Targeting of HIV gp120 by oligonucleotide-photosensitizer conjugates

Light-induced damages

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Abstract Some guanine-rich oligonucleotides inhibit HIV infectivity through interaction with the gp120 glycoprotein. Besides, photoinactivation of viruses attracts attention for blood decontamination. The feasibility of targeting a red light-absorbing chlorin-type photosensitizer to gp120 through covalent coupling with 8-mer phosphodiester oligodeoxynucleotides is investigated. Some conjugates inhibit binding of antibodies directed to gp120. Inhibition is significantly increased upon red light activation. The activity of the conjugates correlates with their ability to self-associate, a process strongly favored by the propensity of the hydrophobic chlorin moiety to dimerize. Thus, the photosensitizer moiety both promotes structures with a higher affinity for gp120 and, upon light activation, can induce site-directed damages to the protein.

Key words: HIV; gp120; Oligonucleotide; Photosensitizer; Chlorin; Self-association

1. Introduction

Recent advances in the knowledge of the mechanism of HIV entry into target cells [1] is opening new ways for prophylactic interventions. The entry is mediated through sequential binding of the HIV glycoprotein gp120 to the cellular CD4 receptor and co-receptors identified as the CXCR4 or CCR5 chemokine receptors, a choice depending on the virus tropism. The recent resolution of the gp120 crystal structure [2] allowed for the identification of the area involved in co-receptor recognition [3]. As surmised, it comprises the gp120 V3 loop that has been shown to be a major determinant of the virus tropism [4]. It is worth noting that the zone near the V3 loop base and the V3 loop itself which delineate the co-receptor binding site are highly positively charged [3,5,6]. Polyanions, such as sulfated polyesters, have long been recognized to inhibit HIV infection in vitro [7,8]. Among other polyanionic compounds, oligonucleotides have attracted attention. Indeed, aptamer strategies make it possible to select oligonucleotide sequences with high affinity for proteins that are not naturally bound by nucleic acids, which considerably extends the range of possible targets for oligonucleotides [9]. Thus, beyond the expected charge effect presented by long homopolymers [10], shorter oligonucleotides with sequence-dependent anti-viral activity have been selected [11]. Although not an absolute requirement [12], the presence of G-tracts favoring the formation of folded structures or strand association involving G-tetrads led to the most efficient compounds [11,13,14]. A phosphorothioate backbone was also required [11]. In assays based on inhibition of HIV infection in vitro without any anticipation on the mechanism of action, gp120 and more specifically the V3 loop were identified as the main targets [11].

However, binding of oligonucleotides to their targets is a priori reversible. Composite molecules associating oligonucleotides with reactive substituents able to produce irreversible damages in the target are worth considering. In particular, photosensitizing substituents [15] that are not toxic in the dark sensitize singlet oxygen formation or react by electron transfer upon light excitation leading to chemical damages to molecules in the vicinity. We focused our attention on photosensitizers belonging to the chlorin family [16,17]. These molecules which absorb light above 620 nm can be activated in a region corresponding to minimal light absorption by blood, a prerequisite for photochemical blood sterilization [18]. We recently reported on the photoinactivation of various strains of the HIV-1 virus by a member of this family [19]. In the present study, we demonstrate the feasibility of targeting a hydrophobic chlorin-type photosensitizer to specific areas of the HIV envelope protein gp120, in particular the V3 loop, through coupling to some G-rich oligonucleotides.

2. Materials and methods

2.1. Chlorin-oligonucleotide conjugates

The chlorin-type photosensitizer referred to as CHEVP (Fig. 1) was prepared from the related vinyl porphyrin by a photochemical method [17]. It was coupled to different phosphodiester deoxyoligonucleotides derivatized at the 3′-position through a hexadiamine linker and the conjugates were purified by high performance liquid chromatography (HPLC) as described elsewhere [15]. The absorption spectrum of the TTGGTTGG-chlorin conjugate is shown in Fig. 1.

2.2. Inhibition of antibody binding

Enzyme-linked immunosorbent assay (ELISA) binding assays were performed using 96 well microtiter plates (Immulon 4 from Dynatech). After the plates were coated with recombinant HIV-1 $gp120_{IIIB}$ (Intracel), oligonucleotides or chlorin conjugates in phosphate buffer saline, pH 7.4, were added to the wells at different concentrations (0, 1×10^{-7}, 1×10^{-6} and 1×10^{-5} M). After incubation (30 min), the plates were irradiated or kept in the dark for the same

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time. Then, the IgG1 anti-gp120 monoclonal antibodies (mAbs) 9284, 9305 and 9301 (Du Pont de Nemours) were added and allowed to react for 15 min. The wells were washed several times before the addition of an anti-mouse goat IgG antibody conjugated to horseradish peroxidase (Bio-Rad) which was used to reveal the presence of the bound mouse antibody directed to gp120. The reaction of the peroxidase substrate (3,3′,5,5′-tetramethylbenzidine from Pierce) was followed at 450 nm where the chlorin conjugate absorption was negligible. Assays carried out in duplicate were found to be reproducible within 10%.

2.3. Light irradiation

The collimated beam from a 1000 W xenon lamp was passed through a water filter to eliminate infrared light and then through an interference filter centered at 670 ± 20 nm matching the absorption of the chlorin molecule. A mirror was used to illuminate the 96 well microplates from above. The optic was adjusted to produce homogeneous light distribution over the working area. The samples were irradiated for 10 min with a total fluence of 4.75 J.

2.4. Dimerization constant determination

The emission fluorescence spectra of the chlorin-oligonucleotide conjugates were measured on a spectrofluorometer (SPEX) with excitation set at 420 nm. The emission maximum was found at 654 nm. The non-linear changes of the fluorescence intensity (F) vs. concentration (C) observed for the solutions in aqueous buffer were analyzed assuming dimerization according to the equation

$$F = \{[1 - \surd(1 + 8K_D C)](f_D/2 - f_M) + 2f_D K_D C\}/4K_D \quad (1)$$

where f_M and f_D are constants characterizing the monomer and dimer fluorescence and K_D the dimerization constant [20]. In all cases, the dimer fluorescence was negligible.

3. Results and discussion

3.1. Specific binding of conjugates to gp120

We investigated the ability of the original oligonucleotides and their conjugates to inhibit the binding of antibodies to the HIV-1 gp120 protein using an ELISA assay. Two mAbs, 9284 and 9305, raised against the peptides NNTRKSIRIQRG and RIQRGPGRAFVTIGK corresponded to the sequences of the V3 loop flanking the GPGR apical motif on the N and C side, respectively [21,22]. The epitopes overlap to some extent at the loop apex as illustrated below on a partial sequence of gp120 (residue number in parentheses).

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(296)                                          (331)      (475)        (486)
┌----------------------------V3 loop-----------------------------┐
...CTRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHC........... MRDNWRSELYKY..
mAb 9284 ***************                          mAb 9301 *****************
                   ****************** mAb 9305
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The mAb 9301 raised against the peptide MRDNWRSELYKY was directed to an epitope located on the α5 helix in the C-terminal region of gp120, remote from the V3 loop [2,23]. It can be noted that this epitope also corresponds to a positively charged area [2].

As shown in Fig. 2a, in the whole range of concentrations investigated, no interaction between the unconjugated oligonucleotides 5′-TTGGTTGG-3′ or 5′-TGTGTGTG-3′ and gp120 was detected whichever antibody was used, as expected in the phosphodiester series [11]. On the other hand, when coupled to CHEVP, the oligonucleotides displayed a dose-dependent inhibitory effect on specific antibodies even in the absence of light (Fig. 2b,c). In the case of the TTGGTTGG-chlorin conjugate, only the two mAbs directed to the V3 loop were affected (Fig. 2b). Moreover, the conjugate appears to inhibit binding of the two mAbs with a similar efficiency in the dark. These results suggest that the TTGGTTGG-chlorin conjugate interacts with a region of the gp120 that is common to the epitopes of the two anti-V3 antibodies. It would be tempting to hypothesize that the RIQRG positively charged sequence next to the highly conserved apex of the V3 loop is concerned.

3.2. Light-induced damages

When the TTGGTTGG-chlorin conjugate was irradiated with red light matching the absorption band of the chlorin (see Fig. 1) while it was in contact with gp120, the inhibition of mAb 9284 binding was increased significantly. In contrast, the effect on mAb 9305 was not amplified. No effect on mAb 9301 binding was observed either in the dark or after light irradiation when the TTGGTTGG-chlorin conjugate was used (Fig. 2b). The epitope of mAb 9284 appears to be specifically concerned by the photodamages. However, effects could be produced by alteration of amino acids somewhat farther away inducing conformational changes. It should be noted that antibodies directed to the N side of V3 loop appear to be more sensitive to the polypeptide chain conformation [24].

Although the TGTGTGTG-chlorin conjugate behaves to some extent as the former conjugate, i.e. it inhibits binding of some antibodies in the dark and more efficiently after light irradiation, its relative efficacy to inhibit binding of the three antibodies tested is clearly different. As shown in Fig. 2c, the effect on the mAb 9305 is null in the dark and hardly discernible after irradiation. Some inhibition of mAb 9284 was observed in the dark and increased after irradiation. The two conjugates clearly differ with regards to their effect on mAb 9301: the TGTGTGTG-chlorin derivative inhibits the binding of this antibody in a light-dependent fashion whereas the TTGGTTGG-chlorin derivative was ineffective. In order to further investigate the role of guanines, a 'control' conjugate, TTTTTTTT-chlorin, was used. The unconjugated oligonucleotide and its chlorin conjugate had no effect on the various antibodies. After light irradiation, some inhibition was observed but it was much lower than with the other conjugates and not specific of any of the three antibodies (Fig. 2d).

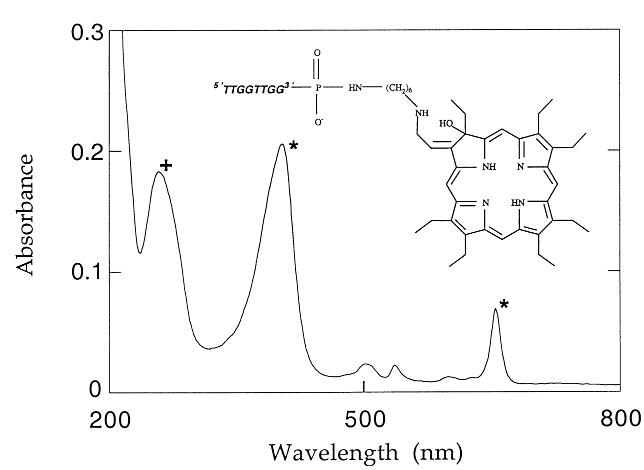

Fig. 1. Structure and absorption spectrum (phosphate buffer, pH 7.2) of the TTGGTTGG-chlorin conjugate. The characteristic absorption bands of the chlorin and nucleic bases are marked by * and +, respectively.

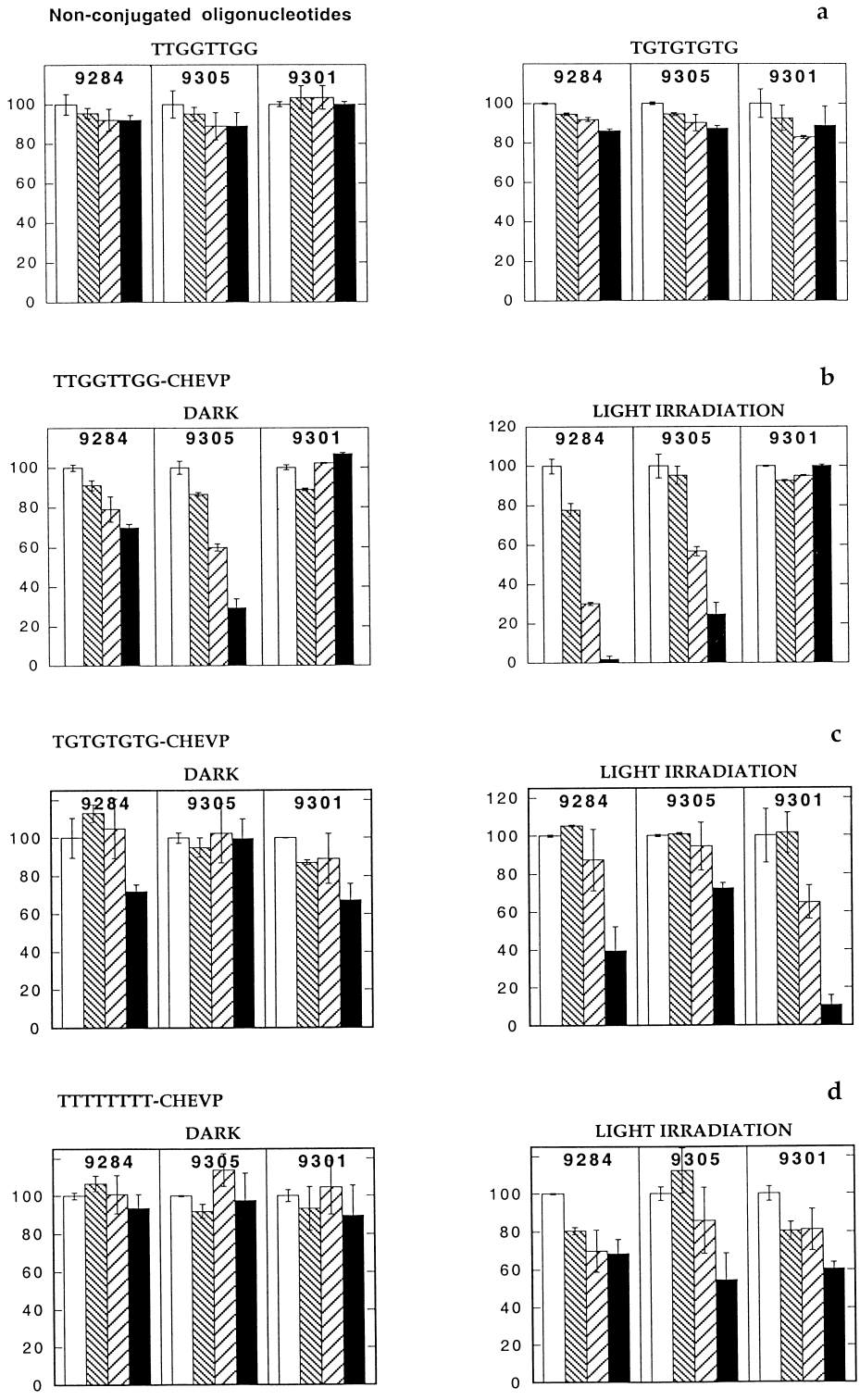

Fig. 2. Dose-dependent inhibition of the binding of gp120-directed antibodies by non-conjugated and chlorin-conjugated oligonucleotides. The antibodies are identified by the numbers 9284, 9305 and 9301 as indicated in the text. Experiments carried out in the dark or involving light irradiation are specified in the figure. In the case of the non-conjugated oligonucleotides (a), only experiments in the dark are shown, light irradiation having no effect, as expected. Experiments with TTGGTTGG-CHEVP, TGTGTGTG-CHEVP and TTTTTTTT-CHEVP are shown in b, c and d, respectively. Antibody binding was quantified by absorbance as described in Section 2, but in order to allow for comparison, data were normalized to 100 in the absence of competitors. The concentrations of the non-conjugated or chlorin-conjugated oligonucleotides are: open, 0; finely hatched, 1×10^{-7} M; broadly hatched, 1×10^{-6} M; filled, 1×10^{-5} M.

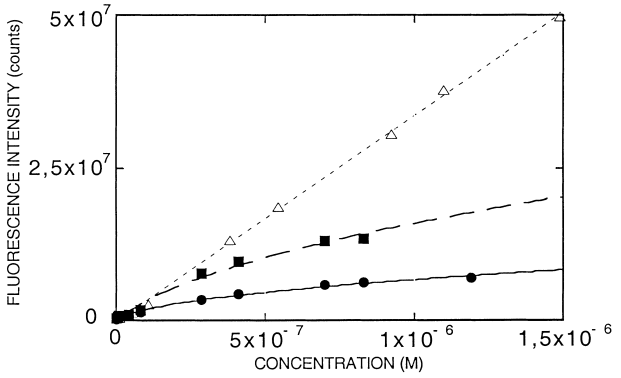

Fig. 3. Concentration-dependence of the fluorescence emission of TTTTTTTT-CHEVP (△), TGTGTGTG-CHEVP (■) and TTGGTTGG-CHEVP (●) in buffer solutions. The data are fitted according to Eq. 1 for a concentration-dependent dimerization process.

3.3. A dual role of the chlorin moiety

Besides its photochemical activity, it clearly appears that the chlorin moiety is required to observe interactions with gp120, even in the dark. Due to its lack of solubility in water, control experiments with the non-conjugated chlorin were precluded, unfortunately. Two hypotheses can be put forward, both based on the hydrophobic character of this molecule. First, it can be anticipated that the chlorin moiety interacts with hydrophobic loci on the protein. Such sites have been identified at the apex of the V3 loop [25] and near its base [2]. A conjunction of hydrophobic and positively charged loci can also be found around the epitope of mAb 9301. Secondly, the chlorin moiety could favor the formation of particular structures owing to its ability to self-associate through π-π interactions.

The second hypothesis was further investigated by monitoring the concentration-dependence of the fluorescence emission spectra of the conjugated oligonucleotides in buffer solutions or in solutions containing a detergent, Triton X-100, able to prevent association. Good linearity was observed for all the conjugates in Triton solutions, indicating that they remain monomeric. As shown in Fig. 3, this is no more true for aqueous solutions. Also, a strong effect of the oligonucleotide sequence is observed. An excellent linearity is seen for the TTTTTTTT-chlorin conjugate, indicating that the presence of the chlorin moiety is not sufficient by itself to promote association. Departure from linearity is clearly seen for the G-containing conjugates. A non-linear concentration-dependence of the fluorescence of hydrophobic porphyrins or chlorins has been observed by several authors and was attributed to dimerization [20]. The shape and the wavelength maxima of the emission and the excitation spectra of the conjugates were found to be independent of the concentration, indicating that only monomers fluoresce. The changes in fluorescence upon concentration were treated accordingly to derive dimerization constants [20]. Theoretical fits assuming dimerization are shown in Fig. 3. The data were not fitted properly assuming other hypotheses such as tetramer formation. The dimerization constant for the TTGGTTGG-chlorin conjugate, $K=(3.3\pm0.8)\times10^7$ M^{-1}, was more than one order of magnitude higher than that measured for the TGTGTGTG-chlorin conjugate, $K=(2.4\pm0.5)\times10^6$ M^{-1}. The higher stability of the TTGGTTGG-chlorin dimer might be due to the formation of two stacked guanosine quartets involving two oligonucleotide strands folded in a hairpin structure [26]. In the case of the oligonucleotide with alternating TG, although the formation of at least one guanine quartet is still possible upon folding as hairpin and dimerization, the absence of two contiguous guanine plateaus would lead to a less stable structure. Taken together, these results indicate that both interactions between chlorin moieties and formation of guanine quartets contribute to the conjugate association. Further characterization of these structures by melting analysis using UV spectroscopy or fluorescence was made difficult by a marked irreversibility. This was apparently due to sticking of the conjugate on the optical cell walls when the temperature was raised. This phenomenon probably arose upon strand dissociation and unfolding exposing the chlorin moiety that could become more prone to adsorption onto the optical glass.

In summary, upon covalent linking with a hydrophobic photosensitizer, some oligonucleotides become able to inhibit binding of antibodies specific of the HIV gp120 glycoprotein. It is worth noting that derivatization of other G-rich oligonucleotides by hydrophobic residues also confers upon them anti-HIV activity [27]. The hydrophobic moiety may promote strand association leading to structures with specific protein affinity. Association is well demonstrated in the case of the planar chlorin macrocycle used in the present study. The stability of the dimer formed also depends on the sequence of the oligonucleotide part and strongly suggests an important contribution of the formation of guanine quartets. The association of conjugates as dimers is a major determinant of activity as illustrated by the 'control' T_8-chlorin conjugate. The comparison of the two G-rich chlorin-oligonucleotide conjugates investigated here shows that the oligonucleotide sequence also comes into play in targeting these molecules to specific regions of gp120. Then, site-directed photochemical damages can be elicited upon irradiation of the conjugates associated to gp120 using red light, which significantly increases their efficiency. In addition to their potential as new anti-viral agents for blood treatment, such conjugates might find use as biochemical tools to explore protein functions. In particular, their ability to produce permanent damages could help in elucidating the role played by protein areas that are temporarily exposed [28].

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