A new class of peptide gene delivery agents were developed by inserting multiple cysteine residues into short (dp 20) synthetic peptides. Substitution of one to four cysteine residues for lysine residues in Cys-Trp-Lys₁₈ resulted in low molecular weight DNA condensing peptides that spontaneously oxidize after binding to plasmid DNA to form interpeptide disulfide bonds. The stability of cross-linked peptide DNA condensates increased in proportion to the number of cysteines incorporated into the peptide. Disulfide bond formation led to a decrease in particle size relative to control peptide DNA condensates and prevented dissociation of peptide DNA condensates in concentrated sodium chloride. Cross-linked peptide DNA condensates were 5–60-fold more potent at mediating gene expression in HepG2 and COS 7 cells relative to uncross-linked peptide DNA condensates. The enhanced gene expression was dependent on the number of cysteine residues incorporated, with a peptide containing two cysteines mediating maximal gene expression. Cross-linking peptides caused elevated gene expression without increasing DNA uptake by cells, suggesting a mechanism involving intracellular release of DNA triggered by disulfide bond reduction. The results establish cross-linking peptides as a novel class of potent gene delivery agents that enhance gene expression through a new mechanism of action.

A variety of nonviral gene delivery carriers have been developed and tested as in vitro transfection agents used to transiently express foreign DNA. Cationic lipids (1, 2), polylysine peptides (3–5), and cationic polymers such as polyethyleneimine (6, 7) bind electrostatically to the phosphate backbone of DNA forming complexes that mediate cellular uptake of DNA in culture.

As opposed to the success of these agents in vitro, attempted in vivo use has revealed many complications related to their toxicity (8), antigenicity (9), complement activation (10), solubility (11), blood compatibility (12), and stability (13). These complications relate to the size and charge of DNA carrier complexes and ultimately to the molecular characteristics of the carrier itself. High molecular weight (HMW)³ DNA carriers can be cytotoxic (8), are able to activate the complement system (10), and can elicit an immune response (9). The size and heterogeneity of these polymers also significantly complicates region-specific derivatization with ligands or polyethylene glycol (14).

To circumvent these problems, several low molecular weight (LMW) carrier peptides have been developed that mediate in vitro gene transfer as efficiently as their HMW counterparts (15–17). They offer the advantage of controlled synthesis and defined purity that then allows strategic optimization to increase expression levels and eliminate side effects.

However, when analyzed for in vivo efficacy, LMW peptide DNA condensates lacked sufficient stability to survive circulation, were not able to significantly protect DNA from metabolism, and could not effect targeting (13, 18). One solution to increase LMW peptide DNA condensate stability is to form intraparticle cross-links to inhibit the dissociation of condensing peptides. Glutaraldehyde was evaluated as one type of cross-linking agent that forms Schiff bases between neighboring peptides and increases the metabolic stability of LMW peptide DNA condensates (19). Glutaraldehyde cross-linked DNA condensates were significantly more metabolically stable both in vitro and in vivo (18, 19) and were able to facilitate specific receptor targeting in vivo (18). However, the slow reversal of the Schiff bases resulted in limited in vitro and in vivo gene expression. Other reversible cross-linking agents have also been applied to form caged DNA condensates (20) by template polymerization (21), but thus far they have not been shown to be transfection competent.

In an effort to design stabilized LMW peptide DNA condensates that are reversibly cross-linked and that simultaneously enhance gene expression, we incorporated multiple cysteine residues into LMW condensing peptides to determine whether they would oxidize to form interpeptide disulfide bonds while bound to DNA. Once internalized, the reducing environment of the cell (22) might allow disulfide cross-linked DNA condensates to undergo reduction and release DNA more readily than glutaraldehyde cross-linked DNA condensates.

The following describes the synthesis of a panel of novel LMW cross-linking peptides that not only undergo disulfide cross-linking to form small stabilized DNA condensates but also enhance in vitro gene expression. These findings suggest that cross-linking peptides may be promising candidates for further development into LMW DNA carriers that function in vivo.

**MATERIALS AND METHODS**

N-terminal Fmoc protected amino acids, 9-hydroxybenzotriazole, disopropylearboxydimide, and disopropylethylamine were obtained from Advanced ChemTech (Lexington, KY). Substituted Wang resin for peptide synthesis was obtained from ChemImpex (Wood Dale, IL). Polylysine, 5-bromo-4-chloro-3-indolyl-β-galactopyranoside.
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sine100g was purchased from Sigma. N,N-Dimethylformamide, trifluoroacetic acid (TFA), acetic acid, acetonitrile, and piperidine were purchased from Fisher. Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) was obtained from Pierce. LB medium, LB agar, n-luciferin, and luciferase from *Photinus pyralis* (EC 1.13.12.7) were obtained from Boehringer-Mannheim, Heidelberg. COS-7 cells were from the American Type Culture Collection (Manassas, VA). Inactivated “qualified” fetal bovine serum (FBS) was from Life Technologies, Inc. Bradford reagent was purchased from Bio-Rad, and thiazole orange was a gift from Beckton Dickinson Immucytometry Systems (San Jose, CA). SYBR-Gold was purchased from Molecular Probes, Inc. (Eugene, OR). The 5.6-kilobase plasmid pCMVL expressing the reporter gene luciferase (26) was obtained from the control of the cytomegalovirus promoter was obtained from the University of Michigan core vector laboratory (Ann Arbor, MI). Endotoxin-free plasmids were purified from *Escherichia coli* on a Qiagen ultrapure column used according to the manufacturer’s instructions that typically yielded plasmid DNA that was 50:50 supercoiled and open circular as determined by agarose gel electrophoresis.

Purified peptides were characterized by liquid chromatography mass spectrometry (LC-MS) to determine the isolated yield, which was typically 20%. The particle size and zeta potential of peptide DNA condensates were determined by titrating 7.5–37.5 nmol of peptide with 75 µg of DNA in 1.5 ml of HBM to produce 50 µg/ml DNA condensates with charge ratio of 0.5–2.5. The zeta potential was averaged from 10 determinations.

**Kinetics of Peptide Cross-linking**—The kinetics of disulfide bond formation in solution was monitored by ES-MS. Fully reduced peptide V (80 nmol) (see Table I) was dissolved in 800 µl of 50%/50% v/v n-propanol: ammonia solution (1 mM, pH 7.5) and directly infused via a syringe pump into the electrospray ionization source of a Finnigan LCQ mass spectrometer at 3 µl/min. Single ion monitoring scans were taken at 20-s intervals over 2 h while monitoring the disappearance of the M + H ion of the oxidized species (1256 atomic mass units).

The kinetics of cross-linking within peptide DNA condensates was studied indirectly by monitoring the displacement of SYBR-Gold intercalator dye from DNA condensates as a function of time. Peptide DNA condensates were prepared (200 µl of 50 µg/ml) at a peptide stoichiometry of 0.4 nmol µg of DNA. Immediately after mixing peptide and DNA, a 10–µl aliquot was combined with 990 µl of SYBR-Gold (1×), and the fluorescence intensity (excitation, 495 nm; emission, 537 nm) was continuously monitored for 30 min.

The rate of cross-linking was also examined as a function of peptide concentration. The stoichiometry of peptide IV was increased from 0.4 to 1.2 nmol µg of DNA, and the rate of declining fluorescence intensity was determined as described above.

**Chemical Reactivity of Cross-linking Peptides with DNA**—A synthetic oligonucleotide (5′-CATGCATCC) (Life Technologies, Inc.) was reconstituted in 10 mM ammonium acetate (pH 7.4) and combined with either AlkCWKx, or peptide V resulting in an oligonucleotide concentration of 17 µM and a stoichiometry of 0.4 nmol peptide/µg oligonucleotide. Peptides were allowed to react with the oligonucleotide for 2 h at 37 °C, lyophilized, and then reconstituted in 500 µl of 70%/20% v/v acetic acid:water:triethylamine:acetone to a final DNA concentration of 10 µM. The reaction products were directly infused at 25 µl/min into the electrospray source of a Finnigan LCQ mass spectrometer. The mass of the oligonucleotide and peptide-oligonucleotide conjugates was monitored by acquiring spectra in the negative mode.

**In Vitro Gene Expression**—HepG2 cells (1.5 × 10⁵) were plated on 6-well plates using 35-mm wells and grown to 40–70% confluency in MEM supplemented with 10% FBS, penicillin, and streptomycin (10,000 units/ml), sodium pyruvate (1 mM), and 5% FBS, and then cultured in MEM (2 ml/35-mm well) with 2% FBS and 80 µg/ml chloroquine. Peptide DNA condensates (200 µl of 50 µg/ml) were formed and incubated at room temperature for 30 min and then combined with 0–200 µl of 5 µl sodium chloride and normalized to 400 µl with HBM to achieve a final sodium chloride concentration of 0, 0.2, 0.4, 0.8, 1.0, 1.5, 2.0, and 2.5 mM. Each sample was sonicated for 30 s with a 100 W Microson XL-2000 ultrasonic probe homogenizer (Kontes, Vineland, NJ) with a vibrational power density of 0.17 W/ml. DNA condensates were digested with 30 µg of trypsin for 12 h at 37 °C and then electrophoresed on an agarose gel and visualized by ethidium bromide staining.

**Clear Stress Stability of Cross-linked DNA Condensates**—Peptide DNA condensates (200 µl of 50 µg/ml) were formed and incubated at 37 °C for 1 h prior to treatment with 2% FBS and 80 µg/ml chloroquine. Peptide DNA condensates (10 µg of DNA in 0.2 ml of HBM) were added dropwise to triplicate wells. After a 5-h incubation at 37 °C, the medium was replaced with MEM supplemented with 10% FBS. After 24 h, cells were washed twice with ice-cold phosphate-buffered saline (calcium- and magnesium-free) and then treated with 0.5 ml of ice-cold lysis buffer (25 mM Tris chloride, pH 7.8, 1 mM EDTA, 8 mM magnesium chloride, 1% Triton X-100, 1 mM dithiothreitol) for 10 min. The cell lysate was scraped, transferred to 1.5-ml microcentrifuge tubes, and centrifuged for 7 min at 13,000 × g at 4 °C to pellet debris. Lysis buffer (300 µl), sodium-ATP (4 µl of a 180 mM solution, pH 7, 4 °C), and cell lysate (100 µl, 4 °C) were combined in a test tube, briefly mixed, and immediately placed in the luminometer. Luciferase relative light units were recorded on a Lumat LB 9501 (Berthold Systems, Munich, Germany) with 10 s of integration after automatic injection of 100 µl of 0.5 µM n-luciferin (prepared fresh in lysis buffer without dithiothreitol). The relative light units were converted into fmol using a standard curve generated by adding a known amount of luciferase (0.01–100 fmol with specific activity of 2.5–3.5 munits/fmol) to 35-mm wells and incubating 40–70% confluency HepG2 cells for 2 h in HBM. DNA concentrations were determined as described above resulting in a standard curve with an average slope of 7.8 ± 0.5 relative light units/fmol of enzyme. Protein concentrations were measured by Bradford assay using bovine serum albumin as a standard (27). The amount of luciferase recovered in each sample was normalized to milligrams of protein and reported as the mean and standard deviation obtained from triplicate transfections.
The specific activity of the procedure described above. The radioactive medium was removed, and the nuclear-stained blue cells were counted with a light microscope under 10× power magnification by averaging the number of transfectants from nine fields. Values were analyzed for significance by one-way analysis of variance.

Cell Uptake Studies—Iodinated plasmid DNA (pCMVL) was prepared with specific activity of 300 nCi/μg of DNA as described previously (28). Prior to forming DNA condensates, the specific activity of the 125I DNA was adjusted to 4.5 nCi/μg of DNA by combining with unlabeled plasmid. DNA condensates were prepared using AlkCWK18 or peptide II as described above. Peptide 125I-DNA condensates (10 μg) were used to transfect HepG2 and COS 7 cells for 5 h according to the procedure described above. The radioactive medium was removed, and cells were washed once with 2 ml of 1 × sodium chloride and three times with 2 ml of cold phosphate-buffered saline and then harvested with 1 ml of lysis buffer. The cell-associated radioactivity from triplicate transfections was quantified by γ counting.

**RESULTS**

Substituting cysteine residues into CWK18 produced a panel of cross-linking peptides (II, III, IV, and V) possessing 2–5 cysteine residues, respectively (Table I). Following binding and condensation of plasmid DNA, cross-linking peptides could potentially form interpeptide disulfide bonds that would stabilize the DNA carrier complex (Fig. 1). Hence, the number of cysteine residues would influence the number of disulfide bonds formed and the overall stability and transfection efficiency of the peptide DNA carrier complex.

Despite the presence of multiple cysteine residues, each cross-linking peptide released from resin produced essentially a single peak on RP-HPLC, indicating that the cysteines remained in a reduced form during acidic cleavage and work-up. Purified cross-linking peptides each produced molecular ions

| Name         | Sequence                      | Mass (observed/calculated) |
|--------------|-------------------------------|----------------------------|
| AlkCWK18     | Alk-S-Cys-Trp-Lys18           | 2672.5/2672.5              |
| DiCWK18      | Lys18-Trp-Cys-S-S-Cys-Lys18    | 5227.5/5227.9              |
| CWK18        | Cys-Trp-Lys18                 | 2614.1/2614.6              |
| II           | Cys-Trp-Lys18, Cys            | 2588.9/2588.6              |
| III          | Cys-Trp-Lys18, Cys-Lys, Cys   | 2564.2/2564.6              |
| IV           | Cys-Trp-Lys18, Cys-Lys, Cys   | 2539.2/2539.6              |
| V            | Cys-Trp-Lys18, Cys, Cys, Cys, Cys | 2514.0/2514.6 |
| Polylysine1007| Lys1007                      | ND/150,000                 |

* Masses are calculated as the average mass of the free base.

**TABLE I**

Mass spectral analysis of DNA condensing peptides

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LipofectAce™ (Life Technologies, Inc.; 1:2.5 w/v dimethyl dicitadecylammonium bromide and dioleoyl phosphatidylethanolamine) was used to mediate gene transfection according to the manufacturer’s instructions. The ratio of DNA to LipofectAce was optimized for HepG2 cells. An optimal DNA/LipofectAce ratio was achieved by dissolving 10 μg of DNA in 100 μl of serum-free medium followed by adding 60 μl of LipofectAce prepared in 140 μl of serum-free medium. The LipofectAce DNA complex was then diluted with 1.7 ml of serum-free medium and used to transfect HepG2 cells for 5 h followed by replacing the transfecting medium with medium supplemented with 10% FBS. The cells were incubated for a total of 24 h, harvested, and analyzed for luciferase activity.

COS 7 cells (72,000) were plated in 35-mm wells and grown to 50% confluency in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with penicillin and streptomycin (10,000 units/ml), L-glutamine (200 mM), and 10% FBS for 24 h. The cells were transfected with medium supplemented with 10% FBS. The cells were incubated for a total of 24 h, harvested, and analyzed for luciferase activity as described above.

RESULTS

Peptide DNA condensates are formed instantly through ionic binding of the peptide to the DNA backbone followed by interpeptide oxidation to form disulfide bonds that reversibly stabilize the DNA condensates on ES-MS that matched the anticipated mass of the fully reduced peptide (Table I). The peptides were maintained in their reduced form at low pH by removing oxygen; however, at neutral pH, the cysteine residues became very reactive in forming disulfide bonds. When cross-linking peptide V was adjusted to pH 7.5 and directly infused into ES-MS, the parent ion completely disappeared within 25 min with a t1/2 of approximately 10 min resulting in the formation of an intramolecular disulfide bond peptide as the primary oxidation product (Fig. 2).

When bound to DNA, cross-linking peptides are less flexible such that intermolecular peptide cross-linking may be the preferred route of oxidation. To determine whether this occurs, the time course of interpeptide disulfide bond formation for peptides bound to DNA was studied using a continuous fluorescence assay. A SYBR-Gold DNA intercalator dye was used because it maintains its fluorescence even with peptide bound to DNA (Fig. 3A). When prepared at a charge ratio of 2:1, both AlkCWK18 and polylysine1007 instantly form DNA condensates and produce a constant SYBR-Gold fluorescence intensity over time with magnitude inversely related to the peptide binding affinity for DNA (Fig. 3A). In contrast, the fluorescence intensity decreased over time when peptides II–V were used to form DNA condensates at a charge ratio of 2:1 (Fig. 3A), because of displacement of the intercalator dye as cross-linking proceeds and peptide binding affinity increases. The kinetic decrease in SYBR-Gold fluorescence was interpreted as the approximate time course of disulfide formation for peptides bound to DNA. Interpeptide cross-linking of V occurred in less than 30 min, similar to that predicted by ES-MS. Increasing the number of cysteine residues in the series II-V increased both the time required to achieve complete cross-linking (t1/2 = 1–10 min) and
Likewise, the rate of kinetic decrease in SYBR-Gold fluorescence and the final fluorescence intensity were not significantly influenced by the cross-linking peptide concentration (Fig. 3B), indicating that the disulfide bonds did not appreciably quench the SYBR-Gold fluorescence.

To establish the peptide to DNA stoichiometry needed to achieve maximal condensation, the ability of peptides II–V to displace thiazole orange from DNA were compared. Titration of 5–75 nmols of peptides II–V with 50 μg of DNA, followed by a 2-h incubation to allow cross-linking, resulted in a decrease in the thiazole orange fluorescence (Fig. 4). At 0.4 nmol of peptide/μg of DNA or greater, each peptide was able to fully condense DNA as determined by a plateau in the residual fluorescence intensity (Fig. 4).

The particle size of each cross-linked peptide DNA condensate was evaluated by quasi-elastic light scattering at both 15 and 90° scatter angles to minimize the influence of internal motion that is detected at 90° (29). At a charge ratio of 2:1, CWK18 and peptides II–V each formed essentially a single population of DNA condensates determined by quasi-elastic light scattering at both 15 and 90° to approximate a mean diameter of 40–60 nm (Table II). A slightly larger population of particles with a mean diameter of 70–100 nm resulted when AlkCWK18, DiCWK18, and polylysine1007 were used to condense DNA (Table II).

The role of disulfide bond stabilization was investigated by treating DNA condensates with TCEP, which caused a negligible change in mean particle size when either AlkCWK18 or polylysine1007 were used as condensing agents. In contrast, TCEP reduction of peptide DNA condensates prepared with DiCWK18, CWK18, and II–V more than doubled the mean particle size (Table II).

Because sodium chloride has been shown to increase the particle size of peptide DNA condensates in a time-dependent fashion (17), we examined the particle size stability of AlkCWK18, CWK18, polylysine1007, and peptide II–V DNA condensates prepared in 0.15 M sodium chloride (Table II). After 15 min the particle size grew significantly because of the formation of a large (>5 μm) population that represented 10–20% of the DNA with 80% maintained as small (<100 nm) particles. After 24 h of incubation in sodium chloride, each of the peptide DNA condensates formed large floculates.

**Fig. 2.** Kinetics of peptide oxidation in solution. ES-MS was used to monitor the intrapeptide disulfide bond formation of peptide V in solution. The loss of peptide ion (M+2H+ at 1258 atomic mass units) was continuously monitored along with the formation of the intrapeptide disulfide bonded product at 1256 atomic mass units. The kinetic profile indicated complete loss of the parent ion and formation of the oxidized peptide within 25 min.

**Fig. 3.** Kinetics of cross-linking DNA condensates. A illustrates the fluorescence intensity of intercalated SYBR-Gold continuously monitored over time during the formation of peptide DNA condensates prepared with AlkCWK18 (○), CWK18 (▼), II (●), III (▲), IV (■), V (●), and polylysine1007 (▲). The results indicate a decrease in fluorescence over time for cross-linking peptides but not for AlkCWK18 and polylysine1007, which are not able to form cross-links. B demonstrates the influence of increasing peptide IV stoichiometry (0.4 (●), 0.8 (○), and 1.2 (▲) nmol/μg of DNA) on the apparent rate of the cross-linking reaction.

**Fig. 4.** Binding of cross-linking peptides to DNA. Thiazole Orange was used in a fluorescent dye exclusion assay to determine the relative binding affinity of each peptide to plasmid DNA. Condensates were formed with AlkCWK18 (○), CWK18 (▼), II (●), III (▲), IV (■), and V (●) at an increasing peptide to DNA stoichiometry. Each peptide formed fully condensed DNA at a peptide to DNA ratio of 0.3–0.4 nmol of peptide/μg of DNA corresponding to a (NH₄PO₄) charge ratio of approximately 2:1.
The stability of cross-linking peptide DNA condensates were studied by determining their sonication shear stress stability as a function of increasing sodium chloride concentration. AlkCWK18 DNA condensates protected DNA from fragmentation in sodium chloride up to 0.4 M, above which higher salt concentrations led to the dissociation of the peptide and fragmentation of the DNA (Fig. 5A). The generation of DiCWK18 when using CWK18 as a condensing agent led to a negligible change in the shear stress stability because peptides with either 18 or 36 lysine residues dissociate from DNA at sodium chloride concentration between 0.4 and 0.8 M (Fig. 5B) (26). However, the substitution of a second cysteine into CWK18 resulted in peptide II DNA condensates that were stable to sonication shear stress in sodium chloride concentrations up to 1.0 M (Fig. 5C). Increasing the number of cysteine residues to 3, 4, and 5 produced very stable DNA condensates that were protected from sonication fragmentation up to 2.5 M sodium chloride (Fig. 5D–E) and could not be dissociated even with saturated (4 M) sodium chloride (not shown). However, reduction of peptide V DNA condensates with TCEP reverted the DNA condensate stability back to 0.4 M sodium chloride (Fig. 5G). Likewise, when V was allowed to fully oxidize prior to making peptide DNA condensates, it also failed to protect DNA from sonication shear stress above 0.4 M sodium chloride (Fig. 5H). Polylysine1007 only protected DNA from shear stress up to 1 M sodium chloride (Fig. 5I), which was equal to the stability afforded by peptide II.

Peptide IV DNA condensates were prepared at charge ratios of 0.5–2.5 and analyzed for particle size, zeta potential, and sonication shear stress stability to determine whether the degree of interpeptide cross-linking was a function of peptide DNA stoichiometry (Fig. 6). Peptide IV formed small (62 nm) electron-dense (−18 mV) DNA condensates at a charge ratio of 0.9 that resisted fragmentation up to 2.5 M sodium chloride (Fig. 6B, inset), indicating that even at low stoichiometry a sufficient number of peptides were bound to DNA to effect cross-linking. However, it is interesting to note that the DNA was almost completely converted to open circular, indicating partial susceptibility of the DNA to shear stress-induced strand breaks as a result of peptide vacancies.

To determine whether cross-linking peptides could react covalently with DNA, mass spectrometry was used to study the reaction of a deca-oligonucleotide with peptides. Both control peptide (AlkCWK18) and peptide V yielded identical spectra to the oligonucleotide alone, producing only a (1480)M−2 m/z ion corresponding to the predicted mass of the oligonucleotide. There was no evidence of higher mass products representing a covalent reaction between the cross-linking peptide and the oligonucleotide.

The enhancement in gene expression mediated by cross-linking peptide II–V DNA condensates was cell type-dependent. Transfection of COS 7 cells only resulted in a 2.5–10-fold increase in gene expression over AlkCWK18, similar to the level mediated by DiCWK18.

In contrast, cross-linking peptide II mediated 60-fold higher gene expression in HepG2 cells relative to AlkCWK18 DNA condensates (Fig. 7A). Cross-linking peptides III and V also yielded higher levels (4–10-fold) of reporter gene expression than AlkCWK18 DNA condensates but not as high as peptide II.

The enhancement in gene expression mediated by cross-linking peptide II–V DNA condensates was cell type-dependent. Transfection of COS 7 cells only resulted in a 2.5–10-fold increase in gene expression over AlkCWK18 (Fig. 7B) compared with the 4–60-fold increase observed in HepG2 cells.

The magnitude of gene expression mediated by AlkCWK18, CWK18, and peptides II–V was also chloroquine-dependent. However, chloroquine enhanced the gene expression of peptide II DNA condensates approximately 5-fold more than AlkCWK18 DNA condensates.

Because the elevated levels of transgene expression mediated by peptide II could be related to either an increased uptake of DNA by cells or to an improved transduction within...
cells, further experiments were performed to distinguish between these possibilities. Transfection of HepG2 cells with plasmid encoding nuclear targeted β-galactosidase demonstrated approximately 9-fold more cells were transduced using peptide II versus AlkCWK18 as a gene transfer agent (Fig. 8). Likewise, 4-fold more COS 7 cells were positively stained for nuclear targeted β-galactosidase using peptide II versus AlkCWK18 to effect gene transfer (Fig. 8). A 5-h transfection of HepG2 cells with 125I-labeled plasmid DNA revealed that AlkCWK18 was nearly equivalent (9.5% of dose) to peptide II (8.7% of dose) at mediating DNA uptake (Fig. 9). A similar result in COS 7 cells demonstrated a nearly identical amount (7–8% of dose) of 125I-DNA taken up when using either transfecting agent. Together, these data support a hypothesis involving enhanced transduction mediated by peptide II.

**DISCUSSION**

The duration of transient gene expression will in part depend on how long DNA can survive metabolism (30). One function of polylysine and other polymers used as carriers for gene delivery is to protect DNA from premature metabolism (19, 26, 30). Polylysine DNA condensates prepared at a charge ratio of 2:1 or greater are electropositive and resist endonuclease digestion. The degree of metabolic protection afforded to DNA by polylysine is proportional to its molecular weight because longer polylysines bind to DNA with higher affinity and create more stable peptide DNA condensates (19, 26). HMW polylysine also enhances in vitro gene transfer efficiency severalfold relative to LMW peptides because of differences in both protection and release of DNA (19).

Studies that utilize HMW polylysine conjugates for in vivo gene delivery have devised a strategy of controlling the stoichiometry of polylysine to DNA to create partially condensed DNA that has a neutral or negative charge (13). This is essential, because intravenous dosing of electropositive DNA condensates leads to rapid opsonization and nonspecific biodistribution to lung and liver (18). However, this strategy fails when using LMW peptides as DNA condensing agents, because, at all charge ratios, these peptides are rapidly stripped during circulation because of their lower DNA binding affinity relative to HMW polylysine (18). If LMW peptides are to be useful as in vivo gene transfer agents, it is critical to improve their design to form small (<100 nm diameter) metabolically stable DNA condensates with the desired surface charge (31).

We have previously evaluated the relationship between LMW polylysine chain length, DNA condensate particle size, and in vitro gene transfer efficiency (16). These studies revealed that peptides possessing 18 lysines (CWK18) were sufficient to form small (<100 nm) DNA condensates that were efficient in mediating nonspecific in vitro gene transfer, whereas shorter peptides formed large DNA condensates and longer peptides were equivalent in gene transfer to CWK18 (16). Although the tryptophan in CWK18 was originally proposed to contribute to the DNA binding affinity, subsequent
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Heterogeneity and lower molecular weight (32). The single cysteine residue in CWK$_{18}$ has been used as a conjugation site to prepare polyethylene glycol-CWK$_{18}$ (33) and triantennary N-glycan CWK$_{18}$ (34), both of which have been used in DNA formulation for in vivo gene delivery (18).

To improve the stability of LMW peptide DNA condensates for use in vivo, cross-linking agents such as glutaraldehyde were used (18, 19). Although cross-linking of LMW peptide DNA condensates did increase their metabolic stability, it also diminished their ability to mediate gene expression because of the slow reversal of the Schiff bases (18). Other reversible cross-linking agents could be applied to stabilize LMW carrier DNA condensates (20, 21), but these agents require careful control over stoichiometry and have yet to be proven transfection competent.

Because endocytosed macromolecules must pass through a reducing environment (35), we envisioned that disulfide bonds could transiently stabilize LMW peptide DNA condensates and be reduced to initiate the intracellular release of DNA. To test this hypothesis, cysteine residues were substituted for lysine residues in CWK$_{18}$, and the physical and gene transfer properties of the resulting cross-linked peptide DNA condensates were studied.

The results established that disulfide bonds formed rapidly during a 30-min incubation at pH 7.4 when peptide V was either free in solution or bound to DNA. Condensate stability increased coincidentally with the number of cysteine residues as revealed by the indirect fluorescence assay (Fig. 3) and by the salt sonication electrophoresis assay (Fig. 5). The upper limit of shear stress stability afforded to DNA condensates by cross-linking peptides III and V could not be ascertained because they remained intact in 2.5 M sodium chloride and even in saturated 4 M sodium chloride (not shown). Thus, a LMW peptide (20-mer) with as few as three cysteine residues produced DNA condensates that far exceeded the stability afforded by polylysine$_{1007}$. Although the shear stress stability of DNA condensates afforded by peptide II was equivalent to polylysine$_{1007}$, the condensates were surprisingly enhanced in mediating gene expression, as discussed below.

To reach full stability, interpeptide disulfide bonds must form after the peptide binds to DNA because preoxidized peptide V formed primarily intramolecular disulfide bonds as established by mass spectral analysis and shear stress stability studies. Ionic binding of peptides to the DNA backbone may inhibit peptide folding and could promote the desired interpeptide disulfide bond formation (21).

Formation of interpeptide disulfide bonds also appears to decrease the size of peptide II-V DNA condensates resulting in mean particle diameters of 40–50 nm compared with 70–100 nm determined for AlkCWK$_{18}$, DiCWK$_{18}$, or polylysine$_{1007}$ DNA condensates (Table II). We hypothesize that DNA condensates compact when DNA loops within the condensate collide and tether through disulfide bond formation. Consequently, reduction of disulfide bonds within cross-linked DNA condensates would release the DNA loops and cause an increase in particle size. Quasi-elastic light scattering analysis of TCEP-reduced peptide II-V DNA condensates supported this hypothesis by revealing a larger population of DNA condensates.

The time-dependent increase in the particle size of cross-linked DNA condensates prepared in normal saline agrees with previous observations for LMW peptide DNA condensates (17) even though it was anticipated that they would resist aggregation as observed when using other cross-linking strategies (20). However, this should not be a problem because the use of cross-linking peptides for in vivo gene delivery will likely involve attaching polyethylene glycol to the peptide, which dra-
matically improves DNA condensate solubility (33) and inhibits salt induced aggregation.2

It is also apparent that disulfide bond formation is not restricted to electropositive DNA condensates (Fig. 6). The ability to form small (62 nm), electronegative (~18 mV), and highly stable DNA condensates is an important property for delivering them as carriers for in vitro gene delivery.

The level of gene expression mediated by CWK18 and peptides II–V was above that determined for AlkCWK18 DNA condensates. The increase in gene expression mediated by CWK18 over AlkCWK18 is likely due to the formation of DicWK18 within the DNA condensates. Both CWK18 and DicWK18 were still 5-fold inferior to polylysine1007 as a DNA carrier, in agreement with previous correlations established between polylysine length and in vitro gene transfer efficiency (19). Cross-linking peptide III mediated a comparable level of gene expression as polylysine1007, whereas less expression resulted when using peptide IV, which was partially regained when using peptide V as a condensing peptide. Most surprisingly, was the gene expression mediated by peptide II in HepG2 cells, which was 60-fold greater than AlkCWK18 DNA condensates and nearly 5-fold greater than polylysine1007 DNA condensates. The increase in gene expression mediated by II–V was less dramatic in COS 7 cells but still significantly improved over AlkCWK18 as a carrier molecule.

The enhanced expression mediated by peptide II DNA condensates is probably not related to their small particle size (42 nm) because CWK18 DNA condensates were also small (51 nm) but mediated similar levels of expression as DicWK18 DNA condensates (68 nm). It is also clear that the increase in DNA stability afforded by peptides III and V inversely correlates with the gene transfer efficiency. The addition of more than two cysteine residues seemingly exceeds the number of disulfide bonds that can be efficiently reduced in the cell.

The results of luciferase expression, nuclear targeted ß-galactosidase transfection, and radiolabeled DNA uptake studies collectively support a probable mechanism involving enhanced transduction mediated by peptide II. Even though the DNA uptake mediated by AlkCWK18 was the same as peptide II in HepG2 cells (Fig. 9), the number of cells reporting positive for nuclear targeted ß-galactosidase was 9-fold higher (Fig. 8), and the magnitude of the luciferase reporter gene expression was 60-fold higher (Fig. 7) when using peptide II. A possible explanation for these results is the intracellular stability afforded to DNA condensates when using peptide II, resulting in a longer residence time of the internalized DNA relative to AlkCWK18 DNA condensates. The ability of peptide II DNA condensates to undergo reduction provides a release mechanism for DNA relative to polylysine1007 DNA condensates. Upon reduction, peptide II would rapidly convert to a weaker binding peptide that could dissociate and release the DNA intracellularly, whereas the dissociation of polylysine1007 from DNA would presumably occur more slowly. The finding that chloroquine enhanced the expression of peptide II DNA condensates more than AlkCWK18 DNA condensates may relate to a previously reported pH dependence of reduction within cells (35).

Disulfide bond formation has been previously used by Behr and co-workers (36), who incorporated a single thiol into a cationic lipid causing its dimerization in the presence of DNA. Here, we have extended this hypothesis by substituting multi-

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2 K. Y. Kwok, R. C. Adami, K. C. Hester, Y. Park, S. Thomas, and K. G. Rice, submitted for publication.
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