Communication

Nuclear Targeting of Chlorin e\textsubscript{6} Enhances Its Photosensitizing Activity*\textsuperscript{a,b}

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Although photosensitizers, molecules that produce active oxygen species upon activation by visible light, are being extensively used in photodynamic therapy to treat cancer and other clinical conditions, problems include normal cell and tissue damage and associated side effects, which are attributable in part to the fact that cytotoxic effects are largely restricted to the plasma membrane. We have previously shown that the photosensitizer chlorin e\textsubscript{6} has significantly higher photosensitizing activity when present in conjugates containing specific ligands and thus able to be internalized by receptor-expressing cells. In this study we use insulin-containing conjugates to which variants of the simian virus SV40 large tumor antigen nuclear localization signal (NLS) were linked to target chlorin e\textsubscript{6} to the nucleus, a hypersensitive site for active oxygen species-induced damage. NLSs were either included as peptides cross-linked to the carrier bovine serum albumin or encoded within the sequence of a \textbeta-galactosidase fusion protein carrier. The results for photosensitization demonstrate clearly for the first time that NLSs increase the photosensitizing activity of chlorin e\textsubscript{6} maximally reducing the EC\textsubscript{50} by a factor of over 2000-fold. This has wide-reaching implications for achieving efficient cell type-specific photodynamic therapy.

Photosensitizers such as porphyrins are molecules that produce active oxygen species upon activation by visible light and are currently being extensively used in photodynamic therapy to treat cancer and other clinical conditions (1–3). Because normal cells are able to accumulate porphyrins, however, and porphyrins are only excreted slowly from the body, prolonged skin photosensitization as well as other effects can be a problem (4, 5), leading to normal cell and tissue damage (6). A high priority with respect to photodynamic therapy is accordingly to increase the specificity of the uptake of photosensitizers in particular target cells, thereby enabling the active dose of porphyrins administered to patients to be reduced. We have previously shown that the photosensitizer chlorin e\textsubscript{6} has significantly higher photosensitizing activity when present in conjugates containing specific ligands such as insulin or concanavalin A and thus is able to be internalized by receptor-expressing cells (7–9). Photosensitization could be competed by incubating cells in the presence of an excess of unconjugated ligand (7–9), indicating that cellular uptake was receptor-dependent. Because only cells expressing specific receptors are targeted in this approach (see Refs. 7–9), it is clear that it enables selectivity in terms of the cell types targeted for photosensitization.

Due to the fact that injury induced by singlet oxygen comprising 80% of all of the active oxygen species generated upon porphyrin activation is localized within less than 0.1 \mu m of the site of its production, the effect of photosensitizers is integrally dependent on their site of cellular accumulation (1). Although most porphyrins such as chlorin e\textsubscript{6} and hematoporphyrin derivatives localize largely at the plasma membrane (10, 11), it is known that intracellular sites, and particularly the nucleus, are much more sensitive sites for photodynamic damage (10, 12, 13). Consistent with this, our previous results (7–9) indicate that the enhancement of photosensitization effected by internalizable conjugates is directly attributable to their ability to be internalized (and thereby damage intracellular sites), because treatments preventing internalization severely reduce photosensitization. The directed delivery of photosensitizers to particularly sensitive subcellular organelles such as the nucleus using specific targeting signals would seem to be a key to performing efficient photodynamic therapy.

Here we use insulin-containing conjugates to which variants of the simian virus SV40 large tumor antigen (T-ag)\textsuperscript{1} nuclear localization signal (NLS) (14) were linked to target the photosensitizer to the nucleus. NLSs were either included as peptides cross-linked to the carrier bovine serum albumin (BSA) or encoded within the sequence of a \textbeta-galactosidase fusion protein carrier. Results for photosensitization demonstrate clearly for the first time that NLSs increase the photosensitizing activity of chlorin e\textsubscript{6} maximally reducing the EC\textsubscript{50} by a factor of over 2000-fold, and confirm that the nucleus is a hypersensitive site for photodynamic action. These observations have implications for achieving efficient cell type-specific photodynamic therapy.

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\textsuperscript{1} The abbreviations used are: T-ag, SV40 large tumor antigen; NLS, nuclear localization signal; BSA, bovine serum albumin; P10, NLS-containing \textbeta-galactosidase fusion protein; CKII, casein kinase II; CMCS, cyclo-hexyl-3(2-morpholinomethyl)carboximidemethy-4-toluene sulfonate; DCFD, 2',7'-dichlorofluorescin diacetate; SDP, N-succinimidyl 3-[2-pyridylidithio]propionate; CLSM, confocal laser scanning microscopy; MBS, 3-maleimidobenzoyl-N-hydroxysuccinimide ester.

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Fig. 1. Schematic representation of the internalizable NLS-containing photosensitizing conjugates used in this study. Conjugates, prepared as described under "Material and Methods," are as follows: A, BSA-P101Lys-(chlorin e₆)-insulin and BSA-P101Thr-(chlorin e₆)-insulin, where peptides and chlorin e₆ are coupled to one BSA carrier, insulin is linked to a second BSA molecule, and the two BSA molecules are subsequently linked. B, BSA-P101Lys-(chlorin e₆)-insulin and BSA-P101Thr-(chlorin e₆)-insulin, where peptides, chlorin e₆ and insulin are linked to a BSA carrier. C, P10-(chlorin e₆)-insulin and β-galactosidase-(chlorin e₆)-insulin, where insulin and chlorin e₆ are linked either to the NLS-containing β-galactosidase fusion protein P10 (10) or to β-galactosidase as carriers.

RESULTS AND DISCUSSION

Construction of NLS-containing Internalizable Photosensitizing Conjugates—The goal of this study was to enhance the photodynamic activity of chlorin e₆ by directing its specific delivery to the nucleus through its incorporation into an internalizable construct containing an NLS. We used either BSA (Fig. 1, A and B) or β-galactosidase/β-galactosidase fusion protein P10 (Fig. 1C) as carriers to which other components were covalently attached in three types of conjugates (see Fig. 1). NLSs were included either as peptides covalently coupled to BSA or within the coding sequence of P10. The NLSs were selected based on our previous work, which showed that nuclear targeting effected by the T-ag NLS (amino acids 126–132) is enhanced markedly by the casein kinase II (CKII) phosphorylation site (Ser₁₁₁/₁₁₂) (14) but inhibited by phosphorylation by the cyclin-dependent kinase cdc2 at Thr₁₂⁴ adjacent to the nuclear targeting enhancer (NTE) and by the short nuclear localization signal (NLS) (15). Images of these constructs are shown in Fig. 1.

Preparation of Conjugates—The basic design of the conjugates used is shown schematically in Fig. 1. BSA was used as the carrier in the case of the conjugates shown in A and B, whereas β-galactosidase or the β-galactosidase fusion protein P10 were the carriers in C (see below). In the case of the BSA-P101Lys-(chlorin e₆)-insulin and BSA-P101Thr-(chlorin e₆)-insulin conjugates (Fig. 1A), BSA was cross-linked to chlorin e₆ using cyclo-hexyl-3-2-morpholinethiocarbodimide-n-male-4-toluene sulfonate (CMCS, Serva) in 10 mM sodium phosphate buffer, pH 7.5 (buffer A), at a ratio of BSA to chlorin e₆ to CMCS of 1:30:300. The peptides P11Lys (NH₄-Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro-Tyr-Cys-COOH) and P11Thr (NH₄-Pro-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro-Tyr-Cys-COOH) were linked individually to the amino groups of BSA-(chlorin e₆) using 3-maleimidobenzoyl-N-hydroxysuccinimide ester (Sulfo-NHS-SPDP; Sigma) in buffer A containing 0.5 mM EDTA, whereas insulin (Sigma) was separately linked to BSA using glutaraldehyde (Merek) (15). BSA-(chlorin e₆)-peptide and BSA-(chlorin e₆)-insulin were then reacted individually with N-succinimidyl 3-[2-pyridyldithiophosphate (SPDP, Sigma) in 10 mM Heps, 150 mM NaCl, pH 7.5, to yield PDP derivatives. PDP-BSA-(chlorin e₆)-peptide was reduced with 50 mM dithiothreitol to yield HS-BSA-(chlorin e₆)-peptide, which was then reacted with PDP-BSA-insulin to form the conjugate BSA-(chlorin e₆)-peptide-BSA-insulin (Fig. 1A). The final conjugates contained 3–4 peptide, 4–5 chlorin e₆, and 4–5 insulin molecules per BSA molecule, respectively.

In the case of the BSA-P101Lys-(chlorin e₆)-insulin and BSA-P101Thr-(chlorin e₆)-insulin conjugates (Fig. 1B), BSA was conjugated with chlorin e₆ as above, and the peptides P101Lys (NH₄-Cys-Gly-Pro-Gly-Met-Asp-Pro-Ala-Ala-Glu-Ala-Ala-Glu-Ala-Ala-Lys-Lys-Lys-Arg-Lys-Val-Glu-Asp-Pro-Tyr-Cys-COOH) and P101Thr (NH₄-Cys-Gly-Pro-Gly-Met-Asp-Pro-Ala-Ala-Glu-Ala-Ala-Glu-Ala-Ala-Glu-Ala-Ala-Glu-Ala-Ala-Glu-Ala-Ala-Ala-Asp-Ala-Gln-His-Ala-Ala-Pro-Pro) were initially reacted with 1,6-diaminohexane (Sigma) using CMCS (CMCS ratio of chlorin e₆ to 1,6-diaminohexane to CMCS of 1:100:100 in 10 mM sodium phosphate buffer, pH 7.0, and CMCS was subsequently used to link aminoochlorin e₆ with the P10β-galactosidase carrier in the same buffer (ratio of carrier to aminoochlorin e₆ to CMCS of 1:30:300). After successive reaction of insulin with citracyanic hydride (18) (Serva) and SPDP (ratio of 1:6) in 25 mM Heps, 150 mM NaCl, pH 7.5 (buffer B), citracyanic-insulin-PDP was linked to P10-chlorin e₆ or β-galactosidase-chlorin e₆ (ratio of 1:5:1) in buffer B, after which the citracyanic groups were removed (18). Two series of constructs were made: P10-(chlorin e₆)-insulin (1:5:7), β-galactosidase-(chlorin e₆)-insulin (1:5:7), and P10-(chlorin e₆)-insulin (1:3:8) and β-galactosidase-(chlorin e₆)-insulin (1:2:8).

The average ratio of components in conjugates was calculated using both the molecular weight estimation from polyacrylamide gel electrophoresis and the optical density measurement at 400 nm (for chlorin e₆). The average ratio of components in conjugates was calculated using both the molecular weight estimation from polyacrylamide gel electrophoresis and the optical density measurement at 400 nm (for chlorin e₆). The conjugates were purified by dialysis or column chromatography.

Visualization and Quantitation of Phototoxic Activity in Situ—Cells were incubated with conjugates or chlorin e₆ for 18 h at 37 °C, washed (1×) with RPMI 1640 medium (without phenol red, containing 2% heat-inactivated FBS, 25 mM Heps, pH 7.5), incubated with 2′,7′-dichlorofluorescein diacetate (DCF, Serva) for 5 min at 37 °C, and then washed in the same medium and irradiated using a slide projector. Fluorescence due to the production of 2′,7′-dichlorofluorescein from DCFD through reaction with reactive oxygen species (19) was visualized either as described previously (9) or using CLSM (Bio-RAD MRC-600 CLSM) (16, 17, 20, 21). Image analysis of CLSM files was performed using the NIH Image public domain software (16, 17, 20, 21). Equivalent measurements were made with fluorescence in the nucleus (Fn), and cytoplasm (Fc) in cells treated without (background fluorescence) or with free chlorin e₆ or chlorin e₆-containing conjugates. Background was subtracted from all values to yield specific fluorescence. The nuclear/cytoplasmic ratio (Fn/Fc) represents the fold accumulation in the nucleus (16, 17, 20, 21).

Determination of Cell Viability—Two assays were used as previously (8, 9). Cells were plated either in 12-well plates (80000 cells/well) or in 35-mm dishes (5000 cells/dish), 1 day after which they were incubated for 18 h at 37 °C with various concentrations of conjugates or chlorin e₆, then washed, and irrigated using a slide projector (96 kJ/m²). In the case of the former, cells were trypsinized 1 h later and replated in 35-mm dishes, and the number of colonies were counted after 10 days, whereas in the latter, cells were not replated but simply fixed after 10 days and stained with methylene blue. Colonies were then either counted in the case of PLC/PRF/5 cells or dye extracted with 1% N-lauroyl-sarcosine (Sigma) in phosphate-buffered saline and absorbance at 630 nm measured (22) in the case of C6 cells.
Nuclear Targeting Enhances Chlorin $e_6$ Activity

**TABLE I**

The photodynamic activity of NLS-containing chlorin $e_6$ conjugates

| Conjugate $^a$ | EC$_{50}$ ± S.D. (nM) | PLC/PRF/5 cells $^b$ | C6 glioma cells $^c$ |
|----------------|------------------------|----------------------|---------------------|
| Chlorin $e_6$  | 350 ± 28               | >1000                | ND                  |
| P10-(chlorin $e_6$)-insulin (1:5:7) | 7.5 ± 0.5            |                      |                     |
| $\beta$-Galactosidase-(chlorin $e_6$)-insulin (1:5:7) | 38 ± 6              |                      |                     |
| BSA-P101Lys-(chlorin $e_6$)-insulin (1:4:7:3) | 23.0 ± 0.5          |                      |                     |
| BSA-P101Thr-(chlorin $e_6$)-insulin (1:4:7:3) | >150                |                      |                     |
| BSA-P11Lys-(chlorin $e_6$)-insulin (2:4:5:4) | 29 ± 6              |                      |                     |
| BSA-P11Thr-(chlorin $e_6$)-insulin (2:3:4:5) | 80 ± 9              |                      |                     |
| **PLC/PRF/5 cells** | **C6 glioma cells** |                      |                     |
| Chlorin $e_6$  | 320 ± 2               | >1000                | ND                  |
| P10-(chlorin $e_6$)-insulin (1:3:8) | 0.13 ± 0.06          | ND                  |                     |
| $\beta$-Galactosidase-(chlorin $e_6$)-insulin (1:2:8) | 2.0 ± 0.4           | ND                  |                     |
| BSA-P101Lys-(chlorin $e_6$)-insulin (1:11:12) | 50 ± 8              | 28 ± 3              |                     |
| BSA-P101Thr-(chlorin $e_6$)-insulin (1:1:8:2) | 104 ± 21            | 74 ± 29             |                     |

$^a$ The ratio of components in the conjugates is indicated in parentheses.

$^b$ Cells were incubated with varying concentrations of conjugates or free chlorin $e_6$, then washed, irradiated, and replated on dishes; after 10 days the number of colonies was counted.

$^c$ One day after plating into 35-mm dishes, cells were incubated for 18 h at 37 °C with various concentrations of conjugates or free chlorin $e_6$ and then washed and irradiated. After 10 days, cells were fixed and stained with methylene blue, and the number of colonies were counted in the case of PLC/PRF/5 cells (A and B), or dye extracted and absorbance at 630 nm were measured in the case of C6 cells (C). The conjugates were P10-(chlorin $e_6$)-insulin (1:3:8) and $\beta$-galactosidase-(chlorin $e_6$)-insulin (1:2:8) (A) and BSA-P101Lys-(chlorin $e_6$)-insulin (1:1:12) and BSA-P101Thr-(chlorin $e_6$)-insulin (1:1:8:2) (B and C). Standard deviations are indicated. $\bullet$, free chlorin $e_6$; $\bigcirc$, $\beta$-galactosidase-(chlorin $e_6$)-insulin; $\times$, P10-(chlorin $e_6$)-insulin; $\mathbf{v}$, BSA-P101Thr-(chlorin $e_6$)-insulin; $\square$, BSA-P101Lys-(chlorin $e_6$)-insulin.

NLS (16, 17). The NLS peptide P101Lys (see “Materials and Methods”) includes the CKII site but contains nonphosphorylatable alanine residues in place of Ser$^{120}$, Ser$^{123}$, and Thr$^{124}$, whereas the control peptide P101Thr is identical to P101Lys except that it contains threonine in place of the critical Lys$^{128}$ residue, which abolishes nuclear targeting activity (14, 23). Shorter peptides containing the wild type (P11Lys) or Thr$^{128}$ derivative (P11Thr) of the T-ag NLS alone (“Materials and Methods”) were also used. All peptides contained either N- or C-terminal Cys residues for cross-linking. The P10 $\beta$-galactosidase fusion protein contains T-ag amino acids 111–135 with Ala substitutions at Ser$^{120}$, Ser$^{123}$, and Thr$^{124}$ identical to those of P101Lys (14, 16, 17). Insulin was included in the constructs to confer binding to and internalization by receptor-containing target cells (7–9), such as those of the PLC/PRF/5 human hepatoma (8) or C6 rat glioma cell lines (24).

NLSs Confer Nuclear Localization of Internalizable Conjugates Containing Chlorin $e_6$—Subcellular localization of the NLS peptide constructs was visualized by confocal laser scanning microscopy (CLSM) using DCFD (Fig. 2; B and D), which interacts with reductive oxygen species to yield a fluorescent product, thus enabling visualization of the precise subcellular sites of photoactivation/photo-oxidation in living cells (9). PLC/PRF/5 cells treated with the NLS-containing BSA-P101Lys-(chlorin $e_6$)-insulin construct showed nuclear levels of specific fluorescence (Fn of 3.47 arbitrary units) 15 times higher than those treated with either chlorin $e_6$ alone or the BSA-P101Thr-(chlorin $e_6$)-insulin construct that contains a nonfunctional NLS (Fn of about 0.2). The extent of nuclear accumulation (Fn/c) relative to that in the cytoplasm was more than 85% higher than that for the functional NLS-containing construct.
with P101Lys (Fn/c of 0.88 compared with 0.48 for the P101Thr construct). Similar analysis for the NLS-containing P10-(chlorin e<sub>6</sub>)-insulin construct indicated nuclear levels significantly higher than those for the NLS-deficient β-galactosidase-(chlorin e<sub>6</sub>)-insulin construct (not shown).

Enhanced Photosensitization Conferred by Nuclear Targeting Internalizable Conjugates Containing Chlorin e<sub>6</sub>-Photodynamic activity was measured in colony formation tests using PLC/PRF/5 and rat glioma C6 cells, photoactivation (at a dose of 96 kJ/m<sup>2</sup>) being carried out 18 h after the addition of conjugates or free chlorin e<sub>6</sub>. In all experiments, the NLS-containing constructs were more effective than those lacking NLSs or containing nonfunctional (Thr<sup>128</sup>-substituted) NLSs, exhibiting much lower EC<sub>50</sub> values (see Table I). The photodynamic activity of the P11Lys-containing conjugate (EC<sub>50</sub> of 29 nM) was more than 10 times higher than that of chlorin e<sub>6</sub> alone (EC<sub>50</sub> of 350 nM), whereas the NLS-deficient P11Thr peptide-containing conjugate exhibited reduced activity (EC<sub>50</sub> of 80 nM) (Table I). The photodynamic activity of P101-containing conjugates was higher (EC<sub>50</sub> of 23 nM for PLC/PRF/5 cells) than that of those containing P11Lys, whereas the substitution of Lys<sup>128</sup> by Thr greatly increased the EC<sub>50</sub> (>150 nM) (Table I). Similar results were obtained using C6 cells (Fig. 3C and Table I). The fact that the P11Lys-containing BSA construct showed reduced efficiency compared with the P101Lys-containing construct implied that the additional T-ag sequences present in P101Lys enhanced nuclear import, presumably as a result of the presence of the CKII site. Consistent with this, the most potent photosensitizing conjugate was P10-(chlorin e<sub>6</sub>)-insulin, exhibiting an EC<sub>50</sub> value of 0.13 nM, compared with 2 nM for the NLS-deficient β-galactosidase-(chlorin e<sub>6</sub>)-insulin construct, in colony formation tests with PLC/PRF/5 cells, which is over 2400 times lower than the value for free chlorin e<sub>6</sub> (Fig. 3A and Table I). Like the P101Lys peptide, P10 contains the CKII site that enhances the rate of nuclear import by about 50-fold (14, 16).

We measured the photodynamic activity of our conjugates using colony formation tests and applied the DCDF test permitting the determination of the subcellular localization of conjugates. Although the data are insufficient to enable a definitive conclusion to be drawn, it is interesting that the number of molecules of chlorin e<sub>6</sub> within the conjugate does not appear to be critical for its photodynamic activity, e.g. the EC<sub>50</sub>s for β-galactosidase-(chlorin e<sub>6</sub>)-insulin (2 chlorin e<sub>6</sub> residues) and for BSA-(chlorin e<sub>6</sub>)-insulin (16 chlorin e<sub>6</sub> residues) were approximately the same (Table I; see Ref. 9). In contrast, the number of insulin and NLS moieties does appear to be critical in determining photodynamic activity, where at least three moieties of each are preferable (see Table I).

In summary, targeting of chlorin e<sub>6</sub> to the nucleus through incorporation in NLS-containing conjugates increases its photodynamic activity by more than 2000-fold, confirming that the nucleus is a hypersensitive site for photodynamic action (10, 12, 13). NLS-containing photosensitizing constructs such as those described here should have important application in cell type-specific photodynamic therapy. Future work will include optimizing nuclear targeting through modification of NLS-function modulating flanking sequences such as phosphorylation sites (20, 25, 26) and substituting insulin with alternative ligands to target photosensitizers specifically to particular tumor cell types.

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