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Cysteine-Tailed Class I-Binding Peptides Bind to CpG Adjuvant and Enhance Primary CTL Responses

Peter J. Wettstein,*† Nancy D. Borson,* Jewn G. Park,‡ Kelly T. McNallan,§ and Ann M. Reed$

Immunostimulatory CpG motifs in synthetic oligonucleotides can be effective adjuvants for the priming of CTLs. We first observed that a single male-specific peptide (KCSRNRQYL) (HY2) was more efficient than another male-specific peptide (WMHHNMDLI) (HY1) at priming IFN-γ-secreting CTLs in vivo when combined with lipid A and CpG and that it also visibly precipitated CpG. The addition of the six N-terminal residues (KCSRNR) from HY2 to HY1 yielded a peptide, KCSRNR-HY1, that both precipitated CpG and primed increased numbers of HY1-specific CTLs. We refer to this type of peptide as a primotope that includes a class I binding peptide tailed with amino acids that increase priming. Ala residues were substituted for the Arg/Lys residues (ACSANA-HY1), and these substitutions did not reduce in vivo priming potential. However, the substitution of Ala for Cys (KASRNR-HY1) resulted in the complete loss of priming, demonstrating the importance of Cys for in vivo priming when mixed with CpG. This result suggested that increased priming was based in disulfide bonding between Cys residues and internal phosphorothioate groups of synthetic CpG. The addition of Cys-bearing primotopes to radiolabeled CpG with a single thioate group resulted in the appearance of a new band that was inhibited by 1) Cys > Ala substitution and 2) reduction and alkylation of CpG. These results reveal a novel mechanism for complexing class I binding peptides and CpG adjuvant for development of new peptide-adjuvant combinations for vaccines for cancer and infectious diseases. The Journal of Immunology, 2005, 175: 3681–3689.

The products of class I genes mapping to the MHCs of mammals bind and present processed peptides to CTLs. Class I protein expression is dependent on the stable binding of these proteins to β2-microglobulin as well as to peptides that are derived from endogenous proteins processed by proteosomes, with the resulting peptides being transported to the endoplasmic reticulum (ER) for binding to class I molecules (1, 2). The majority of peptides bound to products of individual class I genes are endogenous in origin and exhibit binding motifs that control the specificity of binding to class I molecules (3, 4). However, class I molecules have also been shown to efficiently present exogenous peptides through a process of indirect or cross-presentation in which professional APCs present exogenous class I-binding peptides that are either fully processed or derived from ingested proteins, cell lysates, or whole prokaryotic/eukaryotic cells (reviewed in Ref. 5). The generation and presentation of class I-binding peptides from exogenous proteins and cells clearly require a complex network of processing by the degradative machinery in endosomes/lysosomes and phagosomes with coordinated proteosomal degradation and transport of peptides to the ER for binding to class I molecules (reviewed in Refs. 5, 6).

The ability of APCs to indirectly present exogenous peptides has spurred considerable interest in using class I-binding peptides in vaccines to activate and expand CTLs specific for cancer and infectious diseases. The majority of effort in basic and applied studies has been in the use of fully processed peptides with optimal motifs for binding to class I molecules. The use of optimally binding peptides focuses on cell surface events where exogenous peptides must compete with previously bound, endogenous peptides for class I binding sites or bind to empty class I molecules that, except for mouse H2L molecules (7), have relatively short half-lives. An exception is the development of extended “Trojan” peptides, which include class I-binding peptides that have been tailed with positively charged amino acids derived from either the Droso-

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1 This work was supported by National Institutes of Health Grant AI-16052.
2 Address correspondence and reprint requests to Dr. Peter J. Wettstein, Department of Immunology, Mayo Clinic, College of Medicine, Rochester, MN 55905. E-mail address: wettstein.peter@mayo.edu
3 Abbreviations used in this paper: ER, endoplasmic reticulum; MiHAg, minor histocompatibility Ag; MPL-AF, monophosphoryl lipid A-aqueous formulation; TBE, Tris-borate-EDTA.

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activate TLR9 (13) are particularly relevant to the research described in this communication. TLR9 activation drives the release of Th1-like cytokines and the up-regulation of costimulatory molecules by professional APCs to promote priming and expansion of CTLs (14–16). Although adjuvants have been simply combined with peptides and Ags for isolated functions of the two vaccine components, a number of studies have investigated the complexing of Ags with CpG using positively charged amino acids in cationic peptides (17, 18) and chemical cross-linking agents (19–21). CpG adjuvant-protein complexing has been pursued to deposit the two components at anatomic sites for extended activity and reduced, systemic inflammatory activity of free CpG adjuvant (17).

The experiments described in this communication were based on preliminary results of testing of bacterial adjuvants for their capacities to increase the frequencies of CTLs specific for peptides from minor histocompatibility Ags (MiHags). We observed that the most immunogenic peptide, as indicated by CTL frequencies, was also the only peptide that precipitated mixtures of lipid A plus CpG synthesized with a phosphorothioate backbone (hereafter referred to as CpG). We subsequently confirmed that precipitation required only the peptide and CpG, and precipitation of CpG required Arg/Lys residues that could be added to the N termini of class I-binding peptides, as predicted by previous results (18). An unexpected and important finding was that maximal immunogenicity depended on the inclusion of Cys residues in the terminal tails. We propose that Cys residues are capable of forming disulfide bonds with the phosphorothioate backbone of synthetic CpG oligonucleotides.

Materials and Methods

**Mice**

C57BL/6 (B6) and C.B10-H2b (BALB.B) mice were used in these experiments and were purchased from The Jackson Laboratory. All mice were housed under specific pathogen-free conditions in the Mayo Clinic Section of Veterinary Medicine.

**Peptides**

Peptides were synthesized to 75% purity by A&A Laboratories using Fmoc chemistry; purity and sequence identity were assayed by mass spectrometry. Peptides were dissolved in DMSO at concentrations of 40 μg/μl and stored at –70°C before use.

**Adjuvants**

The 1826 CpG sequence of TCCATGACGTTCCTGACGTT that is specific for mouse TLR9 (22) was chosen for study. CpG oligonucleotides were synthesized by the Mayo Clinic Molecular Biology Core with phosphorothioate diester linkages using tetraethylthiuram disulfide on instrument. Synthetic CpG oligonucleotides were also synthesized with single phosphorothioate diester linkages between nucleotide positions four and five. The monophosphoryl lipid A-aqueous formulation (MPL-AF) adjuvant was generously provided by Dr. M. Lacy (Corixa, Seattle, WA).

**Precipitation of CpG oligonucleotides**

Peptides (20 nmol) and CpG oligonucleotides (6 nmol) with phosphodiester or phosphorothioate linkages were dissolved in water and mixed in triplicate in 25-μl total volumes. These mixtures were incubated at room temperature for 15 min and were centrifuged at 10,000 × g. Aliquots of supernatants were diluted 1/100 for measurements of OD at 260 nm.

**Analysis of peptide-CpG complexes**

CpG oligonucleotides were end labeled with [γ-32P]ATP (PerkinElmer) using T4 polynucleotide kinase (Promega). End-labeled CpG oligonucleotides were dissolved in 0.1 M sodium phosphate buffer for reduction and alkylation according to the published method (23) with tris(2-carboxyethyl)phosphine (Pierce) and iodoacetamide (both at concentrations of 1.25 mM) for 22 h at 50°C. Alkylation without reduction was performed with 1.25 mM iodoacetamide for the same time and at the same temperature. Reaction products were precipitated with ethanol and washed with 80% ethanol. Treated and untreated CpG oligonucleotides (7.5 pmol) were then mixed with peptides (12 nmol) in sodium phosphate buffer in total reaction volumes of 10 μl for overnight oxidation at room temperature. Untreated CpG oligonucleotides (3 nmol) were also mixed with peptides (20 nmol) in 10 μl 50 mM Tris (pH 8.0) with 1 mM glutathione (1:1 mix of oxidized and reduced forms of glutathione). Samples were separated by electrophoresis in polyacrylamide gels (15% acrylamide/bis at 29:1) in 1× Tris-borate-EDTA (TBE) buffer at 80-W constant power.

**ELISPOT**

Required doses of peptide plus adjuvant were mixed in Dulbecco’s modified PBS. Recipients were injected with 40 μl of peptide plus adjuvant at the base of the tail. Splenocytes and lymph node cells were aseptically harvested from sentinel lymphoid cells at varying time points after priming. Frequencies of IFN-γ-secreting CTLs were estimated by primary and secondary ELISPOT assays. Stimulators for both types of assays included gamma-irradiated allogeneic and syngeneic splenic cells and RMA/S cells (24); syngeneic spleen cells and RMA/S cells were pulsed with titrated concentrations of peptides for 30 min at room temperature before culture. CD8+ CTL responders for primary ELISPOT assays were enriched from lymph node and spleen populations using magnetic bead-based negative separation kits (Miltenyi Biotec). Later experiments included the depletion of B cells by panning over anti-mouse Ig-coated plastic dishes before enrichment of CD8+ CTLs (25). CD8+ responders (2–4 × 105 splenic cells/well and 2–4 × 106 lymph node cells/well) were mixed with stimulators (5 × 107/well for splenocytes and 2 × 107/well for RMA/S cells) in IMDM plus 5% FCS in wells of ELISPOT plates that had been precoated with diluted anti-IFN-γ capture Ab (Mabtech). Primary cultures were incubated for 40–48 h at 37°C in a 5% CO2 atmosphere. Spots were developed by first washing the wells with PBS + 0.05% Tween 20 and then adding biotinylated anti-IFN-γ detection Ab (Mabtech). Secondary ELISPOT cultures were incubated for 2–3 h at 37°C. Wells were washed, and streptavidin-conjugated HRP (Vector Laboratories) was added for 2 h at room temperature. Following multiple washes with PBS + Tween 20 and PBS alone, 3-aminooxy-ethylcarboxylate substrate was added for a 5-min incubation, after which the wells were washed exhaustively with tap water. Wells were counted by first obtaining digitized images for analysis using Immunospot software from C.T.L. Analysts and software from AID.

Secondary ELISPOT assays were performed with CTLs that had been expanded in primary mixed lymphocyte cultures. Responder spleen cells and irradiated stimulators were mixed in equal numbers in IMDM + 5% FCS + 2-ME in bulk culture for incubation at 37°C as previously described (26). Stimulators included allogeneic spleen cells and syngeneic spleen cells pulsed with 1 μM peptide. Expanded cells were harvested after 7 days, and CD8+ CTLs were enriched as described above. ELISPOT assays were performed as described above with the reduction in numbers of CD8+ CTLs/well (1–4 × 105/well) and incubation for 16–20 h.

**In vivo staining with peptide and CpG**

Peptides were labeled with the amine-reactive dye Alexa 488 according to the manufacturer’s protocol (Molecular Probes). CpG oligonucleotides were stained with Texas red maleimide through the use of the EndTag nucleic acid labeling system that uses T4 polynucleotide kinase (Vector Laboratories). Labeled peptide and CpG were mixed in PBS to deliver 10 μg of each component in 40 μl per recipient. Recipients were anesthetized with sodium pentobarbital (0.05 mg/g body weight) and injected in both rear footpads. Draining lymph nodes were aseptically harvested for in vivo staining with peptide and CpG. 1 × 104 lymph node cells at varying time points after priming. Frequencies of IFN-γ-secreting CTLs were estimated by primary and secondary ELISPOT assays. Stimulators for both types of assays included gamma-irradiated allogeneic and syngeneic splenic cells and RMA/S cells (24); syngeneic spleen cells and RMA/S cells were pulsed with titrated concentrations of peptides for 30 min at room temperature before culture. CD8+ CTL responders for primary ELISPOT assays were enriched from lymph node and spleen populations using magnetic bead-based negative separation kits (Miltenyi Biotec). Later experiments included the depletion of B cells by panning over anti-mouse Ig-coated plastic dishes before enrichment of CD8+ CTLs (25). CD8+ responders (2–4 × 105 splenic cells/well and 2–4 × 106 lymph node cells/well) were mixed with stimulators (5 × 107/well for splenocytes and 2 × 107/well for RMA/S cells) in IMDM plus 5% FCS in wells of ELISPOT plates that had been precoated with diluted anti-IFN-γ capture Ab (Mabtech). Primary cultures were incubated for 40–48 h at 37°C in a 5% CO2 atmosphere. Spots were developed by first washing the wells with PBS + 0.05% Tween 20 and then adding biotinylated anti-IFN-γ detection Ab (Mabtech). Secondary ELISPOT cultures were incubated for 2–3 h at 37°C. Wells were washed, and streptavidin-conjugated HRP (Vector Laboratories) was added for 2 h at room temperature. Following multiple washes with PBS + Tween 20 and PBS alone, 3-aminooxy-ethylcarboxylate substrate was added for a 5-min incubation, after which the wells were washed exhaustively with tap water. Wells were counted by first obtaining digitized images for analysis using Immunospot software from C.T.L. Analysts and software from AID.

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**Results**

**Priming capacities of HY peptides**

The WMHHNMDLI (HY1) and KCSNRQYL (HY2) peptides are derived from proteins encoded by murine Y-linked genes and...
are presented by H2D\(^b\) molecules to CTLs (27, 28). Based on a previous report of the additive effects of lipid A and CpG adjuvants (29), these two peptides were mixed with combined MPL-AF plus CpG adjuvants and tested for their capacities to prime CTL responses. These two peptides were also compared for in vivo priming efficiency with an immunodominant H60 peptide (LT-FNYRNL) (30). B6 females were primed s.c., and spleens were harvested 10 days after immunization. CD8\(^+\) CTLs were enriched by negative selection and stimulated with 1) syngeneic female spleen cells, 2) syngeneic male spleen cells for CTLs specific for HY1 and HY2, 3) allogeneic (BALB.B) spleen cells for anti-H60 CTLs, and 4) peptide-pulsed RMA/S cells in primary ELISPOT assays to estimate the frequencies of IFN-\(\gamma\)-secreting CTLs. Syngeneic male and allogeneic stimulators were used to estimate the frequencies of peptide-specific CTLs with sufficient affinities to recognize physiological levels of target peptides. RMA/S cells were used for pulsing with peptides because their deficiency in TAP2 expression results in efficient loading of class I molecules with exogenous peptides (31). On the basis of frequencies of responding CTLs, the HY2 peptide was the most efficient primer of CTLs (Fig. 1), whereas the H60 peptide was the most inefficient. The ranking of HY2 > HY1 > H60 was surprising in light of the reports that H60 is an immunodominant minor Ag peptide (32) corresponding to the originally described CTT-5 Ag (33).

An additional observation was made in this experiment: HY2 was the only peptide that created visible precipitation when added to the mixture of MPL-AF and CpG in PBS; it was subsequently observed that precipitation required only CpG and HY2. The ability of proteins and amino acid polymers with multiple, positively charged residues to precipitate synthetic CpG oligonucleotides has been previously reported (17). The HY2 peptide includes two Arg residues and a single Lys residue that are expected to be fully charged at the pH of PBS in comparison to the two His residues in HY1 that are predicted to be uncharged at this pH. We investigated the effects of positively charged residues on immunogenicity in our immunization regimen by synthesizing the HY1 peptide with two different tails. First, the six N-terminal residues, KCSRNR, of HY2 were synthesized on the N terminus of HY1. Second, HY1 was synthesized with an N terminus of six amino acids, RKKRRQ, that are included in the membrane-translocating portion of the HIV-1 tat protein, which has been shown to increase transmembrane transport of class I binding peptides (10).

B6 females were primed with mixtures of CpG adjuvant and HY1, KCSRNR-HY1, RKKRRQ-HY1, and HY2. It should be stressed that doses of tailed peptides were increased to maintain equal molarity with 100-\(\mu\)g doses of HY1 and HY2. Spleens were harvested 10 days thereafter for enrichment of CD8\(^+\) CTLs for primary IFN-\(\gamma\) ELISPOT assays with peptide-pulsed RMA/S cells as stimulators (Fig. 2). In accord with the results included in Fig. 1, HY2 primed for greater frequencies of CTLs than HY1 at all tested peptide concentrations. The linkage of the KCSRNR tail with the HY1 target peptide resulted in frequencies of HY1-specific CTLs that were comparable to those obtained with HY2. The RKKRRQ tail increased priming efficiency to an intermediate level compared with HY1 vs KCSRNR-HY1. The HY2, KCSRNR-HY1, and RKKRRQ-HY1 peptides all precipitated CpG oligonucleotides (data not shown). The increased priming efficiency of KCSRNR-HY1 indicated that the addition of this six-amino acid sequence carrying three positively charged residues increased HY1-specific priming. However, the intermediate efficiency associated with the RKKRRQ-HY1 peptide with five positively charged residues suggested that increasing the number of positively charged amino acids was not associated with further increased priming. We will hereafter refer to tailed class I-binding peptides as primotopes, a term that connotes increased priming efficiency. The portions of primotopes that are presented by class I molecules will be referred to as recognition peptides.

**FIGURE 1.** Frequencies of IFN-\(\gamma\)-secreting CTLs specific for three MI-HAg peptides. Primary ELISPOT assays were performed with CD8\(^+\) splenocyte responders from recipients of single immunizations with 100 \(\mu\)g of peptide plus 100 \(\mu\)g of CpG and 10 \(\mu\)g of MPL-AF. Stimulators included syngeneic and allogeneic (B6 male for HY1 and HY2 and BALB.B for H60) spleen cells (A) and RMA/S cells pulsed with the respective target peptides at concentrations of 10 nM (B).

**FIGURE 2.** Positively charged tails increase the immunogenicity of the HY1 peptide. B6 female mice were primed with 100 \(\mu\)g of CpG mixed with HY2, HY1, KCSRNR-HY1, and RKKRRQ-HY1 (doses adjusted to equalize peptide molarity) according to the standard method. CD8\(^+\) CTLs were mixed with RMA/S cells pulsed with the respective peptide (HY2 or HY1) at titrated concentrations (1 \(\mu\)M to \(>1\) pM) in a primary IFN-\(\gamma\) ELISPOT assay.
Ala screen of active residues

The involvement of positively charged residues in increasing HY1-specific priming was directly tested by substituting Ala residues for the three Arg/Lys amino acids in the KCSRNR-HY1 primotope and using this altered primotope mixed with CpG to prime B6 female mice. Spleen cells were harvested at 10 days and CD8⁺ CTLs were enriched for quantitation in primary and secondary IFN-γ ELISPOT assays. As in prior experiments, priming with KCSRNR-HY1 resulted in higher frequencies of HY1-specific CTLs in primary ELISPOT assays than priming with the fully processed HY1 peptide (Fig. 3). This ranking was observed with both syngeneic male (Fig. 3A) and HY1-pulsed syngeneic female spleen cell stimulators (Fig. 3B). To our surprise, substitution of Ala for all Arg/Lys residues in the ACSANA-HY1 primotope did not result in the loss of priming efficiency, although it did result in the loss of the ability to precipitate CpG. In fact, peptide-pulsed female cells stimulated higher frequencies of HY1-specific CTLs from mice primed with ACSANA-HY1 than with KCSRNR-HY1 (Fig. 3B). Confirmatory results were obtained in secondary ELISPOT assays of CTLs expanded by stimulation with syngeneic male spleen cells in primary MLCs (Fig. 4). Comparable frequencies of HY1-specific CTLs were stimulated with syngeneic male spleen cells and peptide-pulsed spleen cells when responder CTLs were derived from mice primed with KCSRNRHY1 and ACSANA-HY1.

The apparent importance of the flanking Cys residue was interesting in light of the fact that synthetic CpG oligonucleotides are synthesized with phosphorothioate linkages to reduce susceptibility to nucleases in vivo (34), and their capacity to bind to plasma proteins in vivo is well documented (35). The simplest explanation for the preeminent role of Cys in driving in vivo priming is the formation of disulfide bonds between the Cys-containing HY1 primotopes and synthetic CpG oligonucleotides. The importance of the Cys residue for in vivo priming with the KCSRNR-HY1 and ACSANA-HY1 primotopes was confirmed by additional Ala substitutions. The KASRNR-HY1 primotope (Cys/Ala) exhibited virtually no priming potential, as evaluated by both primary and secondary ELISPOT assays (Figs. 3 and 4). However, the KASRNR-HY1 primotope still retained the capacity to visibly precipitate CpG (data not shown). The AASANA-HY1 primotope was similar to KASRNR-HY1 in its lack of in vivo priming capacity. These results supported the hypothesis that the single Cys residue was required for optimal HY1-specific priming, potentially through the ability to cross-link multiple CpG molecules. An HY1 primotope was synthesized with N and C tails carrying single Cys residues: ACSANA-HY1-ANASCA. This primotope was combined with CpG for priming of B6 females with frequencies of HY1-specific CTLs estimated by primary and secondary ELISPOT assays. A significant increase in priming efficiency was observed relative to priming with the ACSANA-HY1 primotope as revealed by frequencies of HY1-specific CTLs that responded to syngeneic male stimulators in primary ELISPOT assays (Fig. 3). Furthermore, comparable frequencies of HY1-specific CTLs were observed in secondary ELISPOT assays of CD8⁺ CTLs from mice primed with all three primotopes tailed with Cys residues, i.e., ACSANA, KCSRNR, and double AC SANA tails (Fig. 4).
Titration of peptide + CpG

To this point, in vivo priming with fully processed and tailed HY1 peptides had been performed with fixed doses of peptide and CpG adjuvant. We sought to determine whether increased priming efficiency with ACSANA-HY1 could still be observed with reduced doses of primotope and CpG. B6 females were primed with mixtures of CpG plus HY1 and ACSANA-HY1 at doses of 100, 40, and 10 μg of each of the two components. Spleens were harvested 10 days later for enrichment of CD8+ CTLs for primary and secondary ELISPOT assays with syngeneic male and female spleen cell stimulators, the latter of which were either untreated or pulsed with HY1. In primary ELISPOT assays (Fig. 5), priming efficiency was maintained with 40-μg doses of ACSANA-HY1 peptide but not with the fully processed HY1 peptide when tested with syngeneic male stimulators (Fig. 5A) and female stimulators pulsed with 10 nM HY1 peptide (Fig. 5B). No priming was detectable in primary ELISPOT assays with 10 μg of either ACSANA-HY1 or HY1.

Duration of priming

We have observed that the presence of Arg/Lys residues in HY1 primotope tails promotes precipitation of CpG, and other investigators have observed that poly-Arg precipitates CpG oligonucleotides that remain deposited at injection sites for extended periods of time with associated, extended priming of CTLs (17). We hypothesized that such extended deposition would occur with KCSRNR-HY1, which would then prime for longer periods of time than ACSANA-HY1 that does not precipitate CpG oligonucleotides but strongly primes primary CTL responses. B6 females were primed with CpG plus KCSRNR-HY1 or ACSANA-HY1, and spleens and draining lymph nodes were harvested at 15, 29, and 50 days for primary ELISPOT assays with peptide-pulsed syngeneic female stimulators (Fig. 6). These results showed that ACSANA-HY1 was most efficient at priming CTLs in both spleen and draining lymph nodes harvested at 15 days with stimulators pulsed with 10 nM (Fig. 6) and 1 μM HY1 peptide (data not shown). However, this distinction faded at 29 days with KCSRNR-HY1 and ACSANA-HY1 priming for similar frequencies of HY1-specific CTLs in both lymph nodes and spleens. This similarity between ACSANA-HY1 and KCSRNR-HY1 continued through 50 days with comparable levels of priming. Testing at 50 days revealed a shift toward higher frequencies of HY1-specific CTLs in draining lymph nodes than spleens, suggesting retention of the two primotopes at the sites of injection through this time point with no consistent difference between ACSANA-HY1 and KCSRNR-HY1.

Migration of Langerhans cells

The activity of CpG adjuvant is focused on dendritic cells, including Langerhans cells, that present class I-bound peptides to CTLs. Activation of dendritic cells by CpG requires TLR9 expression and results in increased expression of cytokines and costimulatory molecules as well as migration to draining lymph nodes. As an adjunct to analyses of CTL frequencies, we investigated the effects of Arg/Lys and Cys residues in tailed HY1 peptides on in vivo migration of Langerhans cells. Three B6 females/group were injected in the hind footpads with Texas red-stained CpG mixed with Alexa 488-conjugated HY1, KCSRNR-HY1, and ACSANA-HY1. Draining popliteal lymph nodes were harvested after 24 h, and lymphoid cells were dissociated to estimate the frequencies of doubly stained cells by fluorescent microscopy. The results presented in Fig. 7 indicate that the addition of KCSRNR and ACSANA tails increased the frequencies of doubly stained cells ~2.5-fold relative to the frequencies stimulated by HY1 peptide + CpG.
Primotope and CpG complexing

The extent of precipitation encountered when mixing CpG oligonucleotides with HY1 peptides tailed with positively charged amino acids was investigated. The first experiments were designed to directly assess the roles played by Arg/Lys and Cys residues in precipitating CpG from free solution. HY1 primotopes carrying KASRNR, ACSANA, and AASANA tails were mixed with CpG, and the mixtures were pelleted at 10,000 × g before spectrophotometric measurements of CpG remaining in solution. As shown in Fig. 8, KASRNR-HY1 precipitated ~90% of soluble CpG, and ACSANA-HY1 and AASANA-HY1 had no significant effects on CpG levels. These results support the contention that the initially observed particulate precipitation is the function of interactions between the highly polar nucleic acids and the charged Arg/Lys amino acids of the primotope tails.

Cys-mediated complexing of primotopes and CpG

The potential role of cysteine-bearing peptides in complexing with CpG was investigated by means of gel shift assays using 33P-labeled CpG. CpG oligonucleotides (S1-CpG) were synthesized with only a single phosphorothioate linkage (between the fourth and fifth residues) for these assays due to the complex band patterns observed in electrophoretic separations of CpG with all phosphorothioate linkages (data not shown). A complex band pattern was still observed with S1-CpG in contrast to native phosphodiester CpG, which migrates as a single product on a PAGE gel (Fig. 9 and data not shown). The multiple bands observed with S1-CpG are possibly due to variable, complex interactions between 1) disulfide bonding between S1-CpG molecules and 2) secondary structures that are determined by the nucleotide sequence of the 1826 CpG oligonucleotide and the presence of the phosphorothioate group. Molar excesses (12 nmol) of the ACSANA-HY1 and AASANA-HY1 primotopes were mixed with 33P-S1-CpG (7.5 pmol) before air oxidation and separation by electrophoresis. The addition of ACSANA-HY1 resulted in the appearance of a new band that was not observed with the mixture of S1-CpG and AASANA-HY1 (Fig. 9). The presence of this band was reduced by alkylation of S1-CpG and eliminated by reduction and alkylation of S1-CpG before mixing with ACSANA-HY1 (Fig. 9). These results suggest that the presence of this band required a Cys residue in the primotope and was dependent on bonds between primotopes and S1-CpG oligonucleotides that could be blocked by reduction and alkylation as would be expected for disulfide bonds between Cys residues and the phosphorothioate groups.

We considered that the relatively low amount of S1-CpG/primotope complex that formed when the components were simply mixed and air-oxidized was perhaps due to preferred interpeptide complexing of ACSANA-HY1 peptides at the relatively high concentrations of peptide used for the experiment. Therefore, the reactions were repeated with S1-CpG and ACSANA-HY1 components that were first subjected to mild reduction in 5% 2-ME followed by removal of the 2-ME by evaporation under vacuum with subsequent, overnight air oxidation. The amount of complexing was increased under these conditions with an accompanying decrease in unbound S1-CpG (Fig. 10). The addition of glutathione

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** The addition of primotope tails to the HY1 peptide increases migration of Langerhans cells from injection sites to draining lymph nodes. B6 female mice (three per group) were anesthetized and injected in the footpads with Texas red-conjugated CpG plus the HY1, KCSRNR-HY1, and ACSANA-HY1 peptides that had been conjugated with Alexa 488. The recipients were sacrificed after 24 h, and the popliteal lymph nodes were dissociated. The frequencies of doubly stained cells were estimated by fluorescence microscopy.

![FIGURE 8](http://www.jimmunol.org/)

**FIGURE 8.** Positively charged residues are required for precipitation of CpG oligonucleotides. CpG oligonucleotides (6 nmol) were mixed in triplicate with HY1 primotopes (20 nmol) for 15 min at room temperature. Precipitates were pelleted at 10,000 × g, and supernatants were diluted 1/100 for spectrometric analysis at 260 nm.

![FIGURE 9](http://www.jimmunol.org/)

**FIGURE 9.** Interactions between Cys residues and S1-CpG with single phosphorothioate groups can be disrupted by reduction and alkylation. 33P-labeled S1-CpG with a single phosphorothioate group was mixed and incubated overnight with AASANA-HY1 (lane 1), ACSANA-HY1 (lanes 2–4), or no peptide (lanes 5–7) following one of three treatments: prior reduction and alkylation of S1-CpG (lanes 2 and 5), prior alkylation of S1-CpG with 1.25 mM iodoacetamide only (lanes 3 and 6), or no treatment of S1-CpG (lanes 1, 4, and 7). Mixtures were electrophoresed in a polyacrylamide gel (15% acrylamide/bis at 29:1) in TBE buffer.
To maximize peptide processing, presentation, and CpG adjuvant-mediated activation, the Ag and adjuvant components should be delivered to the same APCs by coordinated mechanisms. Cationic peptides and DNA are known to complex via a combination of electrostatic, hydrogen, and van der Waals bonds (41–44). In particular, the highly polar side chains of Arg and Lys enable these amino acid residues to engage in numerous hydrogen bonds with all four DNA bases (44). The function of positively charged residues in binding to DNA is exemplified by proteins such as transcription factors and capsid proteins that include binding domains that are characterized by relatively high representations of Arg and Lys amino acids. Inspection of the binding regions of several transcription factors from Drosophila and humans indicates that the percentage of positively charged residues (Arg and Lys) ranges from 14 to 35% within the DNA-binding regions. Therefore, it is not surprising that cationic peptides and polymers efficiently precipitate CpG oligonucleotides. These precipitations have been achieved with both poly-Arg polymers (17) as well as cationic tails on Trojan peptides with eight Arg/Lys residues derived from the HIV-1 tat protein (18), and the precipitations have been associated with increased in vivo priming.

We report here that three Arg and Lys residues in HY1 primotopes (and HY2) are capable of efficiently precipitating CpG oligonucleotides, but the inability of KASRNR-HY1 primotopes to prime female recipients indicates that precipitation of CpG and peptide is not sufficient for increased immunogenicity in our experimental system. The difference between these results and those obtained with cationic peptide tails may lie in the effects of the number/percentage of charges. Trojan peptides that include class I-binding epitopes fused with eight positively charged residues from the HIV-1 tat protein and seven charged residues from the Drosophila antennapedia protein exhibited increased transport with accompanying increases in CTL frequencies without the co-administration of CpG (8, 10, 45). However, the addition of synthetic oligonucleotides with either immunostimulatory or non-stimulatory CpG motifs could increase the efficiency of priming with class I-binding peptides fused to positively charged residues from the HIV-1 tat protein (18). The fact that increased priming was observed with either immunostimulatory or nonstimulatory CpG oligonucleotides (18) suggested that the increased priming efficiency was principally dependent on increased transport of the peptides across cell membranes. The contribution of the precipitated DNA may have been to reduce toxicity associated with increased peptide transport (18) to increase survival of peptide-pulsed APCs and subsequent activation of CTLs. Even though the KASRNR-HY1 peptide includes sufficient numbers of positively charged residues to precipitate CpG, it may have an insufficient number of positive charges to increase transport and subsequent peptide processing. Support for this proposal comes from the demonstration that more than four Arg residues are required for internalization of Trojan peptides (46).

The effects of substitutions of Ala residues into KCSRNR tails of the HY1 peptide on priming capacity clearly showed that the elimination of the single Cys residue, regardless of the presence or absence of Arg/Lys residues, eliminated priming of HY1-specific CTLs. This is the first demonstration that Cys residues in tails attached to class I-binding peptides can increase priming when they are combined with synthetic CpG adjuvant. We have observed that the addition of Cys-containing tails increases the priming capacities of multiple, additional peptides, including MiHag and melanoma-associated peptides when mixed with CpG oligonucleotides (data not shown). The involvement of internal nucleotidic phosphorothioates in synthetic oligonucleotides in the formation of disulfide bonds with peptides and/or proteins has not been observed with unbound S1-CpG were increased following the addition of glutathione. Collectively, these findings support the hypothesis that Cys-bearing peptides can form a sulfur-dependent complex with CpG oligonucleotides that carry phosphorothioate groups.

Discussion

Peptides that are presented by class I molecules have great potential for vaccines for infectious diseases and cancer due to their ability to focus CTL responses on peptides that are specifically expressed by infectious agents and tumor cells. Peptides and proteins in vaccines are taken up by professional APCs by fluid-phase macropinocytosis (36) and, in the case of fully processed peptides, by direct binding to cell surface MHC molecules. Neither of these processes is particularly efficient in providing peptides for sustained presentation, but the uptake of peptides can be increased by the addition of cationic tails from the HIV tat protein (18) and Drosophila antennapedia proteins for increased transport across plasma membranes (9, 37). Even if peptide/protein uptake by APCs were efficient, this uptake would not per se activate APCs to efficiently prime CTL responses.

Professional APCs can be activated by either cross-linking of costimulatory molecules, e.g., CD40 (38), or treatment with adjuvants that function by activation of members of the family of TLRs (reviewed in Ref. 12). Synthetic oligonucleotide analogues of bacterial CpG motifs have attracted considerable attention due to their capacity to drive Th1-like T cell responses and the simplicity of their synthesis. Such synthetic CpG oligonucleotides directly activate dendritic cells through TLR9 to increase production of Th1-associated cytokines and chemokines, expression of costimulatory molecules, and migratory function (39). Unlike peptides and proteins, CpG oligonucleotides appear to be taken up by receptor-mediated endocytosis (21, 40) with subsequent delivery to the endosomal/lysosomal compartment for binding to TLR9 molecules (40). Therefore, in vivo immunizations with peptides/proteins and CpG adjuvant as separate components involve their independent delivery to either the same or different APCs.

FIGURE 10. Complexes of S1-CpG and ACSANA-HY1 can be disrupted by competition for disulfide bonding. 32P-labeled S1-CpG was mixed with either AASANA-HY1 (lanes 1 and 3) or ACSANA-HY1 (lanes 2 and 4) and subjected to a mild reduction for 1 h with 5% 2-ME that was removed by evaporation under vacuum. These primotope-CpG mixtures were incubated overnight at room temperature with no additional treatment (lanes 1 and 2) or incubation with 1 mM mix of oxidized and reduced forms (1:1) of glutathione (lanes 3 and 4). Mixtures were electrophoresed in a polyacrylamide gel (15% acrylamide/bis at 29:1) in TBE buffer.

To a mixture of S1-CpG and ACSANA-HY1 inhibited the formation of this complex, presumably due to competition for disulfide bond formation (Fig. 10). Furthermore, the intensities of bands observed with unbound S1-CpG were increased following the addition of glutathione. Collectively, these findings support the hypothesis that Cys-bearing peptides can form a sulfur-dependent complex with CpG oligonucleotides that carry phosphorothioate groups.
described previously. It is well-known that disulfide bonds can form between Cys-bearing proteins and 5′-thiol groups of synthetic DNA (47–49). The formation of disulfide bonds between Cys residues and internal phosphorothioate groups would be expected to be strongly affected by steric hindrance and nucleophilic properties. Steric hindrance for internal thio groups may be relieved in sulfurized CpG because phosphorothioate groups perturb the DNA structure and lead to a widening of the minor groove in sulfurized DNA (50). In regard to nucleophilic properties, the sulfur atoms at internal positions should be more nucleophilic than the terminal sulfurs due to their respective electronic environments. Having more neighboring electron-releasing groups, e.g., alkyl groups, internal sulfurs have greater electronic density and should be more nucleophilic than their terminal counterparts and, therefore, more likely to form disulfide bonds (51).

The potential to form disulfide bonds between Cys residues and internal phosphorothioate groups in synthetic CpG is consistent with our observations that the addition of Cys-bearing primotopes to CpG oligonucleotides carrying single internal phosphorothioate groups results in the appearance of a new CpG band in electrophoretic separations. The presence of this band requires a Cys residue and can be blocked by reduction and alkylation of CpG oligonucleotides. The ability of Cys-bearing primotopes to complex with S1-CpG is apparently not limited to HY1 primotopes because we have recently documented complexing of S1-CpG with an additional MIIHAg primotope (data not shown). The most plausible explanation for these results is that disulfide bonds form between Cys residues in peptides and internal phosphorothioate groups in CpG oligonucleotides. Although the Cys-dependent band was minor in intensity compared with the other S1-CpG oligonucleotide bands, considerably more extensive complexing would be expected with fully sulfurized CpG oligonucleotides. There are many reasons that such bonds may have escaped previous detection by others: 1) disulfide reactions are readily reversible under slightly changing conditions of oxygen availability due to changes in temperature and air pressure, 2) peptides and DNA oligonucleotides have different requirements for optimal solubilization, and 3) there are multiple, possible disulfide-bonded complexes, including DNA-DNA, peptide-peptide, and DNA-peptide complexes that compete for CpG and peptide and complicate analyses.

The Cys-driven increase in immunogenicity is seemingly in conflict with results reported by other investigators. The recognition of melanoma-associated peptides carrying two Cys residues was significantly increased by Cys > Ala/Ser substitutions at one of the two positions (52). Subsequent studies with subdominant influenza peptides showed that cysteinylation of Cys residues and the involvement of these residues in dimerization resulted in the loss of recognition in in vitro assays and altered specificity of in vivo priming (53). We have indeed observed responses in the analysis of ACSANA-HY1 by reverse-phase HPLC that could be explained by dimerization (data not shown). Importantly, these responses were not observed when sulfurized CpG oligonucleotides were mixed with ACSANA-HY1, suggesting that binding to these oligonucleotides reduces dimerization. Therefore, the combination of Cys-bearing primotopes with synthetic CpG oligonucleotides for in vivo priming may mitigate the deleterious effects of peptide dimerization.

The apparent binding of peptides to internal phosphorothioate groups of synthetic CpG oligonucleotides by disulfide bonds may have profound effects on the mechanisms whereby these two components are delivered to APCs. The complexing of these two components by disulfide bonds should ensure that they are concomitantly taken up by the same cells for presentation of peptides by class I molecules and activation by CpG adjuvant. Evidence has been reported indicating that peptides are normally ingested by macrophagocytosis (36), whereas CpG oligonucleotides appear to be ingested via receptor-mediated endocytosis (21, 40). Experiments are in progress to determine whether the binding of primotopes with CpG by Cys residues alters the mechanisms of uptake of the peptides and oligonucleotides. Primotope and CpG components should be dissociable within APCs because disulfide bonds are normally reduced as a prerequisite for efficient proteolytic degrada-
dation of Ags (reviewed in Ref. 54). Enzymatic machinery is available for reduction of disulfide bonds to liberate the two components because specialized enzymes required for reduction of disulfide bonds are present in endosomes in different stages of maturation (54) and in lysosomes (55). Alternatively, the lowered pH in endosomes and lysosomes may be sufficient for liberation of the two components.

Effective vaccines should be expected to either generate persistent CTL memory or sustain priming over extended periods of time through deposition at injection sites. The results presented in Fig. 6 suggest that ACSANA-HY1 exhibits a greater capacity than KC SRNR-HY1 to prime CTLs in spleens and lymph nodes at early time points, i.e., 10–15 days. However, extending the time before CTL harvest results in equivalent priming with these two primotopes, with higher frequencies of HY1-specific CTLs in draining lymph nodes than in spleens. These results are consistent with the hypothesis that precipitation of CpG oligonucleotides by positively charged amino acids in primotopes has an inhibitory effect on the priming of initial CTL responses but extends the duration of priming through the stable deposition of CpG-primotope complexes at sites of injection. Testing of this hypothesis will require extension of the duration of priming beyond 50 days when ACSANA-HY1 and KCSRNKR-HY1 sustained comparable frequencies of HY1-specific CTLs, and these experiments are in progress.

The results reported in this communication document a previously unreported effect of Cys residues on interactions between peptides and CpG oligonucleotides that contain phosphorothioate linkages. Rather than exhibit inhibitory effects on recognition of class I-binding peptides as observed when these residues are included in the actual class I-binding peptide and not combined with CpG adjuvant (52, 53), inclusion of Cys residues in peptides carrying class I-binding peptides can result in augmented priming of CTLs in vivo when combined with synthetic CpG adjuvant with a phosphorothioate backbone. This novel interaction between peptides and synthetic CpG should provide the basis for new, alternative designs of immunogenic peptides to be used in combination with CpG adjuvants in vaccines for cancer and infectious diseases.

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**Disclosures**

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**References**


