific, inhibiting protein synthesis in human fibroblasts but not in human amnion or HeLa cells. In order to assess the effects of the ricin-binding subunit on toxicity mediated through the Man6*P* receptor, Man6*P*-diphtheria toxin fragment A was also constructed.

The results of these experiments show that hybrids can be powerful cell-type-specific reagents and useful probes in discerning the mechanisms of receptor-mediated protein transport into cells.

MATERIALS AND METHODS

Crude diphtheria toxin was obtained from Connaught Medical Research Laboratories, Toronto, Canada, L-[*U*-^{14}C]leucine in 0.01 M HCl (290 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) was from New England Nuclear, and nicotinamide [*adenine-U*-^{14}C]dinucleotide (280 mCi/mmol) was from Amersham. Ricin was purified from seeds of *Ricinus communis* kindly supplied by Deutsche Rizinus Oelfabrik Boley, 415, Krefeld-Uerdingen, Germany, according to Nicolson *et al.* (16) as described (4). Diphtheria toxin fragment A was purified based on the method of Collier and Kandel (17) and Drazin *et al.* (18) as reported (2). Rat liver elongation factor 2 was partially purified at 4°C via the pH 5 enzyme of Moldave (19) as reported (2).

Cell Cultures. Cultured human fibroblasts were kindly supplied to us by Elizabeth Neufeld, National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, MD. All cells were grown in monolayer and used before the 20th passage. Cell cultures were maintained in a 5% CO_2 atmosphere at 37°C in 490-cm^2 roller bottles (Corning) containing 50 ml of Eagle's minimal essential medium with Earle's salts, modified to contain 1.6 g of $NaHCO_3$ per liter and supplemented with nonessential amino acids; penicillin (100 units/ml); streptomycin (100 μg/ml); aureomycin (50 μg/ml); mycostatin (10 units/ml); and 10% non-heat-inactivated fetal calf serum (Associated Biomedic Supplies, Buffalo, NY). L-Glutamine was added to 2 mM immediately before use. Cells were dissociated from stock flasks with 0.075% trypsin in Puck's saline/EDTA (NaCl, 0.14 M/KCl, 5.4 mM/glucose, 5.5 mM/$NaHCO_3$, 4 mM/disodium EDTA, 0.5 mM), and 2×10^5 cells were seeded into 60-mm culture dishes (Costar) containing 2.5 ml of the above growth medium. Experiments were normally done on confluent cultures 7 days after seeding. Human amnion cells were obtained from HEM Research, Bethesda, MD, plated at 2×10^5 cells per dish in 60-mm Costar dishes, and grown to confluence in 14 days in the medium described for fibrobasts. The S3 strain of HeLa cells was obtained from Flow Laboratories. Cells were grown in spinner cultures in Eagle's minimal essential medium with modified Earle's salts for suspension culture (GIBCO) containing 10% fetal calf serum. Cells were maintained between 5×10^5 and 1×10^6 cells per ml.

Protein Synthesis Assay. HeLa cells were resuspended in fresh media at 1×10^6 cells per ml and dispensed in 1-ml aliquots into 35-mm petri dishes (Costar). Fibroblasts or amnion cells in 60-mm dishes were incubated with 2 ml of fresh medium. For assays with 100 mM lactose present, 100 μl of medium from HeLa cells or 200 μl of medium from fibroblasts and amnion cells was removed and replaced with 1 M lactose suspended in medium. Other sugars were added in 50 μl of medium. Toxins were added to the dishes in 50 μl of 10 mM Na_2HPO_4 buffer, pH 7.5. The cells were incubated at 37°C in 5% CO_2 for 3 hr and again for 1.5 hr after adding 100 μl of medium containing L-[*U*-^{14}C]leucine at 9, 18, and 22 μCi/ml for HeLa cells, fibroblasts, and amnion cells, respectively. The medium was removed and the attached monolayer of cells was washed twice with 5% trichloroacetic acid and solubilized with 1 ml or 2 ml (for 35-mm and 60-mm-diameter plates, respectively) of 0.05 M Na_2CO_3/2% sodium dodecyl sulfate/0.01 M EDTA. The samples were counted in toluene/Fluoralloy/Biosolve (Beckman) by using a narrow ^{14}C window.

Preparation of Monophosphopentamannose. Phosphomannan extracted from the yeast *Hansenula holstii* NRRL Y-2448 was a generous gift from M. E. Slodki, Northern Regional Research Laboratory, Peoria, IL. Monophosphopentamannose was prepared essentially as described (20) and has the following structure (20, 21):

P-6-Man-α-(1 → 3)-Man-α-(1 → 3)-
Man-α-(1 → 3)-Man-α(1 → 2)-Man.

After hydrolysis and ethanol precipitation, the isolated Ba^{2+} salt of the pentasaccharide with residual core contamination (3–5%) was applied to a column of Sephadex G-25 superfine (100 × 1.5 cm) and eluted with 0.1 M acetic acid. The slow-moving component was pooled and stored as lyphilized powder. Conversion to Na^+ salt was effected by passing a solution of Ba^{2+} monophosphopentamannose over Dowex 50 (H^+) and then neutralizing the effluent with NaOH.

Coupling of Monophosphopentamannose and Maltotriose to Toxins. Covalent linkage of carbohydrate to toxins was accomplished by reductive amination of the Schiff base between C-1 of the reducing terminal sugar residue and a free amino group on the protein (22). Carbohydrates (0.2 M) were mixed with ricin (15 mg/ml) or diphtheria toxin fragment A (10 mg/ml) and $NaCNBH_3$ (159 mM) in *N,N*-bis(2-hydroxyethyl)glycine (bicine) (50 mM, pH 9) and incubated for 24 hr at 37°C and then dialyzed at 4°C against 10 mM Tris·HCl, pH 7.5.

The phenol/sulfuric acid method (23) was used to analyze carbohydrate content. Protein concentrations were determined by the method of Lowry *et al.* (24) or by ultraviolet absorption at 280 nm. The conversion factor used for A_{280} of 1 mg of protein per ml was 1.18 for both ricin (25) and diphtheria toxin fragment A (26).

RESULTS

Monophosphopentamannose and maltotriose were linked to ricin by reductive amination (22). Carbohydrate analysis revealed Man6*P*-ricin and Glc-ricin to contain 7 and 3 added carbohydrate moieties, respectively.

Man6*P*-Ricin Toxicity is Lactose Insensitive. Ricin, Glc-ricin, and Man6*P*-ricin were equally potent as inhibitors of protein synthesis in cultured human fibroblasts, with 30 ng of toxin per ml required to produce a 50% inhibition (Fig. 1*A*). These data indicate that there is no apparent loss in activity of ricin upon carbohydrate attachment.

Assay of activities of ricin and the two modified derivatives was performed in the presence of 100 mM lactose, a competitive inhibitor of ricin binding (27, 28) (Fig. 1*B*). This revealed that, whereas toxicity of both ricin and Glc-ricin was inhibited by lactose, the effect on Man6*P*-ricin toxicity was minimal. Fig. 1 *A* and *B* shows that 30-fold higher concentrations of ricin or Glc-ricin are required to inhibit protein synthesis by 50% in the presence of lactose than without, whereas only a 2-fold higher Man6*P*-ricin concentration is required. Thus, in the presence of lactose, Man6*P*-ricin is 15-fold more toxic than either ricin or Glc-ricin.

Man6*P*-Ricin Toxicity is Mediated Through Man6*P* Receptors. In the presence of lactose, 100 ng of Man6*P*-ricin per ml inhibited protein synthesis to 20–25% of control levels (Fig. 1*B* and Table 1), whereas the same concentration of ricin had

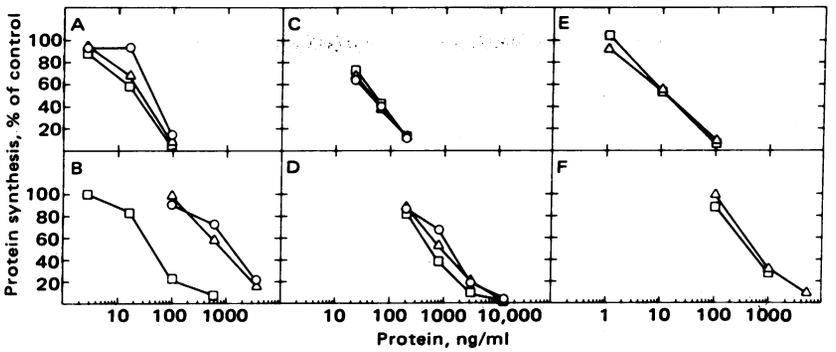

FIG. 1. Dose response curves of inhibition of protein synthesis by ricin (△) Man6*P*-ricin (□), and Glc-ricin (○). Results are shown for cultured cells in the absence of lactose (*A*, *C*, and *E*) and in the presence of 100 mM lactose (*B*, *D*, and *F*) for fibroblasts (*A* and *B*), HeLa cells (*C* and *D*), and amnionic epithelial cells (*E* and *F*). Protein synthesis was determined by L-[*U*-^{14}C]leucine incorporation and was referenced to controls without toxins with or without lactose. Points are the means ± (SD) 10.5% of triplicate samples.

no effect. At this concentration, in the presence of lactose, Man6*P*-ricin toxicity was completely inhibited by 1 mM Man6*P*, partially inhibited by Glc6*P*, and not significantly inhibited by mannose (Table 1). The sugars exhibit the same rank order capacity to inhibit binding and uptake of lysosomal hydrolases into fibroblasts (12, 13). Treatment of Man6*P*-ricin with alkaline phosphatase under conditions that inactivate uptake of lysosomal hydrolases into fibroblasts (12, 13) completely destroyed the ability of Man6*P*-ricin to inhibit protein synthesis in the presence of lactose (Table 1). Thus, the lactose-insensitive capacity of Man6*P*-ricin to inhibit protein synthesis appears to be mediated through the binding of Man6*P* receptors.

Fibroblast Cell-Type Specificity of Man6*P*-Ricin. In the previous sections it was shown that (in the presence of lactose) Man6*P*-ricin is 15-fold more toxic than either ricin or Glc-ricin when assayed in fibroblasts and that this enhanced toxicity is directly due to the presence of the Man6*P* residues on Man6*P*-ricin. When the same experiments were repeated with either HeLa cells or epithelial-like primary human amnion cells, Man6*P*-ricin showed no enhanced cytotoxity over ricin or Glc-ricin (Fig. 1 *D* and *F*). Fibroblasts were markedly more sensitive to Man6*P*-ricin (in the presence of lactose) than were either HeLa or amnion cells. By comparing the concentration of Man6*P*-ricin required for 50% inhibition of protein synthesis in the presence of lactose, we found 56 ng/ml for fibroblasts, 700 ng/ml for HeLa cells, and 440 ng/ml for amnion cells, or a difference of 13- and 8-fold, respectively (Fig. 1). In the absence of lactose the sensitivities of these cell types for Man6*P*-ricin, ricin, or Glc-ricin are within a factor of 4 (Fig. 1 *A*, *C*, and *E*).

The reason for the fibroblast-specific cytotoxicity of Man6*P*-ricin has not been completely elucidated. However, a simple model consistent with the data states that fibroblast surface membranes contain the Man6*P* receptor and transport functions (12, 13) whereas HeLa and amnion cells lack either one or both of these components (not presently known).

Man6*P*-Ricin Toxicity May Require Some Function Provided by the Ricin B Chain. The data in the previous sections indicates that Man6*P*-ricin toxicity (in the presence of lactose) is mediated by Man6*P* receptors. To explore the mechanism of this novel toxicity we attempted to link Man6*P* residues to purified ricin A chain which lacks the binding (B) subunit. The ricin A chain was not stable to the amination procedure. We therefore linked Man6*P* residues to the more stable A fragment of diphtheria toxin. The Man6*P*-diphtheria toxin fragment A was found to contain six Man6*P* residues per toxin molecule. This material had less than 0.1% of the cytotoxic activity of Man6*P*-ricin towards fibroblasts, even though over 25% of the NAD:elongation factor 2 ADP-ribosyl transferase activity was retained. Also, at least 50% of the binding activity towards the Man6*P* receptor was present, as judged by the ability of Man6*P*-diphtheria toxin fragment A to competitively inhibit the toxicity of Man6*P*-ricin in the presence of lactose. Thus, the Man6*P*-diphtheria toxin fragment A appears to differ markedly in cytotoxicity from the Man6*P*-ricin. The reason for this difference remains an important point for further study. One interesting possibility is that the B subunit of ricin is somehow essential for Man6*P* receptor-mediated toxicity of Man6*P*-ricin.

It was considered possible that the above experiment and tentative conclusion was flawed due to two populations of Man6*P*-diphtheria toxin fragment A, a population that bound but was enzymatically inactive and the converse population. Therefore, fractionation of Man6*P*-diphtheria toxin fragment A on hydroxyapatite columns was attempted. Elution with a phosphate gradient gave a single peak at about 0.15 M phosphate for both diphtheria toxin fragment A and Man6*P*-diph-

Table 1. Inhibition of Man6*P*-ricin toxicity in fibroblasts

Additions to medium	Protein synthesis with various additions, % of control				
	Ricin	Man6*P*-ricin	Ricin + phosphatase	Man6*P*-ricin + phosphatase	Glc–ricin
None	10	7	6		10
Lactose (100 mM)	98	25		93	98
Man6*P* (1 mM)	12	6			
Lactose (100 mM) + Man6*P* (1 mM)		99			
Lactose (100 mM) + Glc6*P* (1 mM)		51			
Lactose (100 mM) + mannose (1 mM)		33			

Ricin, Man6*P*-ricin, and Glc-ricin concentrations are 100 ng/ml. Protein synthesis was determined by L-[*U*-^{14}C]leucine incorporation and is referenced to controls incubated without toxins with the appropriate sugars. The values are means ± (SD) 10% of triplicates. Man6*P*-ricin and ricin (0.5 ml, 4 μg/ml) were incubated with 1 unit of alkaline phosphatase (Sigma *Escherichia coli* Type III-R) in 10 mM Tris·HCl (pH 8.2) at 37°C for 1 hr as described (13).

theria toxin fragment A, the latter peak being broadened and slightly retarded. The specific activity of ADP-ribosyl transferase across both peaks was constant, and therefore no evidence for heterogeneity was obtained, although this possibility has not been excluded.

DISCUSSION

This study shows the feasibility of constructing highly toxic cell-type specific reagents by the coupling of the proper receptor-binding moiety to a native toxin. In the present case ricin is used as the native toxin, which exhibits equal toxicity towards fibroblasts and HeLa cells. Monophosphopentamannose is used as the receptor-binding moiety and is an analog for the recognition factor for fibroblast lysosomal hydrolases. The new reagent, Man6*P*-ricin, is incubated with cells in the presence of lactose to prevent binding and entry via the ricin receptor route. Under these conditions Man6*P*-ricin is 13-fold more toxic toward fibroblasts than toward HeLa cells. The separation of dose–response curves is such that at 100 ng of Man6*P*-ricin per ml no toxicity is detected in HeLa cells, whereas protein synthesis is reduced by 80% in fibroblasts. This degree of selectivity should be sufficient to utilize Man6*P*-ricin for the isolation of Man6*P* receptor$^-$ and transport$^-$ mutant fibroblasts.

The utility of this type of hybrid reagent will ultimately depend on the maximum degree of selectivity achievable. In the present study 100 mM lactose does not completely block entry via the recin receptor-mediated transport system, and thus selectivity is limited.

Our data suggest that the ricin B chain may be necessary for Man6*P* receptor-mediated cytotoxicity. This compels us to consider several models. In one, the M6P receptor concentrates Man6*P*-ricin at the membrane where it can interact with the ricin receptor and transport system, and entry is achieved through the normal ricin route. Another model has entry occur through the Man6*P* receptor-mediated transport system. Subsequent steps in this model may involve the B chain of ricin binding to an intracellular ricin receptor which directs specifically bound proteins to the cytosol compartment. Alternatively, the B chain may act solely as a protective agent, preventing inactivation of the catalytic site on the A chain during the entrance to the cytosol. Certain receptors that perform discrete functions at the plasma membrane have also been found localized on intracellular organelles, and possible roles for these intracellular receptors in directing internalized proteins have been discussed (29, 30). It is our belief that hybrid protein technology offers a powerful tool for elucidation of the sequence of events of protein internalization from receptor binding to specific intracellular compartmental localization.

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