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Controlled intracellular localization and enhanced antisense effect of oligonucleotides by chemical conjugation†

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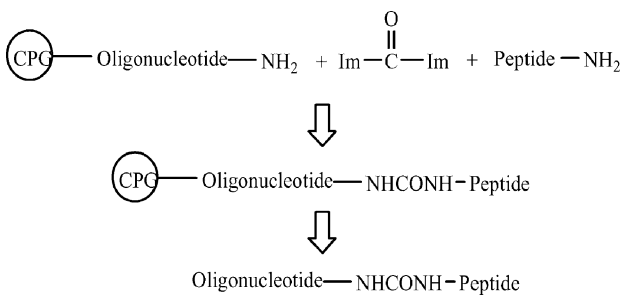
Oligonucleotides can be covalently linked to peptides composed of any sequence of amino acids by solid phase fragment condensation. The peptides incorporated into the conjugates include nuclear localizing signals (NLS), nuclear export signals (NES), membrane fusion domain of some viral proteins and some designed peptides with amphipathic character. Evaluation of biological properties of DNA–peptide conjugates indicated that (a) the conjugates could bind to target RNA and dsDNA with increased affinity, (b) the conjugates were more resistant to cellular nuclease degradation, (c) the conjugate–RNA hybrids could activate RNase H as effectively as native oligonucleotides, (d) the conjugates with fusion peptides showed largely enhanced cellular uptake, (e) the conjugates with NLS could be predominantly delivered into the cell nucleus, (f) the conjugates with NES could be localized in the cytoplasm. As a result, antisense oligonucleotides conjugated with NLS could inhibit human telomerase in human leukemia cells much more strongly than phosphorothioate oligonucleotides.

Cellular uptake and localization of antisense oligonucleotides are crucial problems for their inhibitory effects of genetic expressions.[1] In order to target to mRNA, antisense oligonucleotides should be localized in cytoplasm to inhibit the production of a certain specific protein, while in order to target to DNA, antigene oligonucleotides should be transported and localized into the cellular nucleus to suppress the transcription of a certain specific gene. Various gene delivery agents so far developed can enhance cellular uptake of oligonucleotides but often cause cytotoxicity and cannot control the precise intracellular localization of them. On the other hand, recently, mechanisms of transport of proteins and RNAs between the cell nucleus and the cytoplasm have been revealed in detail.[2] The understanding of the molecular machinery working for the import and export of proteins through nuclear pores prompted us to investigate the control of intracellular delivery and localization of oligonucleotides by using signal sequences in proteins. Recently, studies on synthetic methods of oligonucleotide–peptide conjugates have been reported.[3] For example, Gait and Stetsenko successfully reported an improved synthesis of a variety of oligonucleotide–peptide conjugates by 'native ligation'.[4] Haralambidis and his co-workers achieved sequential synthesis of the conjugates of oligonucleotides and viral fusion peptides.[5] Azhayev's group[6] and Stetsenko and Gait[7] reported an improved method towards a general preparation of oligonucleotide–peptide conjugates. Nevertheless, solid phase synthesis of oligonucleotide–peptide conjugates in a sequential manner in which peptides are usually prepared first and oligonucleotides are prepared next cannot avoid the problem that the coupling efficiencies in peptide synthesis are not always high enough compared with those in oligonucleotide synthesis. Insufficient coupling yields of peptides result in lowering overall yields of desired products. In order to overcome such problems as (1) low overall yields of products and (2) limitation of amino acid components in peptides, several attempts of fragment coupling on solid support have been carried out. In previous studies, amino-functionalized oligonucleotides were coupled with carboxyl-activated peptides by amide bond formation.[8] These methods also have limitations in components of peptides because they employ standard coupling conditions of solid phase peptide synthesis for amide formation. Recently, we attempted to develop a universal method to prepare DNA–peptide conjugates by 'Solid Phase Fragment Condensation (SPFC)'.[9] The strategy of SPFC is that a DNA fragment, having a free amino group prepared on a CPG support, is reacted with α,ω-diisocyanatoalkane or carbonyl diimidazole and then with a peptide fragment bearing a single reactive amino group. The resulting oligonucleotide–peptide conjugate covalently linked to a solid support is cleaved from the CPG and deprotected by treatment with ammonia (Scheme 1). The SPFC allows one to link oligonucleotides covalently to peptides with any component of amino acids in good yields. In the present study, we synthesized oligonucleotide–signal peptide conjugates by solid phase fragment condensation (SPFC) and evaluated the intracellular localization and antisense inhibitory effects of the conjugates.



Scheme 1 Solid phase fragment condensation (SPFC).

Oligonucleotide–peptide conjugates **C1**–**C8** and their fluorescence-labeled counterparts **C1f**, **C2f**, **C4f**, **C5f**, **C8f** were synthesized by SFPC (Scheme 2). Oligonucleotides, whose

† Electronic supplementary information (ESI) available: characterization of products. See http://dx.doi.org/10.1039/b507691a

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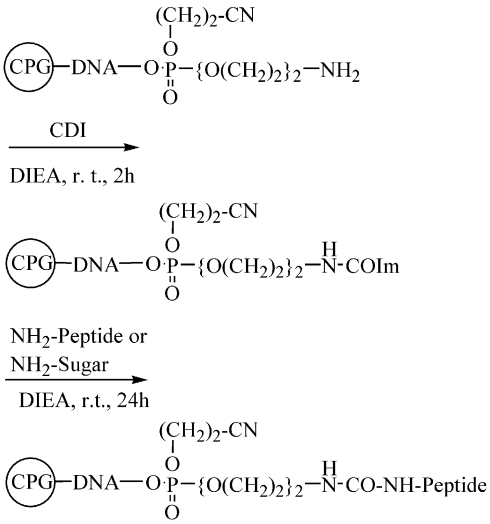
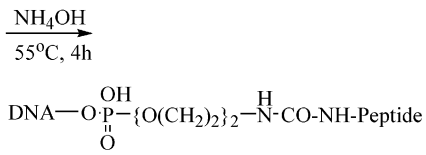


Protective groups in peptide fragments:
-NHtfa (Lys), -SAc (Cys)
free: -OH (Ser, Thr), -COOH(Asp, Glu), -$CONH_2$ (Asn, Gln), -guanidiny l(Arg), -imidazolyl (His)



R-CONHCH$_2$CH$_2$OCH$_2$CH$_2$OPO$_3$-5'-CAGTTAGGGTTAG-3'
N1:5'-CAGTTAGGGTTAG-3'
C1: R = Ac-GPKKKRKVGK-εNH- (SV40 T antigen NLS)
C1f: R = FITC-GPKKKRKVGK-εNH- (SV40 T antigen NLS)
C2: R = Ac-GRKKRRQRRRPPGGK-εNH- (HIV-1 Tat NLS)
C2f: R = FITC-GRKKRRQRRRPPGGK-εNH- (HIV-1 Tat NLS)
C3: R = - NH-βANSAAFEDLRVLS-OH (Influenza Virus nucleoprotein NLS)
C4: R = Ac-LPPLERLTLGK-εNH- (HIV-1 Rev NES)
C4f: R = FITC-LPPLERLTLGK-εNH- (HIV-1 Rev NES)
C5: R = - NH-βALRALLRALLRAL-OH (designed)
C5f: R = - NH-βALRALLRALLRAL-OH (designed)
C6: R = - NH-βARLRLRLRLRL-OH (designed)
C7: R = galactosamine

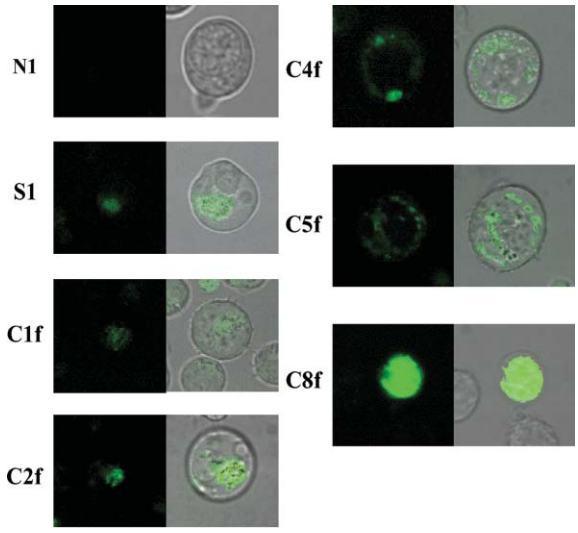
R-CONHCH$_2$CH$_2$OCH$_2$CH$_2$OPO$_3$-5'-s(CAGTTAGGGTTAG)-3'
S1: 5'-s(CAGTTAGGGTTAG)-3'
C8: R = Ac-GPKKKRKVGK-εNH- (SV40 T antigen NLS)
C8f: R = FITC-GPKKKRKVGK-εNH- (SV40 T antigen NLS)

Scheme 2 Synthesis of oligonucleotide–peptide conjugates by SPFC.

sequences are complimentary to the RNA template of human telomerase[10] synthesized on a solid support and modified with an alkyl amino group at the 5′-end, were covalently linked to peptide fragments using carbonyldiimidazole (CDI) as a linker. After cleavage from the solid support and deprotection with ammonium hydroxide, a single HPLC purification gave pure products in 10–50% yield. All of the products were fully characterized by MALDI-TOF MS and HPLC to give satisfactory results (see ESI†). The structures of the conjugates are shown in Scheme 2.

Peptide moieties in the conjugates are derived from a nuclear export signal (NES) sequence of HIV-1 rev protein,[11] a nuclear localization signal (NLS) of SV40 T-antigen,[12] NLS of influenza virus nucleoprotein,[13] NLS of HIV-1 tat protein,[11] and designed amphiphilic peptides.[14,15] Peptides in **C5** and **C6** were proven to form an amphipathic α-helical and an antiparallel β-sheet structure in the presence of DNA, respectively, and both of them could bind to and stabilize dsDNA and a DNA–RNA hybrid.[14,15] Galactosamine was also successfully conjugated to oligonucleotides without any protection (**C7**).

Cellular uptake and intracellular localization of DNA–signal peptide conjugates were evaluated by confocal laser fluorescence microscopy (Fig. 1). Human leukemia cells, Jurkat, were incubated with fluorescence-labeled oligonucleotide–peptide conjugates **C1f**, **C2f**, **C4f**, **C5f**, **C8f** in 10% serum for 48 h. The microscopic observations clearly showed that cellular uptake of oligonucleotide–peptide conjugates was enhanced whereas normal oligonucleotides were hardly taken up into the cells. Phosphorothioate oligonucleotides were taken up into cells but were spread all over the cells (nucleus and cytoplasm). As expected, oligonucleotides conjugated with a nuclear localization signal (NLS) sequence of SV40 large T-antigen (**C1f** and **C8f**) and oligonucleotides conjugated with NLS of HIV-1 tat protein (**C2f**) were delivered and localized into the nucleus. On the other hand, oligonucleotides conjugated with a nuclear export signal (NES) sequence of HIV-1 rev (**C4f**) were localized all over the cytoplasm outside the nucleus. Curiously, the conjugate **C5f**, bearing a designed peptide with a cationic amphipathic character, was localized all over the cytoplasm outside the nucleus just like the NES conjugate **C4f**.



Conditions: Conjugates (1mM) were incubated with Jurkat ($0.5x10^6$ cell/ml) in RPMI medium for 24 h at 37 °C under 5% CO_2.

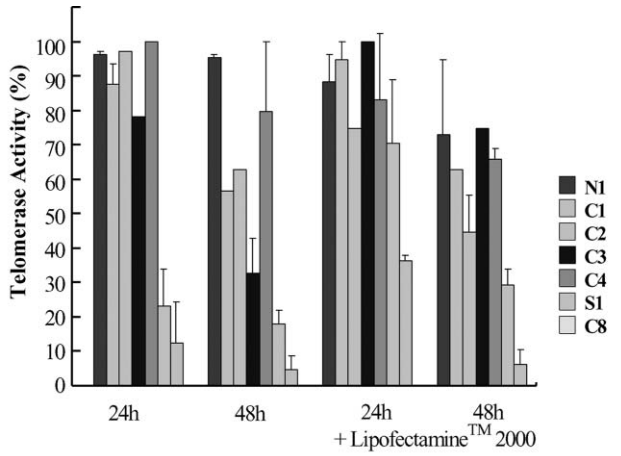
Fig. 1 Cellular uptake and localization of the conjugates.

Antisense inhibition of human telomerase by DNA–peptide conjugates was evaluated by TRAP assay using human leukemia cells Jurkat (Fig. 2). IC_{50} values are summarized in Table 1. The results clearly show that antisense inhibitions of telomere elonganation are dramatically affected by the localization of the oligonucleotides. Oligonucleotide–NLS conjugates **C1**, **C2**, **C3** showed a much higher inhibitory effect (43, 38 and 70% inhibition, respectively after 48 h) than the normal antisense oligonucleotide **N1** (0% inhibition), whereas the oligonucleotide–NES conjugate **C4** showed entirely no inhibitory effect. Furthermore, phosphorothioate oligonucletide–NLS conjugate **C8** completely

Table 1 IC_{50} values of telomerase inhibition

	IC_{50}/μM		IC_{50}[a]/μM	
Antisense oligonucleotide	24 h	48 h	24 h	48 h
S1[b]	1.4	0.94	> 5.00	1.4
C8[c]	1.1	0.15	2.15	0.48

[a] Transfected using Lipofectamine™ 2000. [b] **S1**: 5′-s(CAGTTAGGGTTAG)-3′. [c] **C8**: SV40 T antigen NLS–**S1** conjugate.



Conditions: Oligonucleotide or conjugates (5μM) were incubated with Jurkat cells (0.5x106 cell/ml) at 37 °C under 5% CO_2. Telomerase activities were analyzed by TRAP assay.

Fig. 2 Antisense inhibition of human telomerase by conjugates.

suppressed telomerase activity (99.6% in 24 h and 95.3% in 48 h), while the phosphorothioate oligonucleotide itself inhibited telomerase in 87% in 24 h and 78% in 48 h.

In summary, oligonucleotide–peptide conjugates were shown to be taken up effectively into cells without using any transfection reagents. Controlled nuclear localization was achieved by oligonucleotide–NLS conjugates and cytoplasmic localization was achieved by oligonucleotide–NES conjugates, respectively. Antisense oligonucleotide–NLS conjugates suppressed human telomerase in leukemia cells very effectively. These findings strongly suggest that oligonucleotide–peptide conjugates can be promising candidates for the ideal genetic medicines of the next generation. We believe that intelligent oligonucleotides which have never been produced can be created by linking oligonucleotides to natural and unnatural molecules which will never meet together in nature.

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