F protein induced fusion of Sendai viral envelopes with mouse teratocarcinoma cells through Le^x-Le^x interaction

Mukesh Kumar, Debi P. Sarkar*

Department of Biochemistry, University of Delhi South Campus, Benito Juarez Road, New Delhi - 110021, India

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Abstract The efficiency of membrane fusion between reconstituted Sendai viral envelopes containing only the fusion protein (F-virosomes) and the plasma membrane of mouse teratocarcinoma cells (F9) in culture was assessed using an assay based on the relief of self-quenching of a lipid probe incorporated in the F-virosomes. The potential of F-virosomes was also evaluated for a targeted cytosolic delivery of lysozyme to F9 cells. [^{125}I]Lysozyme entrapped into F-virosomes was taken to examine its fusion-mediated transfer to the F9 cells. Target specificity of the F-virosomes was confirmed by the interaction between the terminal Le^x moiety (Galβ1 → 4(Fucα1 → 3)GlcNAc) of F protein and the Le^x determinant on the membrane of F9 cells. Incubation of the loaded F-virosomes with cells led to fusion-mediated delivery, as inferred from the ability of cells to internalize lysozyme in the presence of azide (a potent inhibitor of endocytosis). These results suggest that carbohydrate-carbohydrate interaction is strong enough for target cell recognition followed by phospholipid bilayer melding induced by fusion glycoprotein of Sendai virus.

Key words: Sendai; Virosome; Le^x/CD15; Membrane fusion

1. Introduction

Le^x or CD15 determinant (α1 → 3 fucosylated type 2 carbohydrate chain) has been shown to function as an adhesion molecule capable of Ca^{2+}-mediated homotypic binding [1]. Cells with high surface expression of Le^x, like mouse teratocarcinoma cells, F9, therefore exhibit strong self-aggregation (based on Le^x-Le^x interaction) in the presence of Ca^{2+} [2]. Le^x and related structures are regarded as human tumor-associated carbohydrate antigens, being highly accumulated in various human tumors [3]. These structures have also been suggested to be involved in the activation of platelets by tumor cells which in turn is correlated with malignancy [4,5]. A monoclonal antibody against carbohydrate antigen Le^y (α1 → 2 fucosylated derivative of Le^x), chemically linked to a genetically engineered form of *Pseudomonas exotoxin,* has been recently demonstrated to have excellent anti-tumor activity against Le^y-positive tumors [6]. The possibility of targeted anti-tumor chemotherapy using Le^x as a ligand is recognized widely.

The fusion protein (F) of the Sendai viral envelope is a glycoprotein containing biantennary chains with Le^x determinants (Galβ1 → 4(Fucα1 → 3)GlcNAc) in the outer chain moieties [7]. We have recently demonstrated that reconstituted Sendai viral envelopes containing the F protein (F-virosomes) can fuse and efficiently deliver entrapped aqueous markers to target cells in the presence of targeting ligand like lectins [8], or by virtue of terminal galactose-asialoglycoprotein receptor interaction in case of liver cells both in vitro [9,10] and in vivo [11]. In the present work, we have prepared F-virosomes containing a fluorescent lipid in its membrane or loaded with [^{125}I]lysozyme. These virosome can efficiently fuse with F9 cells, as exhibited by the lipid mixing assay, and microinject the encapsulated [^{125}I]lysozyme in the cell cytoplasm by means of Le^x-Le^x interaction. The efficiency of F-virosome as a selective carrier of drugs to various Le^x-bearing tumor cells is discussed.

2. Materials and methods

2.1. Reagents

N-4-Nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine (NBD-PE) was purchased from Avanti (Birmingham, AL). SM2 Bio-Beads were obtained from Bio-Rad (Richmond, CA). Triton-X-100 (TX-100) was obtained from Aldrich (Milwaukee, WI). Lysozyme (chicken egg white, IUB No. 3.2.1.17), DTT, sodium azide and EDTA (disodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), fetal calf serum (FCS), trypsin-EDTA, penicillin-streptomycin were obtained from Gibco (Grand Island, NY, USA). Carrier-free Na^{125}I was obtained from BARC, Bombay, India. All other reagents used were of analytical grade.

2.2. Virus

Sendai virus was isolated and its activity was determined as previously described [8].

2.3. Cells

Mouse teratocarcinoma F9 cells (from ATCC, Bethesda, MD, USA) were grown on gelatin-coated plates in DMEM containing 4.5 g/l glucose and supplemented with 10% FCS (v/v). The plates were prepared by incubating 0.01% gelatin in water for 2 h at 4°C followed by washing once with water. Chinese hamster ovary (CHO) cells (Le^x-deficient) were grown at 37°C, 5% CO_2 in DMEM. Single-cell suspensions of F9 and CHO cells were made by lifting them from monolayers with 10 mM EDTA in DPBS. The cells were then washed three times with DPBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and finally suspended in the same buffer.

2.4. Radioiodination of lysozyme

Lysozyme was radiolabeled with the chloramine-T method, using Na^{125}I as the iodine source [12]. The labeled protein was separated from free iodine by gel filtration through a Sephadex G-25 column. The specific activity of the labeled protein was calculated from the TCA-precipitable counts and expressed as cpm/mg protein.

2.5. Preparation of NBD-PE-labeled and loaded F-virosomes

Reconstituted Sendai viral envelopes containing the F protein (F-virosomes) were prepared essentially as described by Bagai et al. [8].

*Corresponding author. Fax: (91) (11) 6885270 or 6886427.
E-mail: BIC_DUSC@DBT.ernet.in

Abbreviations: F, fusion factor; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; TX-100, Triton X-100; DTT, dithiothreitol; NBD-PE, *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine; FCS, fetal calf serum

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NBD-PE was incorporated into the virosomes as described earlier [8]. Briefly, the NBD-PE dissolved in chloroform was dried in a glass vial under nitrogen to form a thin film. The supernatant from the detergent extract of the virus, containing only the F protein and lipids, was added to the NBD-PE film and incubated at 20°C for 30 min with gentle shaking. The detergent was removed by stepwise addition of SM2 Biobeads as described earlier [8]. Loaded F-virosomes containing [^{125}I]lysozyme were prepared as described by Bagai and Sarkar [10]. The unentrapped protein was separated by repeated centrifugation at 100 000 × *g* for 1 h at 4°C. Radioactivity associated with virosomes was estimated using 1275 Minigamma Counter (LKB, Wallac). SDS-PAGE was carried out to test the purity of F-virosomes.

2.6. Spectrofluorometric measurements

The fusion of NBD-PE labeled F-virosomes with F9 cells was carried out essentially as described earlier [8]. The virosomes (25 μg of protein) were mixed with 10^7 cells in 1 ml of DPBS containing 1 mM Ca^{2+} and 1 mM Mg^{2+} and incubated at 4°C for 40 min. The virosome cell complexes were washed three times at 300 × *g* in the same buffer to remove unbound virosomes. This suspension was then incubated for various time points at 37°C. Fluorescence changes as a result of fusion of NBD-PE-labeled F-virosomes with F9 cells, at various time points, were measured with a spectrofluorometer (model RF 540, Shimadzu Corp., Kyoto, Japan). 100 μl of the virosome-F9 cell suspension (after various times of incubation at 37°C) was placed into a cuvette containing 3 ml DPBS and fluorescence measured at 560/590 nm (excitation/emission) wavelength. The fluorescence dequenching (%FDQ) data were normalized and calculated according to the following equation: $\%FDQ = 100 \times (F - F_0/F_T - F_0)$; where F and F_T are the fluorescence (arbitrary) units at a given time point and in the presence of 0.1% TX-100, respectively, and F_0 is the observed fluorescence after incubation at 4°C. As controls, the binding and fusion assays were done in the presence of 10 mM sodium azide, or 50 mM EDTA in DPBS. As another control, heat-treated F-virosomes (incubated at 56°C for 20 min) were used to study binding and fusion with F9 cells in the presence or absence of 10 mM sodium azide. To check the specificity of interaction, F-virosomes were incubated with CHO cells under similar conditions as those for F9 cells.

2.7. Fusion-mediated delivery of [^{125}I]lysozyme into F9 cells

Single-cell suspensions of F9 cells were made as described above and suspended in DPBS to obtain 2×10^6 cells/ml. Viability of the cells was checked by staining with Nigrosin [13]. Loaded F-virosomes (200 μg F protein in 0.2 ml DPBS with 1 mM Ca^{2+} and 1 mM Mg^{2+}, containing 0.8–1 μg [^{125}I]lysozyme, 5×10^7 cpm/mg) were incubated with 1 ml F9 cells (2×10^6) in culture tubes for 40 min in ice. The virosome-cell mixtures were then washed with chilled DPBS thrice to remove unbound virosomes. The virosome-cell suspensions were then suspended in DPBS containing 1 mM Ca^{2+} and Mg^{2+} and incubated at 37°C for 30 min. The cells were then pelleted at 1000 × *g* for 10 min at 4°C and finally incubated for 10 min at 4°C in DPBS containing 50 mM EDTA (stripping) to remove the cell surface bound (unfused) virosomes. The cell pellet after EDTA stripping was solubilized in the extraction buffer (10 mM Tris·Cl, 150 mM NaCl, pH 7.4 containing 1% TX-100 and 1 mM PMSF) and the cell associated radioactivity was determined. As control, binding and fusion was carried out using heat-treated F-virosomes. Sodium azide is known to be an efficient inhibitor of the endocytotic process [14]. Binding and fusion of F-virosomes and heat-treated F-virosomes with F9 cells were studied in the presence of 10 mM azide as described earlier [10].

2.8. Cell fractionation

F-virosomes (untreated or heat-treated, 2 mg F protein in each case) were incubated with F9 cells (2×10^7) at 37°C for 30 min. After washing three times with DPBS and EDTA stripping, the cells were subjected to subcellular fractionation following a published procedure [15]. In brief, the cells were resuspended in isotonic homogenizing buffer (0.01 M Tris·Cl, pH 7.4 containing 0.25 M sucrose) and then dispersed in a Potter-Elvehjem type homogenizer at 4°C. The isolation of nuclear, lysosomal/mitochondrial and plasma membrane fractions was carried out by differential centrifugation at 600 × *g*, 15 000 × *g* and 100 000 × *g* at 4°C for 10, 30 and 60 min, respectively. Cytosolic fraction comprised the supernatant obtained after the final centrifugation (100 000 × *g*). Subcellular fractions were further characterized by marker enzyme analysis [16]. Radioactivity associated with each fraction was analyzed and expressed as percent of total amount of radioactivity associated with the whole cells.

3. Results

3.1. Fusion of F-virosomes with F9 cells

It has been shown earlier by our laboratory that F-virosomes can bind and fuse efficiently with HepG2 cells through the interaction of the terminal galactose of F with the ASGP-R [8–10]. We used a similar system to analyze the binding and fusion of NBD-PE-labeled F-virosomes with F9 cells by incubating the virosomes with F9 cells for 30 min at 37°C, taking binding and fusion in the presence of 10 mM sodium azide or 50 mM EDTA, or taking heat-treated F-virosomes, as controls. Fig. 1 shows that F-virosomes fused efficiently with F9 cells even in the presence of 10 mM sodium azide in DPBS. Incubation of the F-virosome-F9 cells mixtures in

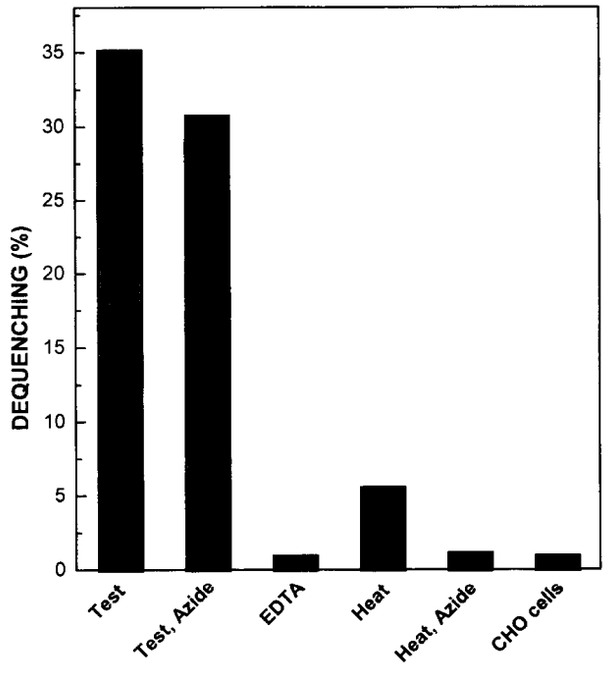

Fig. 1. Fusion of F-virosomes and heat-treated F-virosomes with F9 cells via Le^x-Le^x interactions. NBD-PE-labeled F-virosomes (25 μg of protein) (untreated or heat-treated) were incubated at 4°C for 40 min with 10^7 F9 cells (or CHO cells) in 1 ml of DPBS containing either 1 mM Ca^{2+} and 1 mM Mg^{2+} (without or with 10 mM azide) or 50 mM EDTA. The virosome-cell complexes were washed three times at 300 × *g* (4°C) in the same buffer to remove unbound virosomes. 100 μl of the virosome-F9 cell suspension was placed into a cuvette containing 3 ml chilled PBS and fluorescence was measured at 560/590 nm (excitation/emission) wavelength with a spectrofluorometer (model RF 540, Shimadzu Corp., Kyoto, Japan). The rest of the suspension was incubated at 37°C for 30 min. Fluorescence changes as a result of fusion of NBD-PE-labeled F-virosomes with F9 cells (or CHO cells) were measured as described above. Percent fluorescence dequenching (%FDQ) was calculated as described in Section 2.6. Test, F-virosomes in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+}; Test,Azide, F-virosomes in the presence of 1 mM Ca^{2+}, 1 mM Mg^{2+} and 10 mM azide; EDTA, F-virosomes in the presence of 50 mM EDTA; Heat, heat-treated F-virosomes in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+}; Heat,azide, heat-treated F-virosomes in the presence of 1 mM Ca^{2+}, 1 mM Mg^{2+} and 10 mM azide; CHO cells, F-virosomes incubated with CHO cells in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+}. The values are means of duplicate determinations.

50 mM EDTA led to complete dissociation (>98%) of the bound virosomes from the surface of F9 cells ('EDTA stripping') (data not shown). Hence the presence of 50 mM EDTA led to complete inhibition of fusion. The fusion activity observed with heat-treated F-virosomes is negligible (<2%) in the presence of sodium azide. The little fluorescence dequenching (ca. 5%) observed in the case of heat-treated F-virosomes may be due to endocytosis of bound non-fusogenic virosomes. Negligible fusion activity (<2%) was obtained with CHO cells as target. To provide some indication of the reproducibility and variation between experiments, we performed additional experiments (Fig. 2). Fig. 2 shows that the rate of fusion of the F-virosomes with F9 cells was almost same in the presence or absence of 10 mM sodium azide indicating the occurrence of the fusion event at plasma membrane level. Heat-treated F-virosomes in the presence of 10 mM sodium azide exhibited no fusion activity.

3.2. *Internalization of [^{125}I]lysozyme into F9 cells delivered through F-virosomes: fusion versus endocytosis*

Uptake of [^{125}I]lysozyme by F9 cells at 37°C was examined as a function of dose of F-virosomes. Fig. 3 shows a saturation type curve representing EDTA-resistant cell-associated lysozyme. Half of the maximal cell-associated radioactivity was obtained with about 200 μg of F-virosomes and this was employed in all subsequent experiments. Both F-virosomes and heat-treated F-virosomes were able to bind to F9 cells to the same extents at 4°C (data not shown). This suggests that heat denaturation of F protein has no effect on its recognition by Le^x. However, heat treatment of F protein renders it fusion-inactive [10]. The observed internalization of lysozyme in the presence of 10 mM azide was apparently due to an effective fusion of F-virosomes with F9 cells induced by the F protein at plasma membrane level. Comparing the uptake with F-virosomes (without azide), the internalization by fusion mode was found to be about 80% (Fig. 3). However, in the case of heat-treated F-virosomes the uptake was negligible in the presence of 10 mM azide (Fig. 3).

3.3. *Subcellular distribution patterns of the [^{125}I]lysozyme delivered by F-virosomes and heat-treated F-virosomes: cytosolic delivery of microinjected marker*

As stated above, about 80% of the total amount of radioactivity (EDTA-resistant) associated with the F9 cells was retained in the presence of azide, indicating thereby the vital role played by the fusion mode in the delivery of lysozyme by F-virosomes. Table 1 shows the distribution of radioactivity among various organelles of F9 cells. About 75% of the total cell-associated radioactivity was recovered in the cytosolic fraction using F-virosomes as carrier. Furthermore, the recovered cytosolic radioactivity could be precipitated (>98%) by

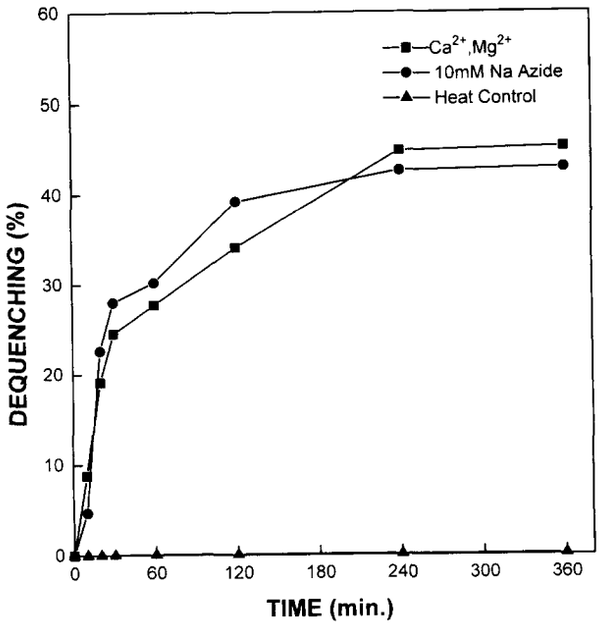

Fig. 2. Kinetics of fusion of F-virosomes with F9 cells in the presence and absence of azide. For details about the incubation of virosomes and fluorescence measurements, see the legend to Fig. 1. ■, F-virosomes in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+}; ●, F-virosomes in the presence of 1 mM Ca^{2+}, 1 mM Mg^{2+} and 10 mM azide; ▲, heat-treated F-virosomes in the presence of 1 mM Ca^{2+}, 1 mM Mg^{2+} and 10 mM azide. Each value is the mean of duplicate determinations.

10% trichloroacetic acid and specific anti-lysozyme polyclonal antiserum. By contrast, 80% of the radioactivity was recovered in lysosomal/mitochondrial fraction with a concomitant decrease (84%) in the cytosolic radioactivity in the case of heat-treated F-virosomes. These results strongly support the fusion-mediated delivery of lysozyme by F-virosomes to F9 cells. Heat-treated F-virosomes being non-fusogenic are likely to be taken up by endocytosis leading to their accumulation and subsequent degradation in lysosomes.

4. Discussion

The present study demonstrates the potential of F-virosomes in the fusion-mediated transfer of entrapped aqueous markers to the cytosol of F9 cells following the corresponding mixing of the two apposing bilayer membranes. F, the only glycoprotein of these virosomes, contains complex type sugar chains, viz. Galβ1→4(Fucα1→3)GlcNAc residues in their outer chain moieties [7]. This is known as the Le^x determinant which can interact specifically with Le^x moieties present on the F9 cells [1]. As a result of this specific attachment, the F-

Table 1
Distribution pattern of microinjected [^{125}I]lysozyme over subcellular fractions of F9 cells

Virosomes	% Recovery of radioactivity			
	Nuclear	Lysosomal/mitochondrial	Cytosolic	Membrane
F-Virosomes	9.6	12.4	75.1	2.1
Heat-treated F-virosomes	3.9	80.1	12.5	4.3

F9 cells (2×10^7) were incubated with F-virosomes or heat-treated F-virosomes (2 mg of F protein) in DPBS containing 1 mM Ca^{2+} and 1 mM Mg^{2+} for 40 min at 4°C followed by washing and further incubation for 30 min at 37°C. The cells were EDTA stripped and cell fractionation carried out. [^{125}I]Lysozyme associated with each fraction was analyzed as described in Section 2. Total radioactivity associated with F9 cells is set at 100%. Each value is the mean of three independent determinations.

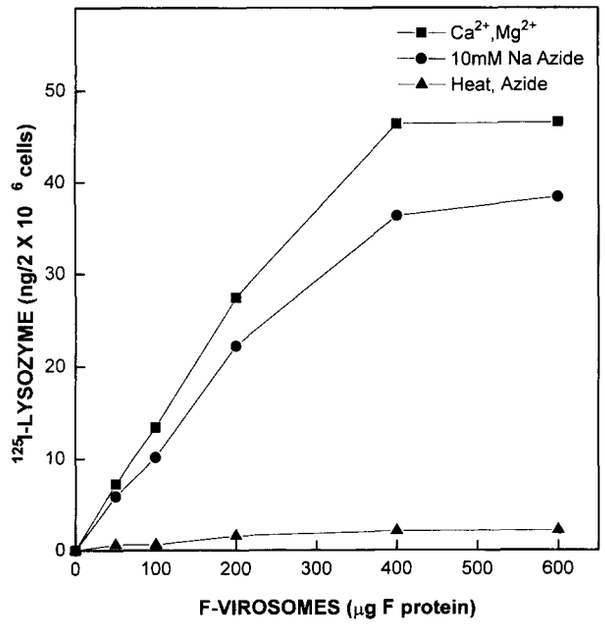

Fig. 3. dose-dependent internalization of [^{125}I]lysozyme into F9 cells delivered through F-virosomes. Different amounts of F-virosomes or heat-treated F-virosomes (in terms of F protein) were incubated with F9 cells (2×10^6) in DPBS (containing 1 mM Ca^{2+}, 1 mM Mg^{2+}; or containing 1 mM Ca^{2+}, 1 mM Mg^{2+} and 10 mM azide) at 4°C for 40 min followed by washing in respective buffer and further incubation for 30 min at 37°C. [^{125}I]Lysozyme associated with the cells was determined after EDTA stripping as described in Section 3.1. ■, F-virosomes in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+}; ●, F-virosomes in the presence of 1 mM Ca^{2+}, 1 mM Mg^{2+} and 10 mM azide; ▲, heat-treated F-virosomes in the presence of 1 mM Ca^{2+}, 1 mM Mg^{2+} and 10 mM azide. Each point is the mean of two independent determinations.

virosomes were found to fuse with F9 cell membrane as judged by both lipid mixing and aqueous connection assays. The report presented here emphasizes (for the first time) the fact that the F protein can be used both as a ligand and as a fusogen for cytosolic delivery of virosomal contents using Le^x-Le^x interaction. Such interactions have been extensively characterized in last few years [1,2]. Involvement of Le^x of F protein in the fusion of virosome-F9 cell membrane is inferred from its observed sensitivity to 50 mM EDTA (Fig. 1) which is known to totally abolish the Le^x-Le^x interaction [2]. Moreover, incubation of F-virosomes with a Le^x-deficient cell line (CHO cells) does not lead to any significant membrane fusion (Fig. 1), as observed earlier [10]. Plasma membrane level fusion of F9 cells with F-virosomes was ascertained from the inability of azide to inhibit this process. Heat treatment of F-virosomes is known to completely abolish the fusogenicity of F protein [8–10]. Very low dequenching of NBD-PE fluorescence of heat-treated F-virosomes with and without the presence of azide reflects the specificity of this fusion assay (Fig. 1). It is interesting to note that the presence of azide during fusion does not alter the initial rate of dequenching and heat-treated virosomes showed negligible fusion activity in the presence of azide (Fig. 2). The most convincing evidence in support of cytosolic delivery mediated by F-virosomes through Le^x-Le^x interaction is presented in Fig. 3 and Table 1. The net internalization (after EDTA stripping) of [^{125}I]lysozyme into F9 cells as a result of membrane fusion appeared to be saturated at a ratio of 400 μg F protein to 2×10^6 cells. This can be explained by the limited availability of Le^x moieties on the F9 cell surface. No significant interference by azide on the amount of internalized radioactivity further ensures the fusion-mediated delivery process. Moreover, the radioactivity associated with internalized [^{125}I]lysozyme could be mostly recovered (>98%) in trichloroacetic acid-insoluble fractions and immunoprecipitates proving thereby the integrity of delivered lysozyme as a result of fusion-mediated process. The studies on subcellular localization of intact [^{125}I]lysozyme demonstrates about 75% of the radioactivity (EDTA-resistant) to be associated with the cytosolic fractions (Table 1). This is in contrast to heat-treated F-virosomes where most of the radioactivity (>80%) is retained in lysosomal/mitochondrial fraction. This provides substantive proof to the fusion-mediated cytosolic delivery.

Le^x-terminated glycosphingolipids and glycoproteins are highly expressed in a variety of human cancers [17–19]. F-virosomes by virtue of Le^x-Le^x interaction may find their use as a potent carrier of cytosol active anticancer drugs.

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