



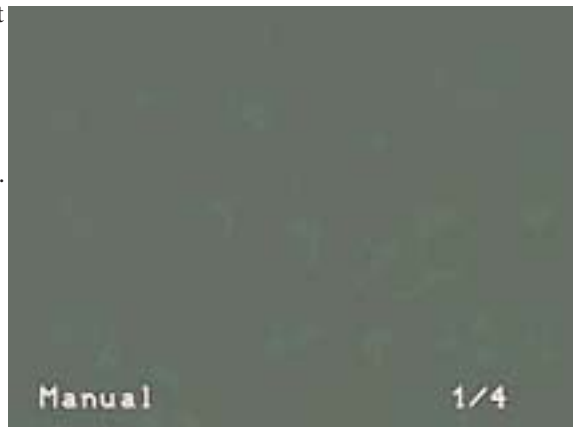
BioPORTER™ Reagent Product Data

Results described below validate the efficacy of BioPORTER™ protein delivery reagent and clearly show that it is very effective for delivering a number of different macromolecules into the cytoplasm of many different cells. The ability of BioPORTER to allow efficient protein delivery into difficult-to-transfect cells such as human dendritic cells demonstrates that it is a great alternative to DNA transfection.

BioPORTER™ reagent can deliver fluorescently labeled antibodies into cells

Figure 1 shows intracellular delivery of a fluorescently labeled antibody in NIH-3T3 cells. The left hand panel (Figure 1A) shows background fluorescence when NIH-3T3 cells were incubated with the fluorescein labeled antibody alone. The panel on the right (Figure 1B) shows the cells that were incubated with antibody associated with the BioPORTER reagent. Clearly, BioPORTER reagent greatly enhanced the uptake of the fluorescein labeled antibody in NIH-3T3 cells compared to the very small amount of uptake that occurred with the antibody alone. A time course study of fluorescent antibody delivery was also examined in NIH-3T3 cells, and optimal delivery was obtained after 4 hours of incubation in serum free medium.

A. Control - Fluorescent Antibody Alone



B. Fluorescent Antibody + BioPORTER

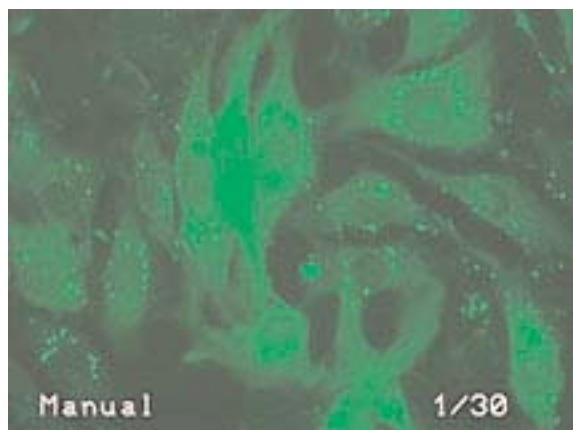


Figure 1. BioPORTER reagent-mediated intracellular delivery of a fluorescently labeled monoclonal antibody.

2 ug of FITC-labeled antibody was delivered by BioPORTER reagent (2.5 ul) into NIH-3T3 cells grown on cover slips in serum free conditions for 4 hours. After washing with PBS, live cells were directly examined with an upright microscope (NIKON E-600) equipped with a 60X objective and a 3-CCD camera video.

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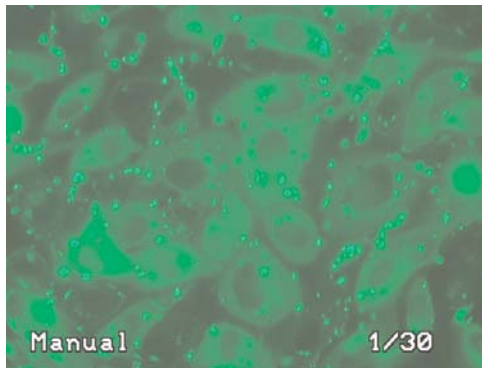
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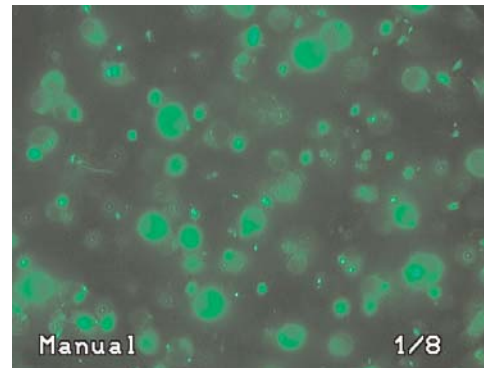
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BioPORTER reagent is effective in a variety of different cell types

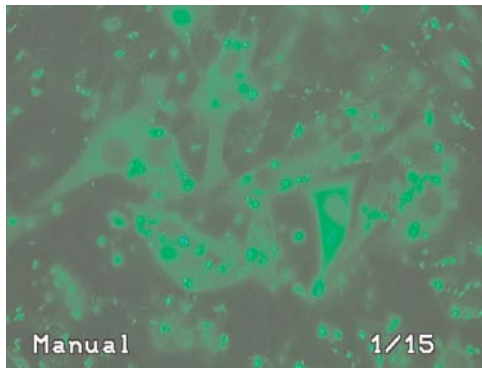
Similar results were obtained while delivering fluorescently labeled antibodies + BioPORTER reagent into the following cell types: Jurkat, HeLa-S3, BHK-21, CHO-K1, B16-F0, and NIH-3T3 (Figure 2). Other cell types, such as 293, MDCK and P19 cells, were successfully used (not shown). Various monoclonal and polyclonal antibodies directed against different antigens (and obtained from different commercial suppliers) gave essentially the same results. In addition an immunoglobulin derived from goat serum was also successfully delivered.



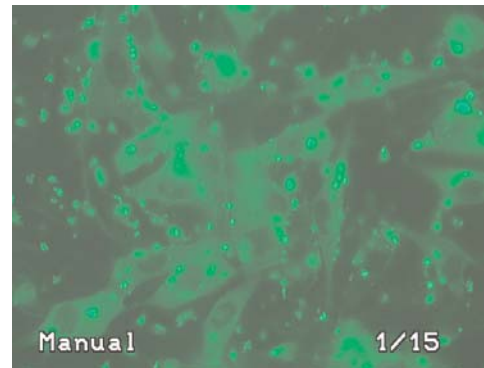
CHO-K1



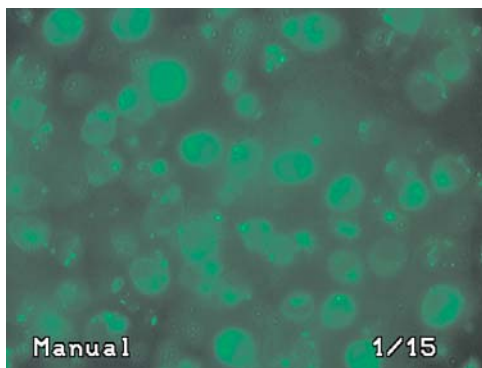
Jurkat



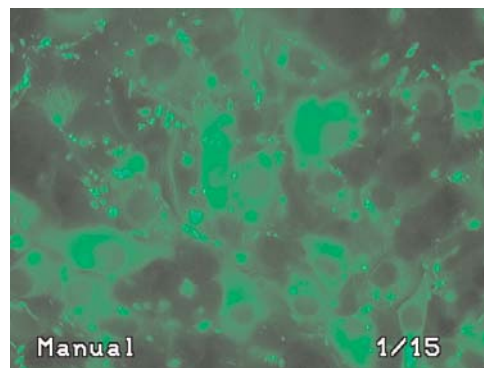
BHK-21



B16-F0



HeLa-S3



NIH-3T3

Figure 2. BioPORTER reagent-mediated intracellular delivery of a fluorescently labeled antibody to 5 cell types. CHO-K1, Jurkat, BHK-21, B16-F0, HeLa-S3 and NIH-3T3 cells were grown on cover slips. 2 ug of FITC-labeled antibody was delivered by BioPORTER (2.5 ul) in serum free conditions for 4 hours. After washing with PBS, live cells were directly examined with an upright microscope (NIKON E-600) equipped with a 60X objective and a 3-CCD camera video system. In all cases, the cells treated with antibody in the absence of BioPORTER were dark as in the previous Figure 1A.

Different macromolecules can be delivered into cells with BioPORTER reagent

1. B-Galactosidase

Results in Figure 3 show that BioPORTER reagent can deliver B-Galactosidase into cells (Figure 3B). In the absence of BioPORTER reagent, there is no cellular uptake of B-Galactosidase (Figure 3A).

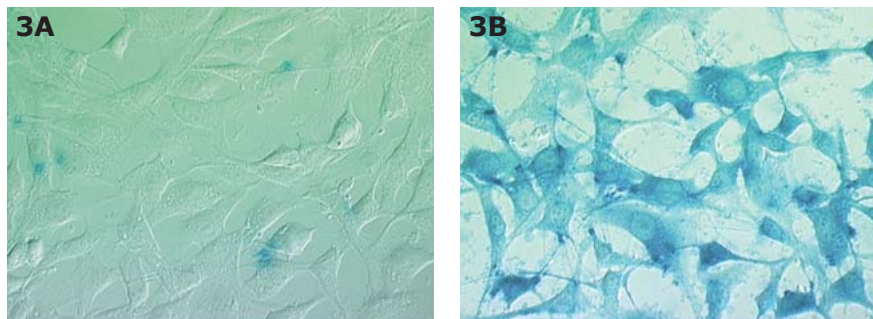


Figure 3. BioPORTER reagent mediated intracellular delivery of B-galactosidase in NIH-3T3 cells. NIH-3T3 cells were grown on cover slips. 0.5 ug of B-galactosidase was delivered with BioPORTER reagent (2.5 ul) in serum free conditions. For B-galactosidase alone, 2 ug was used. After the incubation period, cells were washed with PBS, fixed and stained for B-galactosidase activity with the X-Gal staining Kit (catalog # A10300K). Finally, cells were examined with an upright microscope (NIKON E-600) equipped with a 60X objective, DIC optics and a 3-CCD camera video. Panel A shows cells treated with B-galactosidase without BioPORTER reagent. Panel B shows cells treated with B-galactosidase +BioPORTER reagent.

2. Dextran sulfate

Results in Figure 4 show BioPORTER reagent mediated uptake of high and low molecular weight dextran. Low molecular weight dextran (10 KD) is able to enter into the nucleus of the transduced cells (Figure 4B), whereas, high molecular weight dextran (70 KD) does not enter the nucleus (Figure 4D). In contrast, without the BioPORTER reagent both dextran sulfate 10 KD (Figure 4A) and 70 KD (Figure 4C) do not enter into the cells

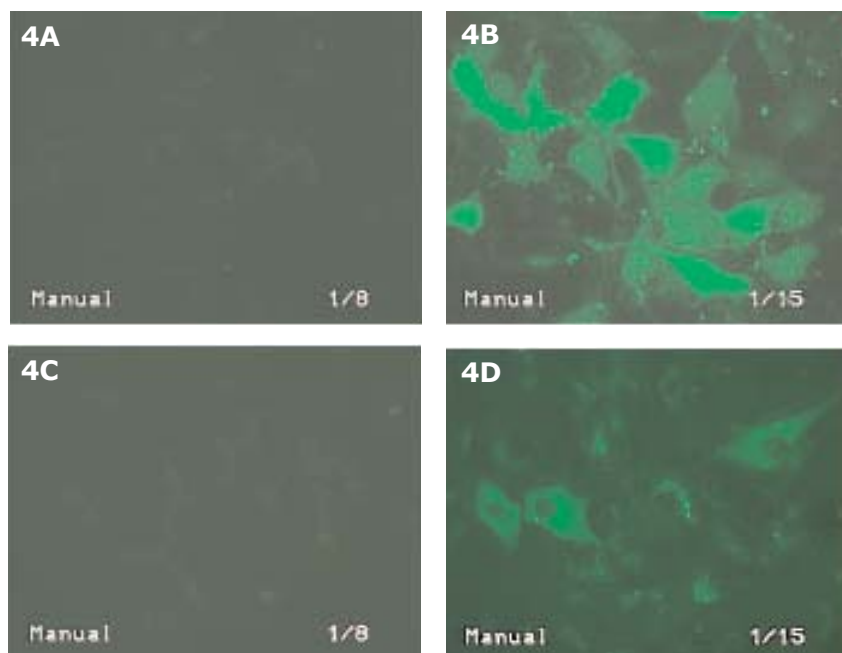


Figure 4. BioPORTER reagent-mediated delivery of high and low molecular weight dextran sulfate into NIH-3T3 cells. BioPORTER reagent was used to deliver fluorescent derivatives of either 10 KD or 70 KD dextran sulfate. 1 ug and 2 ug of Oregon Green 488 labeled dextran sulfate (10 KD and 70 KD, respectively) was delivered with BioPORTER reagent (2.5 ul) into NIH-3T3 cells grown on cover slips for 4 hours in serum free conditions. After washing with PBS, live cells were directly examined with an upright microscope (NIKON E-600) equipped with a 60X objective and a 3-CCD camera video system.

Induction of Apoptosis in different cell types after delivery of various apoptotic proteins with BioPORTER™ reagent

1. BioPORTER reagent mediated delivery of granzyme-B to primary human acute myelocytic leukemia (AML) and Chronic Myelocytic Leukemia (CML) cells leads to apoptosis

Histograms in Figure 5 show flow cytometry analysis of primary human acute myelocytic leukemia (AML) cells that were treated with either phycoerythrin (PE) alone, or with a mixture of PE and granzyme-B. Intracellular uptake of these proteins was accomplished either by electroporation or BioPORTER delivery. Cells appearing above the horizontal line are PE positive. Cells appearing to the right of the vertical line in the histograms have activated caspase 3 (effector caspase that is a key component of the apoptotic pathway and plays a central role in the apoptotic process) because of increased CaspaTag substrate fluorescence (Caspatag Fluorescein Caspase Activity Kit). Therefore these cells are considered to be apoptotic.

Histograms in Figures 5 A and B are negative controls (PE delivery). Figures 5 C and D show that granzyme-B has been successfully delivered into leukemia cells in a biologically active form since it can drive them into apoptosis. The histograms show that electroporation-mediated granzyme-B delivery induced 58% of the cells to enter into apoptosis, whereas BioPORTER reagent-mediated granzyme-B delivery caused 40% of the cells to apoptose. The difference in the extent of apoptosis can be accounted for by differences in the optimization conditions. Similar results were obtained with another primary human Chronic Myelocytic Leukemia (CML) cells (data not shown).

2. BioPORTER reagent-mediated delivery of granzyme-B, caspase-3 and caspase-8 into Jurkat cells

BioPORTER reagent treatment is much less toxic to Jurkat cells than electroporation. An experiment similar to that shown in Figure 5 was conducted in Jurkat cells (acute T cell leukemia). Results in figure 6 show that electroporation treatment was very toxic to Jurkat cells since 90% of the cells were killed. In contrast, BioPORTER reagent treatment affected only 10% of the cell population.

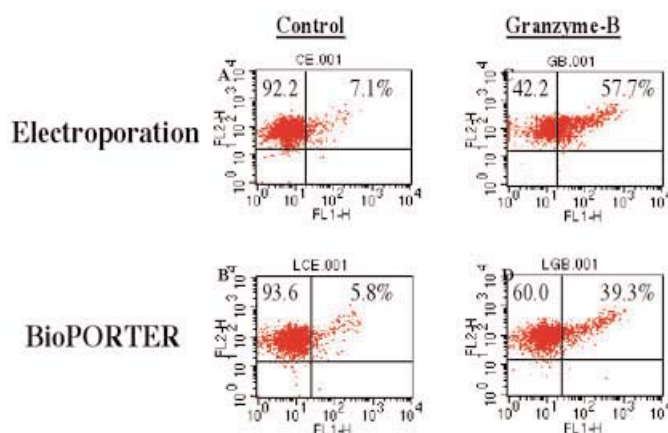


Figure 5. BioPORTER reagent-mediated and Electroporation-mediated delivery of granzyme-B into human acute myelocytic leukemia (AML) cells. A and B) Analysis of cells treated with phycoerythrin (PE). C, D) Analysis of cells treated with a mixture of PE and granzyme-B (1 unit). PE and granzyme B were delivered by electroporation (A, C) or with 2.5 ul of BioPORTER reagent (B, D). The y-axis on these histograms quantifies the amount of the fluorescent PE that enters the cells, and the x-axis quantifies the amount of apoptosis using Caspatag Fluorescein Caspase Activity Kit. Untreated cells are confined to the lower left hand quadrant (data not shown).

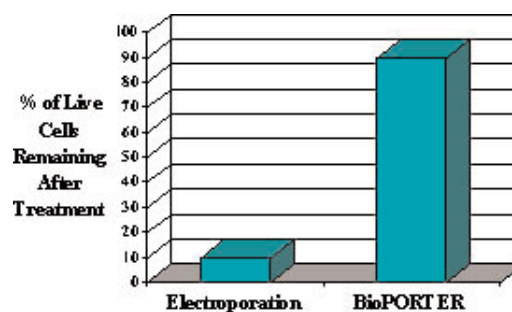


Figure 6. Comparison of cytotoxicity between electroporation and BioPORTER reagent treatment. The graph shows the amount of electroporation-mediated cell death in Jurkat cells compared to treatment with BioPORTER™ reagent. Cell death was monitored by flow cytometry (forward and side scatter) and spontaneous caspase activation.

BioPORTER reagent was used to deliver caspase-3, granzyme-B, and caspase-8 into Jurkat cells. BSA and B-galactosidase were used as negative controls. Two different assays were conducted. The first is the Caspatag Fluorescein Caspase Activity Kit that monitors caspase activation inside cells. The second is Annexin V-FITC/Propidium iodine Apoptosis Detection Kit that monitors later stages in the apoptotic process such as cell death.

With the CaspaTag Fluorescein Caspase Activity Kit, caspase-3 was the most potent apoptosis inducer leading 40% of cells into apoptosis. Granzyme-B and caspase-8 caused about 20% of cells to go into apoptosis, whereas the background level was about 7%. (Data not shown). Because of its high toxicity, electroporation cannot be used adequately to deliver caspases into Jurkat cells, so it is not possible to make a reasonable comparison between electroporation and BioPORTER reagent in these cells. In fact, electroporation is not a generally applicable method for delivering proteins into cells because the treatment itself is usually too toxic. The AML cells described previously are among the exceptions. They seem to resist the toxic effects of electroporation while allowing entry of functional macromolecules.

Delivery of granzyme B was also tested with the AnnexinV-FITC/Propidium Iodine Apoptotic Detection Kit (Figure 7 and 8). As previously observed, granzyme B delivered by BioPORTER reagent induces apoptosis of Jurkat cells. In contrast, BioPORTER reagent alone or complexed to a control protein (B-galactosidase) did not lead to significant apoptosis. Similar results were obtained with BSA (data not shown). While the previous results are very robust and demonstrate the efficacy of BioPORTER reagent, we have also found that it is not effective for all proteins. Indeed, cytochrome C, another powerful effector of apoptosis, failed to lead the cells into apoptosis after delivery with BioPORTER reagent, whereas with electroporation cytochrome C was able to kill the cells (data not shown). This observation illustrates the importance of the intrinsic properties of the molecules to be delivered (see section V). In contrast to granzyme B or B-galactosidase, cytochrome C is very positively charged at neutral pH. This factor may explain the inefficient reaction and interaction with the positively charged lipid formulation.

In general, the efficiency of delivery depends on several parameters such as amount of protein, amount of BioPORTER reagent, cell types, number of cells, sensitivity of the assay, buffer and the volume used to form the protein/BioPORTER complexes. In the case of granzyme B, the extent of apoptosis is dependent on the amount of granzyme B and BioPORTER reagent used. However, when excess BioPORTER reagent was used the induction of spontaneous apoptosis increased, too (data not shown). Optimal dose of BioPORTER reagent will also depend on the number of cells used.

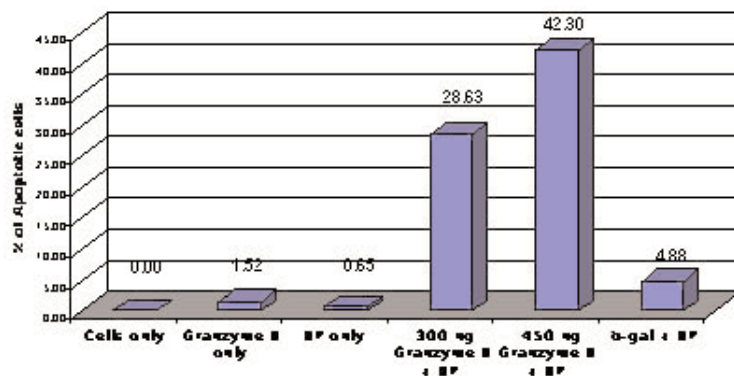


Figure 7. BioPORTER reagent mediated delivery of granzyme-B, cytochrome C and B-galactosidase into Jurkat cells. 100,000 Jurkat cells/well were plated in a 24well-plate. A) Granzyme B (300 and 450 ng) or B-galactosidase (1 ug) were delivered with or without 2.5 ul of BioPORTER reagent (BP) into Jurkat cells for 4 hours in serum-free medium. Serum was then added to each well and cells were incubated for 20 more hours. The next day, cells were assayed for apoptosis using an Annexin V-FITC Apoptosis Detection Kit and directly analyzed by flow cytometry. Data shows the percentage of apoptotic cells (Annexin V-FITC positive and Propidium Iodine positive or negative) after 24 hours of incubation.

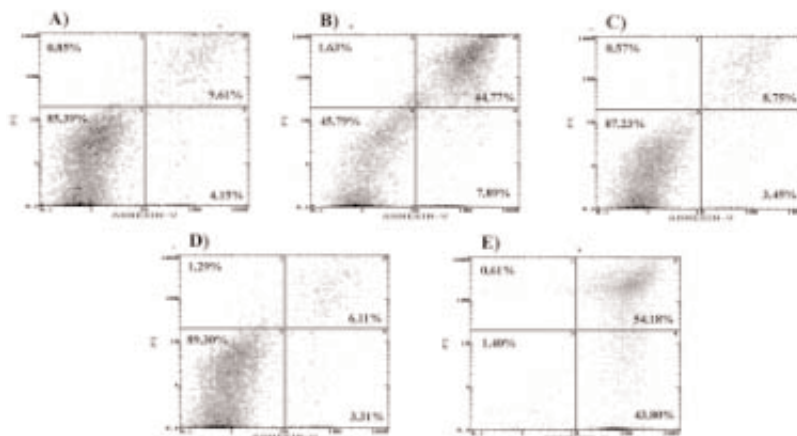


Figure 8. BioPORTER reagent-mediated delivery of granzyme-B, cytochrome C and b-galactosidase into Jurkat cells. Jurkat cells were treated and analyzed as described in figure 7. A) BioPORTER reagent only, B) BioPORTER reagent + granzyme B (450 ng), C) BioPORTER reagent + B-galactosidase (1 ug), D) BioPORTER + cytochrome C (1 ug) and E) Positive control of 1 uM staurosporine was added to the cells for 4 hours in serum-free medium. The y-axis on these histograms quantifies the amount of the propidium iodine taken up by the cells and the x-axis quantifies the amount of Annexin V-FITC bound to the cells.

3. BioPORTER reagent-mediated delivery of granzyme-B, caspase-3 and caspase-8 into Ki-Ras 267 B1 cells (prostate cancer cells) BioPORTER reagent was used to deliver caspase-3, granzyme-B, and caspase-8 into Ki-Ras 267 b1 cells (prostate cancer cells). Two assays CaspaTag Fluorescein Caspase Activity Kit and Annexin V-FITC/Propidium iodine Apoptosis Detection Kit were used as described for Jurkat cells.

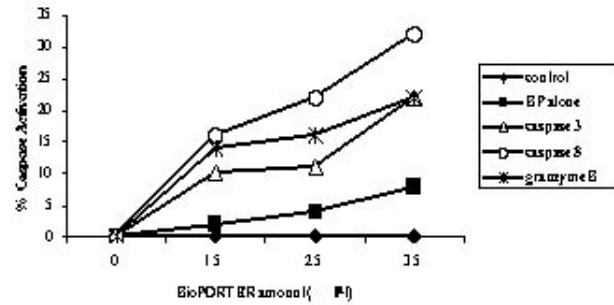


Figure 9. Dose-Response of BioPORTER reagent mediated delivery of caspase-3, granzyme-B and caspase-8 into Ki-Ras 267 B1 cells. 50,000 cells/well were plated in a 24 well plate. Granzyme-B (200 units), caspase-3 (1 unit) and caspase-8 (1 unit) were delivered with various doses of BioPORTER reagent into Ki-Ras 267 B1 cells for 4 hours in serum-free medium. Serum was then added to each well and cells were incubated for 16 more hours. The next day, cells were assayed with CaspaTag Fluorescein Caspase Activity Kit and directly analyzed by Flow cytometry.

With the CaspaTag Fluorescein Caspase Activity Kit, dose-response was studied for BioPORTER reagent (Figure 9). Extent of caspase activation was BioPORTER reagent dependent. However, spontaneous caspase activation was induced by higher doses of BioPORTER reagent. This phenomenon depends on the cell density used during the assay and a similar result was observed with Jurkat cells (data not shown). Optimal dose of BioPORTER reagent will also depend on the number of cells used.

Delivery of granzyme-B and caspase-3 was also tested with the AnnexinV-FITC/Propidium Iodine Apoptotic Detection Kit (Figure10). B-galactosidase was used as negative control and staurosporine was used as a positive control. Granzyme-B and caspase-3 delivered by BioPORTER reagent induced apoptosis of Ki-Ras 267 b1 cells to a similar extent (65% and 66.5%). These two proteins delivered by BioPORTER reagent were as potent in inducing apoptosis as staurosporine (a well-know chemical that lead cells to apoptosis). There is a clear correlation between induction of apoptosis and the use of the BioPORTER reagent. In addition, BioPORTER reagent alone (or when complexed to a control protein, B-galactosidase) did not induce significant cell death. Delivery efficiency depends on several parameters (listed previously for Jurkat cells). Extent of apoptosis was dependent on the amount of granzyme-B and caspase-3 used in this assay (Data not shown).

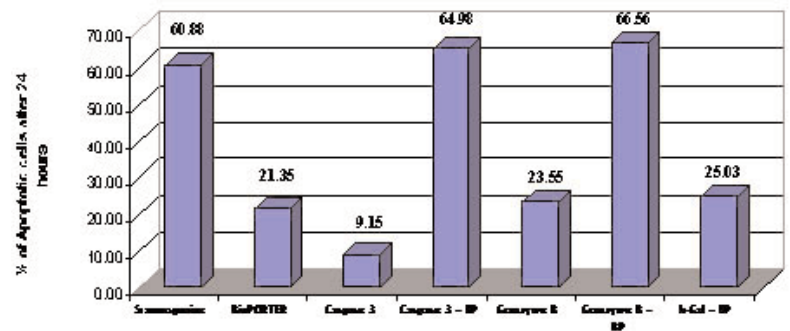


Figure 10. BioPORTER reagent mediated delivery of granzyme-B, caspase-3 and b-galactosidase into Ki-Ras 267 B1 cells. 50,000 cells/well were plated in a 24 well plate. Granzyme-B (450ng), caspase-3 (3.3 ng) or B-galactosidase (2 ug) were delivered with or without 2.5 ul of BioPORTER (BP) into Ki-Ras 267 B1 cells for 4 hours in serum-free medium. Serum was then added to each well and cells were incubated for 20 more hours. For the positive control, staurosporine was added to the cells at 1 uM final concentration for 4 hours in serum-free medium. The next day, cells were assayed for apoptosis using Annexin V-FITC Apoptosis Detection Kit and directly analyzed by Flow cytometry. Data show the percentage of apoptotic cells (Annexin V-FITC positive and Propidium Iodine positive or negative) after 24 hours of incubation.

Conclusion.

We have demonstrated that BioPORTER protein delivery reagent is remarkably effective for delivering a number of different macromolecules into the cytoplasm of cultured cells. Most importantly, molecules delivered to cells with BioPORTER reagent, such as caspases and granzyme-B, are functional since they can drive cells into apoptosis.

Some proteins, such as cytochrome-C or BSA, did not get delivered efficiently into cells. Indeed, in our functional assay, cytochrome-C did not induce apoptosis when complexed with the BioPORTER reagent, as opposed to electroporation, where cytochrome-C was able to induce cells into apoptosis as expected.