Cellular delivery of peptide nucleic acids and inhibition of human telomerase

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Background: Human telomerase has an essential RNA component and is an ideal target for developing rules correlating oligonucleotide chemistry with disruption of biological function. Similarly, peptide nucleic acids (PNAs), DNA analogs that bind complementary sequences with high affinity, are outstanding candidates for inducing phenotypic changes through hybridization.

Results: We identify PNAs directed to nontemplate regions of the telomerase RNA that can overcome RNA secondary structure and inhibit telomerase by intercepting the RNA component prior to holoenzyme assembly. Relative potencies of inhibition delineate putative structural domains. We describe a novel protocol for introducing PNAs into eukaryotic cells and report efficient inhibition of cellular telomerase by PNAs.

Conclusions: PNAs directed to nontemplate regions are a new class of telomerase inhibitor and may contribute to the development of novel antiproliferative agents. The dependence of inhibition by nontemplate-directed PNAs on target sequence suggests that PNAs have great potential for mapping nucleic acid structure and predictably regulating biological processes. Our simple method for introducing PNAs into cells will not only be useful for probing the complex biology surrounding telomere length maintenance but can be broadly applied for controlling gene expression and functional genomics.

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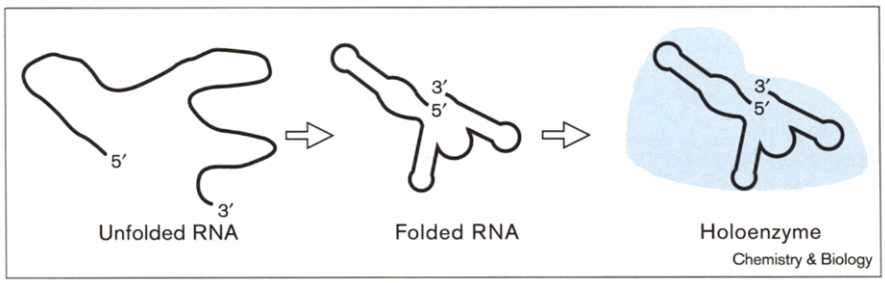
Introduction

Progress in genome sequencing and the use of gene expression arrays as a high-throughput method for examining mRNA levels are expanding our knowledge of the sequence and expression of cellular proteins faster than our understanding of their function. Oligonucleotides, with their inherent potential for specific recognition, can be used to deconvolute the complex role of proteins within cells and sort the diverse interactions of individual proteins. To create 'knock out' phenotypes that can be interpreted, however, oligonucleotides must be delivered across the cell membrane and then bind complementary sequences with high specificity.

Peptide nucleic acids (PNAs), DNA analogs that have N-(2-aminoethyl) glycine linkages rather than a phosphodiester backbone [1], possess properties that make them leading candidates for this task, including a propensity for rapid [2] and high-affinity binding [1,3], resistance to degradation by nucleases and proteases [4], poor affinity for proteins that normally bind nucleic acids [5], and high mismatch discrimination [3]. To take full advantage of these favorable properties it is necessary to develop rules to predict the propensity for hybridization of PNAs to varied nucleic acid sequences and structures. Such rules have already been developed *in vitro*, where hybridization at polypurine–polypyrimidine sequences [6,7], inverted repeats [8,9], AT-rich regions [9] and trinucleotide repeats [10] appear to be most favored. A similar understanding, however, has yet to be developed for hybridization within cells, an environment characterized by an enormous complexity of nucleic acid sequence, chemistry and structure.

Human telomerase [11], a ribonucleoprotein that is responsible for maintaining the length of telomeres, is an excellent model for developing rules correlating oligomer chemistry, target sequence and target structure. The RNA component of telomerase (hTR) [12] contains an 11-base region (nucleotides +46 to +56) that acts as a template for binding and extending telomeres. This critical role requires that this region of hTR be single stranded, making it one of the most accessible of all cellular nucleic acid sequences and an ideal target for oligomer hybridization. Less is known about the remaining 440 bases of hTR, but deletion analysis has shown that much of this sequence is not required for activity ([13,14]; V.M. Tesmer, S.E. Holt, B.C. Frank, X. Yi, D. Aisner, M.M. Ouellette, J.W. Shay and W.E. Wright, personal communication). *In vitro* experiments demonstrate that a catalytic-core region of nucleotides +44 to +325 retains 5% wild-type activity, whereas the region from bases +33 to +325 retains full activity (V.M. Tesmer, *et al.*, and W.E. Wright, personal communication). Telomerase can be reconstituted *in vitro* upon expression of the genes for

Figure 1



A scheme for reconstitution of telomerase holoenzyme from hTR and hTERT. PNAs can inhibit telomerase activity by binding to unfolded RNA, to folded RNA or to RNA bound by protein in the holoenzyme. The structure of hTR is unknown but it is reasonable to expect that some base-paired structure is formed prior to holoenzyme assembly.

hTR and its protein component, hTERT [14,15], allowing a comparison between oligonucleotide-mediated inhibition at three different stages—the unfolded RNA, the folded RNA and the holoenzyme.

There is also great practical interest in developing effective telomerase inhibitors because telomerase activity has been found in many types of human tumors, but not in most types of somatic cells [16,17]. This has led to the hypothesis that activation of telomerase is necessary for sustained proliferation of most tumor cells and that telomerase inhibitors might represent a novel class of antiproliferative agents [18,19]. This hypothesis has been vigorously debated; potent oligonucleotide inhibitors of telomerase could be used to validate the connection between telomerase and cancer because of the stringent control inherent in oligonucleotide hybridization—the match oligomer should have a physiological effect, whereas a mismatch-containing oligomer should not. A phosphorothioate oligomer has recently been approved as a drug to treat CMV retinitis and several other oligonucleotide drugs are in clinical trials [20], suggesting that oligonucleotide-based inhibitors of human telomerase would be lead compounds for the development of chemotherapeutic agents.

We have shown previously that PNAs complementary to the 11-base template of hTR are potent inhibitors of human telomerase when tested *in vitro* [21,22]. We now describe two advances that expand the options for disrupting cellular function using PNAs. We report that PNAs directed to regions of hTR other than the 11-base template are a new class of inhibitor that act by intercepting the RNA component prior to assembly with the protein component. As with any class of drugs, it is likely that inhibitor resistance will arise upon prolonged treatment, and the availability of multiple types of telomerase inhibitors, each possessing a separate binding site, is likely to facilitate effective long-term therapy. We then report the discovery that PNAs can be delivered into eukaryotic cells using a simple protocol and use this protocol to demonstrate that PNAs can sequence-selectively block cellular telomerase activity. The observation that PNAs can be delivered into cells by adapting a widely used technology and that they can then inhibit a cellular RNA target has implications that extend beyond telomerase inhibition to the development of a chemical-based technology for conditional repression of gene expression.

Results and discussion

Effect of target sequence and structure on inhibition of telomerase

The RNA component (hTR) and protein component (hTERT) of human telomerase can be expressed *in vitro* using run-off transcription and rabbit reticulocyte lysate, respectively, and then mixed to generate active telomerase [15]. The ability to reconstitute telomerase from its components permits the individual stages of ribonucleoprotein assembly to be isolated and their susceptibility to PNA binding to be examined and compared. PNAs can bind to hTR as it folds, to hTR that has been permitted to fold in the absence of protein or to the holoenzyme after RNA and protein have associated (Figure 1). Relative levels of inhibition would probably reflect differences in structure and stability of RNA target sites, information that will contribute to a model for the telomerase RNA and advance design of improved antitelomerase agents.

We initiated our studies by correlating ribonucleoprotein assembly with the efficacy of inhibition by PNAs **3**, **4** and **7** (Table 1, Figure 2). These three PNAs were chosen to be complementary to target sequences likely to possess differing degrees of secondary structure and functional importance. PNA **3** is complementary to the 11-base RNA template region, which is both critical for telomerase activity and highly accessible to nucleic acid hybridization because of its role in telomere recognition and elongation. PNA **4** is complementary to a sequence adjacent to the template that is likely to be less accessible than the template region itself. PNA **7** is directed to a sequence 49 bases distant from the template within a region of unknown structure but critical for catalysis ([13]; V.M. Tesmer, *et al.*, and W.E. Wright, personal communication). PNAs were added either to the isolated RNA component after it had been denatured and allowed to refold or to the telomerase holoenzyme after assembly of

Table 1

Potency of inhibition of telomerase by PNAs as a function of template target and holoenzyme assembly.

PNA/sequence/target site	IC_{50} (μM) Pre-assembly	IC_{50} (μM) Post-assembly
46–56 (hTR template region) hTR (1-451) 5′ — 3′		
1. CCTCCGCAACCC (1-12)	0.3	>100
2. GTTAGACAAAAAA (38-50)	0.1	0.5
3. CAGTTAGGGTTAG (46-58)	0.02	0.03
4. CGCCCTTCTCA (57-67)	0.1	2.0
5. CGGCGCCTACGCC (64-76)	1	>100
6. AGAAAAACAGCGC (93-105)	0.1	>100
7. TGAAAGTCAGCGA (105-117)	0.1	>100
8. TGCTCTAGAATGAAC (153-167)	15	>100
9. GCAGCTGACAT (176-186)	0.1	>100
10. ACGGGCCAGCAGCTG (180-194)	0.01	10
11. CTTCGCGGTGGCA (291-303)	0.05	>100
12. GAGCCGAGTCCTG (430-442)	2	>100
13. TTGCTAGCCATC (not complementary to hTR)	30	>100

PNAs are listed amino to carboxyl termini and contain amino-terminal glycine and carboxy-terminal lysine residues.

the RNA and protein components. Telomerase activity was monitored using the polymerase chain reaction (PCR)-based telomere repeat amplification protocol (TRAP) [23].

The IC_{50} values for inhibition of telomerase activity by PNA **3** (20–30 nM) were similar regardless of whether the PNA was added to the holoenzyme or to the isolated RNA component (Table 1). Inhibition of the holoenzyme appears to reflect the high accessibility of the template region that is needed to bind and extend telomeric DNA during normal functioning of the ribonucleoenzyme. By contrast, inhibition by nontemplate-directed PNAs **4** and **7** was acutely dependent on the state of ribonucleoprotein assembly. When **4** was added to the RNA component prior to holoenzyme assembly, inhibition was characterized by a relatively low IC_{50} value of 100 nM. Addition of **4** to the holoenzyme, however, yielded a 20-fold higher IC_{50} value of 2 μM. The difference in IC_{50} values for inhibition by **7** was even more striking. Inhibition of telomerase activity after addition to the isolated RNA was 100 nM, whereas no inhibition was detected upon addition of **7** to holoenzyme.

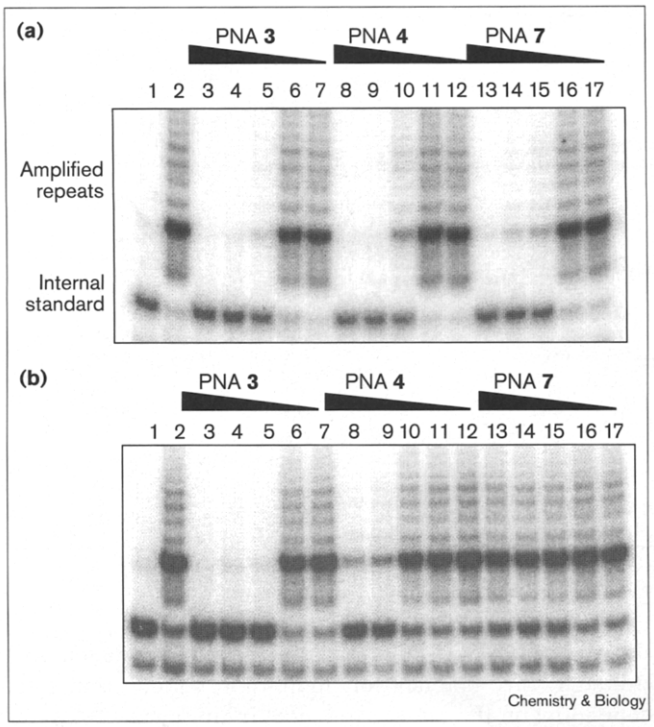
Inhibition of telomerase upon addition of nontemplate-directed PNAs **4** and **7** to hTR encouraged us to test PNAs **1**, **2**, **5**, **6** and **8–12** (Table 1) to determine whether inhibition was a general phenomenon and to probe the accessibility and importance of RNA structure throughout hTR. None of these PNAs was capable of inhibiting telomerase upon addition to holoenzyme, with the exception of **2**, which partially overlapped the template region, and **10**, which inhibited with a modest IC_{50} value of 10 μM. Every PNA, however, inhibited telomerase when added to hTR prior to ribonucleoprotein assembly, although the potency of inhibition varied 1000-fold depending on the target sequence. Inhibition by PNAs **1**, **2**, **6**, **7** and **9–11** was most potent, with IC_{50} values of 10–300 nM. Inhibition by PNAs **5** and **12** was less potent, with IC_{50} values of 1–2 μM, whereas inhibition by PNA **8** was characterized by an IC_{50} value of 15 μM, barely lower than a PNA that is not complementary to hTR (PNA **13**).

PNA-mediated inhibition as a probe for hTR structure and hTR/hTERT assembly

Several conclusions can be drawn from the inhibition of telomerase by nontemplate-directed PNAs. PNA-mediated inhibition is not restricted to PNAs targeted to the intrinsically accessible RNA template, as demonstrated by our observation of IC_{50} values as low as 10–100 nM. Potent inhibition was surprising because we had not previously observed significant levels of inhibition by nontemplate-directed PNAs when using telomerase obtained from extracts of cultured cells, and because there is no obvious reason why nontemplate RNA should be accessible to hybridization with an incoming oligomer. We observe inhibition in these studies because the ability to reconstitute telomerase *in vitro* allows us to test separately the effect of inhibitor binding to hTR alone, a property that should be generally valuable during screening for other classes of telomerase inhibitor.

Accessibility to PNA binding was a common feature of isolated hTR, as seven of the ten nontemplate-directed PNAs examined in these studies inhibited telomerase with IC_{50} values of less than 1 μM. Hybridized PNA either prevented holoenzyme formation or disrupted hTR structure within the holoenzyme, leading to less active

Figure 2



Telomere repeat amplification protocol (TRAP) gels showing inhibition of telomerase as a function of target sequence for PNA binding and holoenzyme structure. **(a)** The effect on telomerase activity of addition of PNAs **3**, **4** and **7** to hTR prior to assembly of holoenzyme. Lane 1, no cell lysate added. Lane 2, no PNA added. Lane 3–7 PNA **3** at the following concentrations: 33 μM, 3.3 μM, 330 nM, 33 nM and 3.3 nM. Lanes 8–12, PNA **4** added at the following concentrations: 33 μM, 3.3 μM, 330 nM, 33 nM and 3.3 nM. Lanes 13–17 PNA **7** added at the following concentrations: 33 μM, 3.3 μM, 330 nM, 33 nM and 3.3 nM. **(b)** Effect on telomerase activity of addition of PNAs **3**, **4** and **7** to reconstituted holoenzyme at the concentrations listed in (a).

enzyme in either case. Conversely, nontemplate-directed PNAs are almost entirely unable to inhibit the holoenzyme, suggesting that binding of hTR to hTERT renders previously open target sequences inaccessible or stabilizes RNA structure sufficiently to prevent recognition by PNAs. The suggestion that the RNA structure is stabilized is supported by the observation that the half-life of hTR is much longer in cells that express hTERT relative to cells that do not [24].

PNA **8**, which is targeted to bases 153–167, inhibited with a comparatively high IC_{50} value of 15 μM when added to hTR prior to holoenzyme assembly. Consistent with our observation Tesmer *et al.* (personal communication), using genetic techniques, report the intriguing result that fragments spanning +33 to +147 and +164 to +325 can be added together *in trans* to yield active telomerase. This result suggests that the region between bases +147 and +164 does not contribute to catalysis. Tesmer *et al.* (personal communication) further suggested that the sequence between nucleotides +62 and +147 forms a nuclease-resistant hairpin structure *in vitro*. Our results support this suggestion. PNA **5**, which is targeted within the putative hairpin stem, is a relatively poor inhibitor with an IC_{50} value of 1 μM. PNAs **6** and **7**, by contrast, are targeted to the predicted loop region, and possess tenfold more potent IC_{50} values of 100 nM.

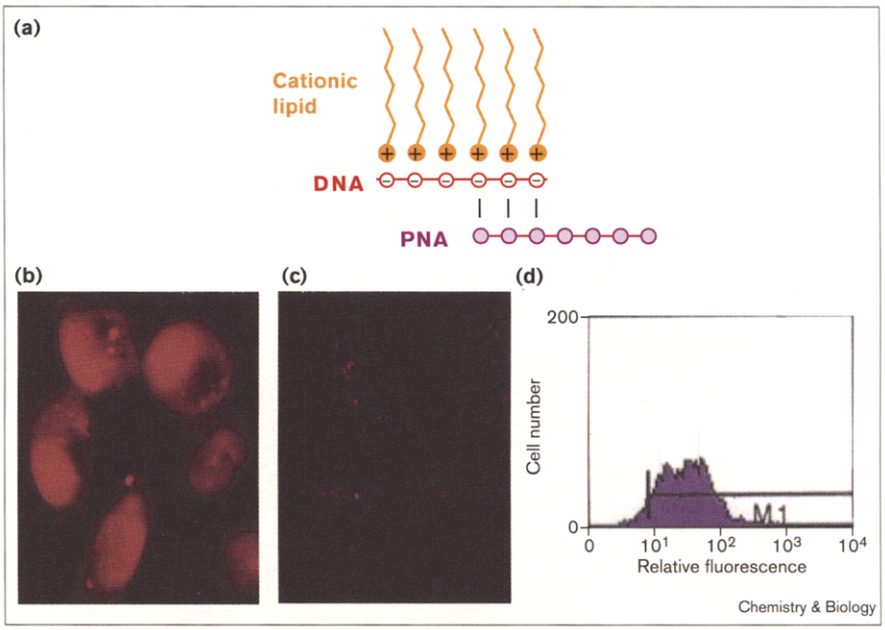
Surprisingly, we observe inhibition by PNAs targeted to regions of hTR that previous studies have reported to be dispensable. For example, PNA **1**, directed to bases 1–12, and PNA **12**, directed to bases 430–442, both inhibit telomerase even though these regions can be deleted without affecting activity. Such results demonstrate that RNA sequences that are unnecessary for catalysis may still be relevant targets for inhibitors designed to disrupt RNA structure. PNAs **10** (+180–194) and **11** (+291–303) had potent IC_{50} values of 10 and 50 nM, respectively. This result is consistent with reports that mutation or deletion of regions near the target sequences for **10** and **11** reduces telomerase activity ([13]; Tesmer *et al.* and W.E. Wright, personal communication) and suggests that these regions are not only functionally important but may form partially single-stranded structures that are highly accessible to PNA binding.

Delivery of PNAs into cells by lipofection

The results described above demonstrate that nontemplate-directed PNAs are valuable reagents for probing RNA and holoenzyme structure within a cell-free system and reinforce the growing body of evidence that PNAs are likely to be widely useful probes for DNA and RNA structure. Many of the most important questions that involve nucleic acid structure and function, however, can only be fully addressed in the context of the cell. To fully exploit the potential of PNAs as probes for structural biology and tools for chemical genetics it was therefore necessary for us to develop an efficient method for delivery of PNAs into eukaryotic cells. This issue is particularly pressing for telomerase because highly selective inhibitors are required to resolve the putative link between telomerase and cancer.

Several methods have been developed for introducing PNAs into eukaryotic cells, including transient permeabilization with streptolysin O [25] and the conjugation of PNAs to peptides known to be internalized [26–28]. We describe here a new approach to PNA delivery that relies on well-established protocols for transfection using cationic lipid. For DNA and RNA the cationic lipid associates with the negatively charged phosphodiester backbone. The lipid–nucleic-acid complex can fuse with cell membranes, allowing oligonucleotide to enter cells through an endocytotic pathway, localize in the nucleus and regulate gene expression [29]. This approach was

Figure 3



Transfection of PNA–DNA heteroduplexes to DU145 cells. **(a)** Scheme showing partially overlapping PNA–DNA heteroduplexes in complex with cationic lipid. **(b)** Fluorescence microscopy of cells upon after addition of rhodamine-labeled PNA **3**–DNA–lipid complex. **(c)** Fluorescence microscopy of cells upon after addition of rhodamine-labeled PNA **3** and cationic lipid. **(d)** FACS analysis of cells transfected with PNA **3**–DNA–lipid complex. The horizontal line delineates fluorescence above background. The mean relative fluorescence for cells treated with DNA–PNA-rhodamine–lipid complex was 37.4. The mean relative fluorescence for cells alone and cells treated with DNA–PNA-rhodamine hybrid in the absence of lipid was 4.1 and 6.7.

attractive to us because cationic-lipid-mediated delivery of DNA and RNA is a simple and widely used method for introducing nucleic acids into cells [29] and because we had previously shown that lipofection could be used for intracellular delivery of 2′-O-meRNA inhibitors of telomerase [30]. Lipofection does not require covalent chemical modification of the PNA, and if it could be employed for PNA delivery it would encourage immediate and widespread examination of the effects of PNAs within cells by researchers who are already familiar with lipid-mediated delivery of nucleic acids.

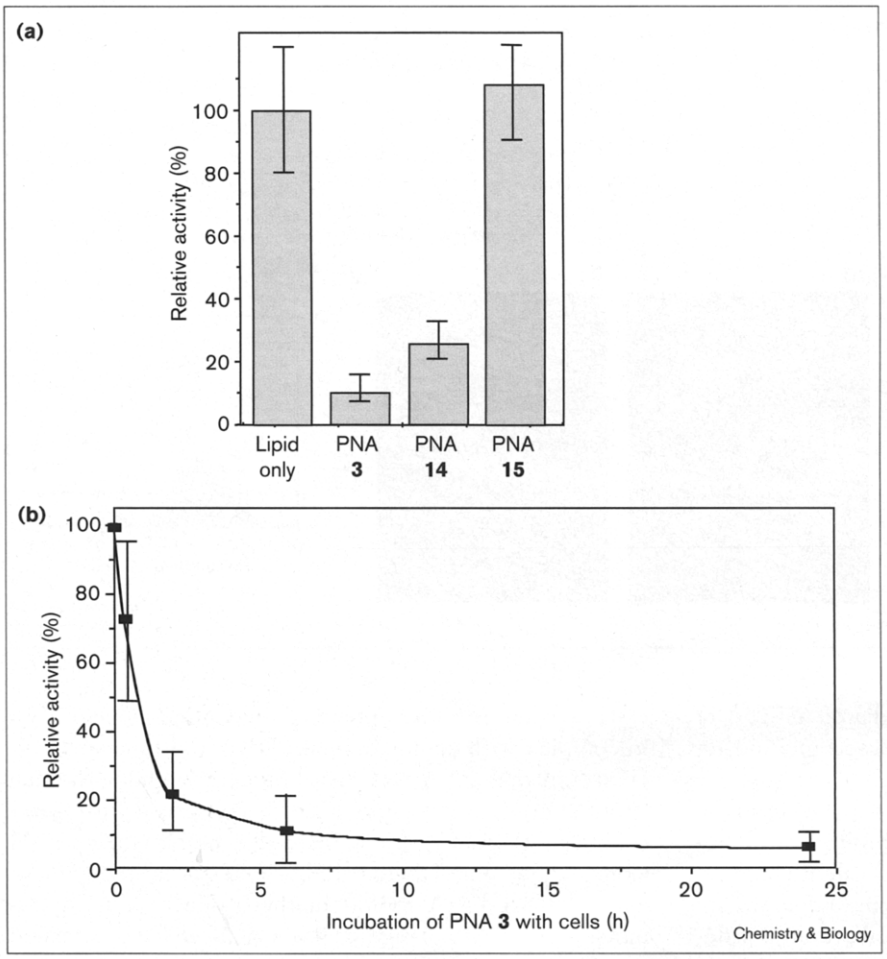
To achieve lipid-mediated delivery of PNAs we hybridized PNAs to overlapping DNA oligonucleotides (Figure 3a), with the 11–15-base transporter DNA designed to have 8–11 bases complementary to the cargo PNA. This complex was then mixed with cationic lipid and added to cells. The rationale for our approach is that the lipid should associate with the DNA strand, allowing the PNA strand to be imported as a passive cargo. Prior to our experiments it was not obvious that this approach would succeed because complexes between lipid and nucleic acids form an ordered structure [31] and it might have been expected that the attached PNA would disrupt lipid assembly. Once within the cell, the PNA should dissociate from the DNA and bind its target. The PNA–DNA hybrids were designed to overlap to promote nuclease degradation of the DNA component and dissociation of the hybrid.

We first examined cellular uptake of PNAs using microscopy and fluorescence-activated cell sorter (FACS) analysis. Microscopy of DU145 prostate-tumor-derived cells revealed that the uptake of rhodamine-labeled PNA in complex with complementary DNA and lipid was efficient and that PNA was distributed throughout the cell (Figure 3b). Similar results were also observed with Cos-7 cells (data not shown). Uptake of rhodamine-labeled PNA alone, rhodamine-labeled PNA–lipid, or a rhodamine-labeled PNA–DNA hybrid in the absence of lipid was much less intense (Figure 3c). FACS analysis revealed that treatment with PNA–DNA–lipid complex resulted in delivery of rhodamine-labeled PNAs to greater than 90% of cells (Figure 3d).

Inhibition of telomerase after lipid-mediated delivery of a template-directed PNA

As an initial test of the ability of lipid-mediated transfection to deliver PNA inhibitors of human telomerase we first examined inhibition by 13-base PNA **3** (Table 1) and 11-base PNA **14** (Gly–CAGTTAGGGTT–Lys). These two PNAs were chosen because they are directed to the intrinsically accessible 11-base template region of hTR and effective inhibitors of the holoenzyme (Table 1, Figure 2). Upon addition of complexes of DNA and lipid with either PNA **3** or PNA **14** to cultured DU145 cells we observed greater than 85% inhibition of telomerase activity (Figure 4a). Inhibition is sequence specific, as transfection of PNA **15** (N-Gly-AAGTTAGACAA-Lys) that contained mismatched bases did not result in inhibition of telomerase. Similar results were observed for inhibition of telomerase in 293 cells (an adenovirus-transformed human cell line). Inhibition was also specific to cells that had taken up PNA. When cells that were treated with the PNA–DNA–lipid complex were mixed with untreated

Figure 4



Inhibition of telomerase upon lipid-mediated transfection of template-directed PNAs. **(a)** Telomerase inhibition by PNAs directed to the template region of human telomerase. Transfection assays were performed for 6 h and included either no inhibitor, template-directed PNA **3**, template-directed PNA **14** or a mismatch-containing PNA **15**. **(b)** Inhibition of human telomerase by a template-directed PNA **3** as a function of incubation time of the PNA–DNA–lipid complex with cells. Telomerase activity was measured after 0, 0.33, 2, 6 and 24 h. All transfection assays were performed in triplicate.

cells we found that telomerase activity corresponded to that expected from the proportion of untreated cells. This result demonstrates that the highly efficient inhibition that we observe cannot be explained by PNA leaking from cells that have taken up PNA into cells that have not.

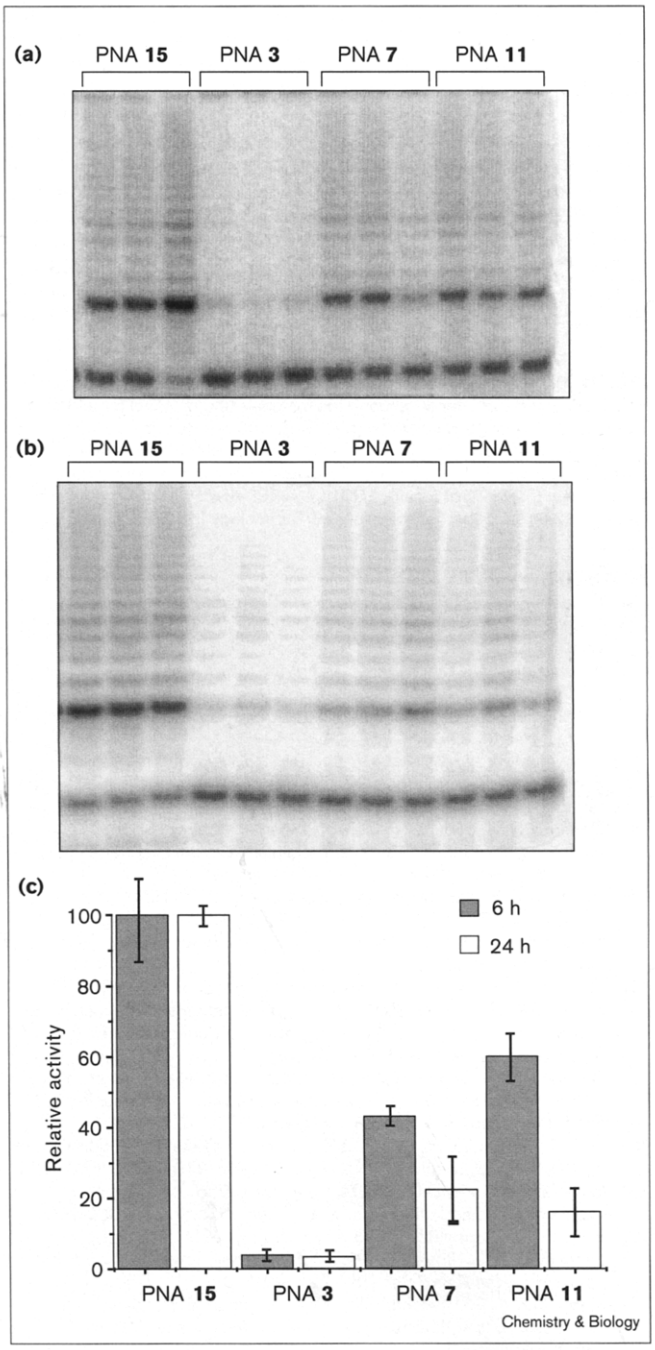
The efficiency of inhibition by PNA **3** was dependent on the time allowed for incubation of the PNA–DNA–lipid complex with cells, and leveled at a maximum after 6 h (Figure 4b). Addition of DNA alone produced no inhibition, nor did addition of the PNA–DNA complex in the absence of lipid or the PNA–lipid mixture in the absence of DNA. None of the PNA–DNA hybrids tested in these studies was toxic to cells upon transfection. This lack of toxicity may be due to the reduced ability of PNAs to bind to proteins that normally bind to nucleic acids, and might be a general advantage for use of PNAs within cells. We continued to observe inhibition of telomerase activity for up to three days (or three population doublings) after transfection. This results suggests that PNAs persist in active form for days and that our DNA–PNA–lipid delivery method is adequate to deliver a dosage of PNA that remains effective after dilution due to repeated cell doublings. After a four-week treatment we observed telomere shortening, confirming that introduced PNAs are able to cause phenotypic change in a sequence-specific manner (A. Pitts, S. Baker and D.R.C., unpublished observations).

Inhibition of telomerase after lipid-mediated delivery of a nontemplate-directed PNA

After establishing that lipid-mediated delivery of template-directed PNAs could inhibit cellular telomerase, we tested whether nontemplate-directed PNAs could also act as inhibitors inside cells. Inhibition by nontemplate-directed PNAs is a more severe test for the ability of PNAs to bind a biological target within cells because, unlike the accessible template region, binding to nontemplate sequences is likely to be hindered by nucleic-acid structure and protein–nucleic-acid interactions in the holoenzyme.

We complexed PNAs **7** and **11** with partially complementary DNA oligomers and lipid and transfected them into

Figure 5



Inhibition of telomerase upon transfection of nontemplate-directed PNAs. Telomerase activity was measured after **(a)** 6 h or **(b)** 24 h incubation time in the presence of PNA **15**, **3**, **7** or **11** complexed with DNA and lipid. Assays were performed in triplicate, and the quantitation is shown in **(c)**. Residual activity was normalized to mismatched PNA **15**.

DU145 cells. We incubated cells with PNA–DNA–lipid complex for 6 h, the period that we had found to be adequate for maximal inhibition by template-directed PNA **3**, and observed 53% inhibition by **7** and 40% inhibition by **11**. These levels of inhibition are significantly lower than those produced by template-directed PNAs **3** and **14** (Figure 4). This result parallels our observations of inhibition of reconstituted telomerase (Table 1), which show that the preformed telomerase holoenzyme present within cells is resistant to inhibition by nontemplate-directed PNAs. To increase the efficiency of inhibition we lengthened the time for incubation of the PNA–DNA–lipid complex with DU145 cells to 24 h. PNA **7** now inhibited 78% of telomerase activity, whereas **11** inhibited 84%, indicating that the barrier to inhibition presented by holoenzyme structure can be overcome within cells (Figure 5a–c).

Inhibition of telomerase within cells suggests that lipid-meditated delivery of PNAs will facilitate antisense inhibition of gene expression by targeting mRNA. Intracellular PNA delivery will also allow testing of the hypothesis that PNAs have the potential to target genomic DNA by strand invasion. PNA binding within cells may also have significant applications beyond antisense inhibition of gene expression. For example, fluorescently labeled oligonucleotides can be used as 'molecular beacons' for identifying mRNA in cells. This approach relies on the loss of fluorescence quenching of two fluorophores in a DNA hairpin upon binding a complementary sequence. We have already shown that PNA hairpins can bind to duplex DNA by strand invasion at 37°C, and it is reasonable to assume that they will also be able to bind RNA and that the insights we gain can be directly applied to this exciting technology.

Significance

Elucidating the rules governing recognition of sequences within nucleic acid structure is fundamentally important for sequence-specific control of gene expression. We demonstrate that recognition of sequences within human telomerase by peptide nucleic acids (PNAs) is stringently dependent on whether hTR (the RNA component of telomerase) is in complex with hTERT (the protein component) and on the precise location of the target site. Sequences are accessible when the RNA is in isolation, and most are inaccessible when present in the context of the holoenzyme. Accessibility of the RNA prior to holoenzyme formation offers an opportunity for inhibitor binding through interception of the uncomplexed RNA. As RNA and protein are synthesized separately within the cell, it is possible to envision this occurring in the intracellular milieu, enhancing the efficacy of nontemplate-directed PNAs and making them lead compounds for controlling telomere length and cell proliferation. This new class of telomerase inhibitors may prove particularly valuable as components of combination inhibitor cocktails that overcome inhibitor resistance and curb the growth of proliferating cells in a clinical setting.

Realizing the full potential of PNAs for control of any biological process, including inhibition of human telomerase,

demands that methods be developed for the delivery of PNAs into cells. Ideally such methods should be simple and general so that the advantages that PNAs have exhibited in cell-free systems [32] can be widely exploited by researchers. We demonstrate a convenient method for intracellular delivery of PNAs using cationic lipids. Cationic-lipid-mediated delivery of nucleic acids is employed by many laboratories and our protocol should be readily adapted for wide use. Intracellular delivery of PNAs is important because progress in genome sequencing is revealing thousands of genes, many of which are closely related isoforms likely to play overlapping but not identical roles in cellular pathways. Convenient intracellular delivery will allow PNAs to join minor-groove-binding polyamides [33] and other chemical genetic strategies [34] for translating the one-dimensional understanding of protein function derived from genomic data into the multidimensional understanding necessary to understand cell signaling and regulation.

Materials and methods

Reconstitution of telomerase

Constructs encoding the human telomerase protein component (XhTERT) and the RNA subunit (pTRC3) [12] were provided by J.W. Shay (University of Texas Southwestern Medical Center, Dallas TX). hTERT was synthesized *in vitro* using a rabbit reticulocyte transcription and translation system (Promega, Madison WI). The RNA component was prepared using the MEGAscript™ T_7 *in vitro* transcription system (Ambion, Austin TX). Reconstitution of hTR and hTERT to form telomerase holoenzyme was performed as described previously ([15]; V.M. Tesmer, *et al.*, and W.E. Wright, personal communication).

Telomerase assays, inhibition and PNA preparation

Telomerase activity from immortal human prostate cancer cell line DU145 was detected using the TRAPeze kit (Intergen, Purchase NY) [24]. PNAs were added to hTR or the telomerase holoenzyme. Solutions containing reconstituted telomerase and PNA were diluted from 6 µl to a final volume of 50 µl with CHAPS lysis buffer provided in the TRAPeze kit and 4 µl was used per amplification reaction. Cells transfected with oligonucleotides were lysed with CHAPS lysis buffer to a final concentration of 50 cells/µl and 200 cell equivalents were used per assay reaction. PNAs were synthesized either using a PerSeptive Biosystems (Framingham MA) Expedite 8909 Synthesizer and Fmoc chemistry [35], or manually using tBoc chemistry [36]. PNAs were purified using reverse phase HPLC and analyzed using time-of-flight mass spectrometry (MALDI–TOF) as described previously [35].

Transfection of oligonucleotides and PNA–DNA heteroduplexes

DU145 cells were plated at 25,000 cells/well on 24-well plates in Dulbecco's MEM containing 10% fetal calf serum and 500 U/ml penicillin and 0.1 mg/ml streptomycin and incubated at 37°C at 5% CO_2 for 3 h. Oligomers (1 µM each) were pre-complexed with 2 µl (7 µg/ml) LipofectAmine (Life Technologies, Gaithersburg MD) in a total of 200 µl Optimem (Life Technologies) for 45 min. The media was replaced with the lipofection-oligomer solution and the incubated 6–24 h. After incubation the transfection mixture was replaced with plating media and the incubation continued 12–15 h. Cells were washed once with 1 × PBS, treated with trypsin, harvested, counted and immediately assayed for telomerase inhibition as described above. DNA oligonucleotides and their partial PNA complements (in parentheses) were as follows 5′-TCTAACTTTAA-3′ (PNA **15**), 5′-TCTAACCCTAA-3′ (PNA **3**), 5′-TCTCGCTGACT-3′ (PNA **7**), 5′-CCACTGCCACCGCG-3′ (PNA **11**), and 5′-AATCCCAATCT-3′ (PNA **14**).

Fluorescence microscopy and FACS analysis

PNA **3** was labeled with amino-terminal tetramethyl rhodamine (Molecular Probes, Eugene OR) and cotransfected with DNA oligomer into DU145 cells as described above. Cells were rinsed four times with phosphate buffered saline (PBS) and fresh media was added. Cells were then incubated for 2 h and rinsed an additional four times with PBS. The intervening incubation was added to allow cells to withstand the extended washing necessary to ensure removal of free rhodamine from cell surfaces. Cells for evaluation using microscopy were rinsed once more with distilled water, transferred onto slides and mounted with GEL/MOUNT (Biomeda Corp., Foster City CA). Cells were visualized using an Olympus fluorescent microscope at 100 × magnification. Cells for FACS analysis were pelleted and resuspended in PBS and were sorted using a FACStarPlus flow cytometer (Becton Dickinson, Franklin Lakes NJ).

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