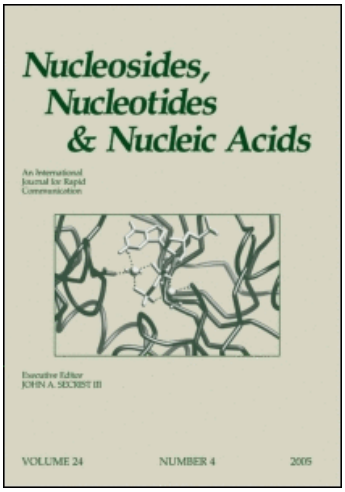

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CHARACTERIZATION OF GPI2A, A POTENT INHIBITOR OF HIV-1 GENE EXPRESSION AND VIRAL REPLICATION

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ABSTRACT. Fully thioated antisense molecules are often cytotoxic and non-specific in action. GPI2A is thioated at 7 base positions. GPI2A posses sequence-specific activity against HIV-1 gene expression and viral replication without significant cytotoxicity. Partial thioation did not compromise its uptake, cellular distribution and nuclease resistance.

INTRODUCTION

New drugs that inhibit gene expression and virus replication are required as tools to study HIV-1 proliferation and as potential therapeutic compounds. These considerations have led us to synthesize and test GPI2A as a possible virus inhibitor.[1,2,3] This is because there is a high probability of designing very specific oligodeoxynucleotide (ODN) sequences in the antisense orientation that target nucleic acid sequences and prevent virus gene expression.[4] GPI2A is a 20-mer that is thioated only at 7 base positions, and is designed to target a highly conserved region of the HIV-1 genome. GPI2A is an excellent inhibitor of HIV-1 gene expression and posesses antiviral activity. Cellular uptake and stability characteristics are important factors in the development of a drug candidate. These properties of GPI2A were evaluated.

MATERIALS AND METHODS

A *cos*-like monkey kidney cell line[5], CMT3 was transfected by the $CaPO_4$ procedure with the pCMV*gagpol-rre-r* and pCMV*rev* plasmids to generate the B4.14 cell line.[2] Human H9 cells are of lymphoid origin, and H9IIIB cells are chronically HIV-1

infected.[1,2,6] ODN sequences were chosen as previously described.[2] GPI2A was synthesized to target nucleotides +1189 to +1208 according to the HIV-1 nomenclature[7], and has the following composition: 5'-G(s)GTTC(s)TTTTG(s)G(s)TCC(s)TTG(s)TC(s)T-3'. The inverse of GPI2A, 5'-T(s)CTG(s)TTC(s)CTG(s)G(s)TTTTC(s)TTG(s)G-3', and GPI2A with two point mutations, 5'-G(s)GTTC(s)TTTTG(s)TG(s)CC(s)TTG(s)TC(s)T-3' were used as the control sequences. The inversed and the mutated sequence were also chemically modified at 7 base positions by substitution of the naturally occuring nonbridging oxygen atoms of the phosphodiester backbone with sulfur atoms as indicated above. These sequences were synthesized on an ABI Model 390 DNA synthesizer by standard phosphoramidite chemistry[8], and were purified by anion exchange chromatography on resource Q (Pharmacia Biotech., Uppsula Sweden).

To demonstrate the antisense activity of GPI2A, B4.14 cells were pretreated with the construct at 37°C in a 5% CO_2 environment. Chronically infected H9/IIIB cells were used at 4 x 10^5 cells/ml. The cells were incubated for 7 days and the inhibition of HIV-1 activity was determined by estimating the intracellular p24 viral core antigen expression, by Western blotting analysis.[1,2] Northern analysis was performed by a modification of a previously described procedure.[9]

The synthesized ODNs have a 5'-OH group and they were labelled using the 5' DNA terminus labelling system according to the manufacturer's instructions (GIBCO Research Products Life Technologies Inc.). For the experiments done in the presence of lipofectin reagent, 6.2 μg of DNA and 10 μg of lipofectin reagent were diluted according to the manufacturer's instructions (GIBCO Research Products Life Technologies Inc.). To determine cell association, B4.14 cells were seeded into 24-well flat bottom (well diameter 16 mm) plastic tissue culture plates and allowed to grow to 70% confluence. [$^{35}\gamma$S]-ATP-labelled ODNs were made up to a final concentration of 1 μM with cold oligomer. Under the experimental conditions for the determination of energy dependence, 10 mM sodium azide was used, while 100 times molar excess of each ODN was used to eliminate non-specific binding. After 1, 2 and 4 hours incubation on ice or at 37°C in the presence or absence of lipofectin reagent, an aliquot of the incubation medium was used to determine the amount of radioactivity in the incubation medium, and the cells were washed three times with cold phosphate buffered saline (PBS), and then lysed with cell lysis buffer [50 mM

Tris-HCl pH 7.2, 150 mM NaCl , 5 mM EDTA, 1% Triton X-100, 0.2% deoxycholic acid]. Protein concentrations of cell lysates were determined by the Bio-Rad protein assay procedure (Bio-Rad laboratories Inc., Hercules, CA), and the cell-associated radioactivity was determined by direct suspension of the labelled cell lysate in liquid scintillation cocktail (Universal, ICN Biomedicals Inc., Irvine, CA).

For cellular uptake, B4.14 cells were pretreated with labelled GPI2A with or without lipofectin reagent as described above, and the cells were lysed by freeze thawing after 4 h. The isolated nuclear pellets were washed with PBS and both the nuclear pellet and the cytoplasmic fraction were digested at 37°C for 12 to 16 hours with 50 μg/ml proteinase K, 10 mM Tris-HCl pH 7.6, 5 mM EDTA, and 0.5% SDS. Phenol/chloroform extractions were carried out on both resulting solutions and the DNA was then ethanol precipitated, resuspended in 9:1 formamide/TBE (0.045 M Tris-borate, 0.001 M EDTA, pH 8.0) buffer, heated at 55°C for 5 min and separated on a 1 x TBE - 20% PAGE/7 M urea gel. Gels were dried and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, N.Y.).

To determine stability in the presence of serum nucleases, incubation medium containing 7% bovine serum (FetalClone III, HyClone, Laboratories, Inc., Logan, Utah, USA) or 7% bovine serum, heat-inactivated at 55°C for 30 min, was added to 24-well flat bottom plastic tissue culture plates without cells. [γ^{35}S]-ATP labelled ODNs were added to the incubation medium to a final concentration of 62 μg/ml (specific activity of 1 to 2 x 10^6 cpm/μg DNA), and were incubated at 37°C in a water bath or in a humidified 5% carbon dioxide environment. At various time points 120 μl of the medium was transferred to an eppendorf tube containing 880 μl of absolute ethanol and stored at -70°C. The collected samples were ethanol precipitated, then resuspended in 9:1 formamide/TBE buffer and heated at 55°C for 5 min. Electrophoresis was performed on a 1 x TBE -20% PAGE/7 M urea gel, that was later dried and exposed to Kodak X-Omat film.

RESULTS AND DISCUSSION

B4.14 cells were transfected with a plasmid carrying the *gagpol* region of HIV-1, under the control of the CMV promoter-enhancer, and therefore these cells constitutively express the HIV-1 protein.[1,2] However, treatment of B4.14 cells with 1 μM GPI2A markedly decreased the synthesis of the viral proteins, in contrast to cells treated with the inverse sequence or GPI2A containing a two-point mutation as controls (Fig. 1A). A

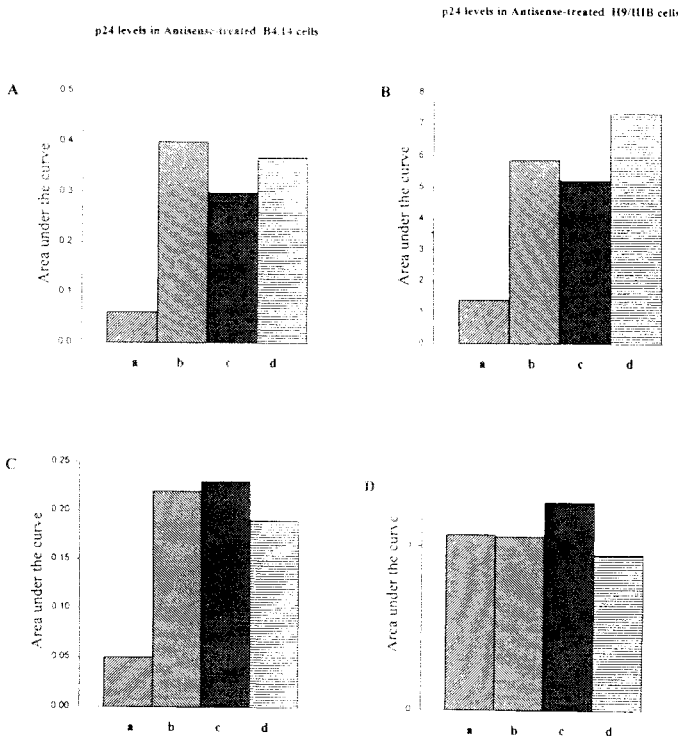

FIG. 1 Densitometry analysis of (A) Western blotting of HIV-1 p24 expression in B4.14 cells: (a) GPI2A, (b) inverse sequence, (c) GPI2A with a two point mutation, and (d) untreated B4.14 cell; (B) in H9/IIIB cells: (a) GPI2A, (b) GPI2A with a two point mutation, (c) inverse sequence and (d) untreated H9/III cells. Densitometry analysis of (C) Northern blotting of HIV-1 mRNA levels: (a) GPI2A, (b) GPI2A with a two point mutation, (c) inverse sequence and (d) untreated H9/IIIB cells; (D) loading control performed with glyceraldehyde-3-phosphate dehydrogenase cDNA for data shown in C. Data shown for A-D are from three separate experiments.

further test for antiviral potential was performed with chronically HIV-1 infected human H9/IIIB cells. Consistent with the observations obtained with B4.14 cells, 1μM GPI2A treatment of H9/IIIB cells clearly reduced the level of expression of the HIV-1 core antigen p24 (Fig. 1B), and decreased the level of viral mRNA (Fig. 1C). Again, in control experiments, the inverse sequence and the two-point mutated version of GPI2A had no significant effect on the levels of p24 protein or virus mRNA in H9/IIIB treated cells (Figs. 1B and 1C). These results are consistent with our earlier observed inhibition of

reverse transcriptase activity in acutely infected cord blood mononuclear cells post GPI2A treatment[2], supporting the view that the mode of action of GPI2A occurs via an antisense mechanism.

Modifications to ODN sequences can significantly affect their cell association characteristics.[10] Therefore, we compared the properties of GPI2A, which is thioated at 7 base positions, with the same 20-mer sequence which had not been thioated. Figs. 2A and 2B show that there was a temperature and time dependent cell association of both the thioated and the non-thioated versions of the GPI2A. Furthermore, the kinetics of GPI2A cell association was similar in both cases, with about double the level of ODN sequence bound at 37°C vs 4°C after 240 min. To determine the proportion of cell associated GPI2A that was energy-dependent and therefore likely to be due to intracellular accumulation of GPI2A,[12] identical experiments were performed in the presence of 10 mM sodium azide, and compared to results obtained in the absence of an energy inhibitor (Fig. 2C and 2D). Energy-dependent uptake was similar for the partially thioated (45%) and the non-thioated forms of GPI2A (55%).

A variety of approaches have been used to improve cellular uptake of ODNs,[12] and frequently DNA is delivered to cells in transfection experiments in the presence of liposomes or cationic lipids.[13,14] Fig. 3 demonstrates that formulation of GPI2A with the commercially available cationic lipid, lipofectin (GIBCO BRL, Life Sci. Inc.), markedly enhances the cell association properties of the antisense molecule, with about a four-fold increase in the presence of lipid after a 60 min incubation at 37°C. In keeping with increased cell association in the presence of the lipid, Fig. 4 shows that the lipid reagent enhances cellular uptake of GPI2A. Furthermore, the antisense ODN was found in both cytoplasmic and nuclear fractions, with the highest concentration located in the cytoplasm, and this was observed when GPI2A was delivered in either the presence or absence of a lipid carrier. These observations suggest that, although GPI2A is modified, it still retains the ability to localize to probable sites of action when a carrier vehicle is used to potentiate its uptake characteristics.

If significant phenotypic changes resulting from inhibition of gene expression are to occur, it is necessary that the inhibitory effects are maintained for a sufficient length of time

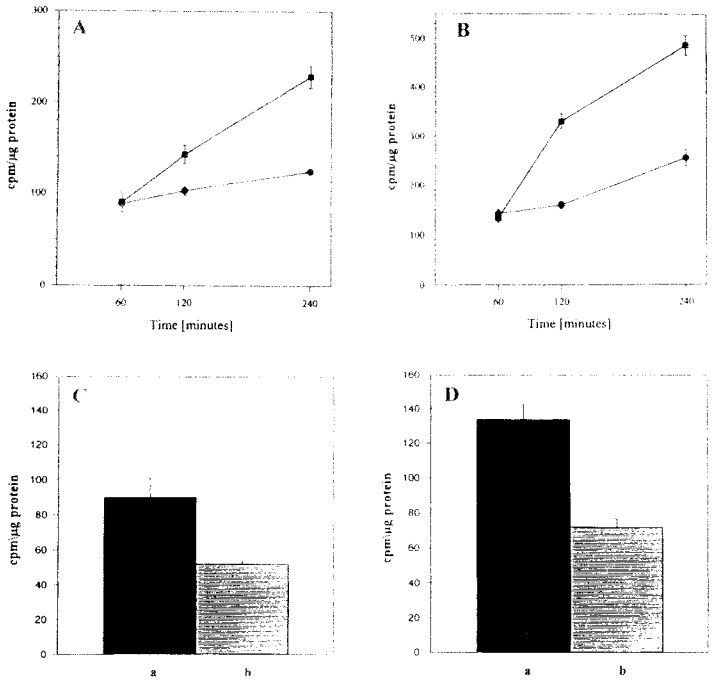

FIG. 2 Cell-associated GPI2A (A) or non-thioated GPI2A (B) performed at 4°C (●) or at 37°C (■). Energy dependent binding of GPI2A (C) or non-thioated GPI2A (D) at 37°C over 60 min: (a) in the absence of an energy-inhibitor, (b) in the presence of NaN_3. Data points in A-D represents the average of at least 4 independent trials.

to allow pre-existing levels of the target protein to decay through turnover within the cell. Breakdown of ODN occurs through the action of nucleases which are found in relatively high concentrations in serum.[15] Therefore, the GPI2A sequence was partially thioated in an attempt to inhibit nuclease digestion and reduce cytotoxicity. To determine whether or not thioation does, as predicted, decrease the rate of nuclease digestion, we have compared GPI2A to the non-thioated version of this sequence as substrates for serum nuclease activity (Fig. 5). In both cases there is a dramatic difference in the nuclease sensitivity of the GPI2A partially thioated sequence when compared to the non-thioated form. In the presence of

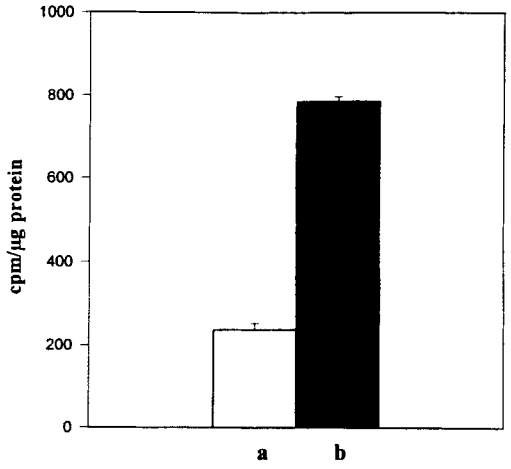

FIG. 3 Cell associated GPI2A in the absence of lipofectin (a) or labelled GPI2A/lipid mixture (b) at 37°C for 60 min. Four independent trials were performed.

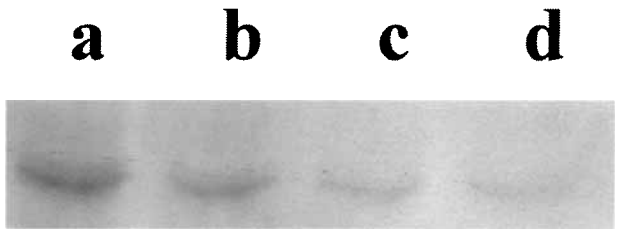

FIG. 4 Detection of labelled GPI2A in the cytoplasmic fraction: (a) delivered in the presence of lipofectin, and (b) in the absence of lipofectin. Detection of labelled GPI2A in the nuclear fraction: (c) delivered in the presence of lipofectin, and (d) in the absence of lipofectin.

7% bovine serum obvious degradation of the non-thioated version was observed after 15 min, whereas it required about 8 hours to observe a significant degradation of the thioated sequence (Fig. 5A). A majority of the non-thioated GPI2A sequence was completely degradated by 8 hours, whereas a significant level of the partially thioated GPI2A sequence remained even after 24 hours. Similar differences in nuclease sensitivity were observed in the presence of heat-treated serum, where a significant level of partially thioated GPI2A sequence was found even after 21 days of incubation, long after the non-thioated form had been degraded (Fig. 5B).

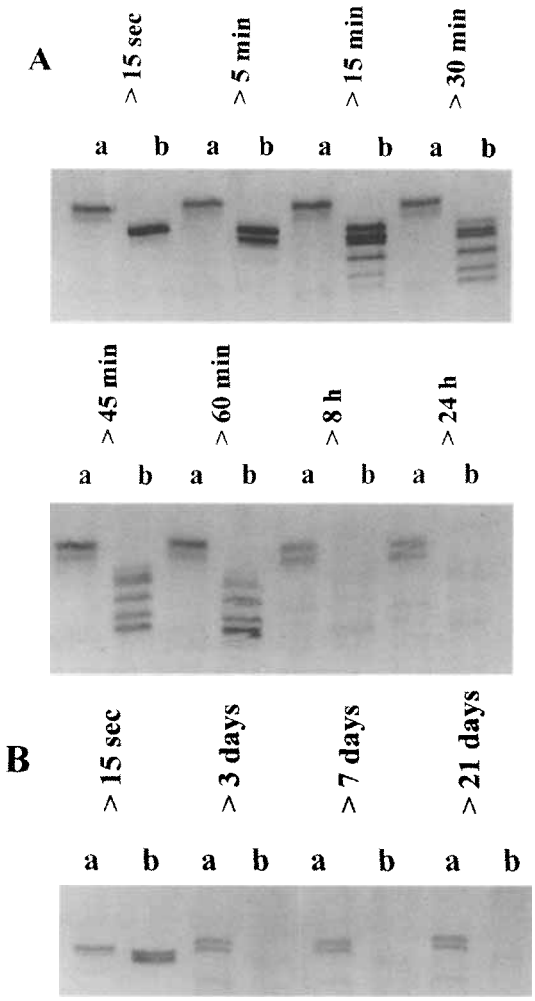

FIG. 5 Time-dependent degradation of GP12A (a) and non-thioated GP12A (b).

In conclusion, the results of this study indicate that GPI2A is an effective inhibitor of HIV-1 gene expression, and that it cell-associates in a time- and temperature-dependent fashion, which is enhanced in the presence of a lipid formulation. Furthermore, it is taken up by cells to both cytoplasmic and nuclear locations, and due to partial thioation, it exhibits significant resistance to the action of nucleases. These characteristics suggest that GPI2A will be a very useful molecular reagent for analyzing HIV-1 proliferation, and may have therapeutic potential.

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