Brief Articles

IFN-γ-Derived Lipopeptides: Influence of Lipid Modification on the Conformation and the Ability To Induce MHC Class II Expression on Murine and Human Cells

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Received February 19, 1999

Two truncated analogues of a previously identified lipopeptide agonist toward the IFN-γ receptor were synthesized in an attempt to determine the minimal compound able to induce expression of MHC class II molecules on murine and human cells and to study the role of the lipid tail. Circular dichroism studies were used to probe the induced conformationnal changes. Our results indicate at least a double role for the lipid modification that contributes to the stabilization of helical organization of the associated peptide and to its passive delivery into the cytoplasm. The persistence of biological activity in a truncated peptide of half of the residues present in the lead compound suggests that the lipid tail could also contribute to the stabilization of the peptide−receptor binding through additional hydrophobic interactions. This study allowed to readjust the minimal requirements for intracellular IFN-γ receptor stimulation. More generally, we suggest that lipidated analogues of functional peptides could be utilized for intracellular target validation in the drug discovery process.

Introduction

The need for therapeutic agents which act unequivocally on the outcome of the establishment of immune balance has led to investigations on the possibility of using cytokines as immunological adjuvants.[1] In this context, interferon-γ (IFN-γ) is perhaps more practical than other cytokines, in that the optimal time of administration is at the same time as the antigen.[2] IFN-γ is a pleiotropic cytokine that plays a central role in promoting innate and adaptative mechanisms of the host's defense. This cytokine is a potent activator of the immune system and is effective at enhancing memory for both cell-mediated immune responses and humoral immunity, when given mixed with the antigen in aqueous solution.[3] This cytokine has been shown to be effective in clinical trials in vaccination against hepatitis B.[4] Its mode of action appears to be via an enhancement of the antigen presentation efficiency, by increasing the expression of class I and class II molecules of the major histocompatibility complex (MHC I and MHC II) on the surface of antigen-presenting cells. However, dose-related side effects limit its effectiveness.

An interesting way of increasing the adjuvant effects of this cytokine would be to limit its delivery to the antigen-presenting cells that are actually presenting the antigen vaccine: this can be achieved through a physical linkage of cytokine to the antigen.[5] Another explored approach has been described recently, based upon the use of small molecule enhancers of IFN-γ activity, that might permit lower doses of IFN-γ to be administered while achieving an equal therapeutic effect.[6]

IFN-γ exerts its biological activity by interacting with its transmembrane receptor (IFN-γR), which is ubiquitously expressed on nearly all cells. Functionally active IFN-γR consists of two distinct subunits: an α-chain that confers binding specificity and a β-chain that participates in signal transduction. Cellular responses result from the ligand-induced coupling of the IFN-γR complex to particular components of the JAK-STAT pathway.[7,8] The initial interaction occurs through the N-terminal domain of the cytokine.[9] Surprisingly, a synthetic peptide derived from the C-terminal extremity of murine IFN-γ (95−133) has been identified as being able to bind the cytoplasmic part of the IFN-γR α-chain[10] and to activate the signal transduction pathway related to this receptor.[11] These findings are in support of previous reports indicating an intracellular action of IFN-γ.[12−15] Interestingly, this intracellular activation defies the well-known species specificity of exogenously applied IFN-γ.[16,17] This synthetic peptide derived from the C-terminal domain of murine IFN-γ was actually shown to be capable of stimulating the IFN-γR, but its activity required a relatively high concentration (0.1 mM) and was limited to pinocytic and phagocytic cells.[18] Helical organization of the peptide and the presence of the polycationic sequence RKRKR are required for efficient binding to the cytoplasmic domain of the receptor and induction of biological activity.[19]

Recently, we have demonstrated[20,21] that lipid-modified peptides were able to reach and modulate the ac-

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10.1021/jm991025f CCC: $18.00
Published on Web 08/21/1999

Table 1. Physicochemical Characterization of the Studied Lipopeptides[a]

peptide	molecular mass		*k′*	
	calculated	$[M + H]^+$ found	C3	C18
mIFN-γ 95−132 Ac-AKFEVNNPQVQRQAFNELIRVVHQLLPESSLRKRKRSR-NH_2	4614	4613	20.81	9.46
L-mIFN-γ 95−132 Ac-K(Pam)AKFEVNNPQVQRQAFNELIRVVHQLLPESSLRKRKRSR-NH_2	4981	4983	26.17	12.68
L-mIFN-γ 113−132 Ac-K(Pam)IRVVHQLLPESSLRKRKRSR-NH_2	2824	2825	21.57	10.88
L- mIFN-γ 122−132 Ac-K(Pam)ESSLRKRKRSR-NH_2	1768	1769	19.85	10.53
SL-mIFN-γ 95−132 Ac-K(Pam)PSRENQNAVKIQKLSVVLRREQKHRVERLAFRNQSLPF-NH_2	4981	4979	22.59	11.24

[a] Molecular masses of the peptides were determined by plasma desorption time-of-flight mass spectrometry. Peptides were analyzed by reversed-phase HPLC in two different systems using either a Vydac C18 (0.01−5 μm) (250 × 4.6 mm) column or a Zorbax C3 (0.03−5 μm) (150 × 4.6 mm) column, eluted at 50 °C with a flow rate of 1 mL/min. Solvent composition: A = 0.05% TFA in H_2O, B = 0.05% TFA in H_2O/acetonitrile (20:80); monitoring at 215 nm. A shallow, 0−100% B over 60 min linear gradient was used. *k′* C3 and *k′* C18 are the capacity factors measured in both systems.

tivity of cytoplasmic pharmacological targets in intact nonphagocytic cells, whereas their nonlipid counterparts could not cross the cytoplasmic membrane and were not active. We have synthesized a construct (termed L-mIFN-γ 95−132), resulting from the coupling of a lipid moiety (palmitoyl-lysine residue) to the C-terminal sequence 95−132 of the murine IFN-γ. This lipopeptide was, to our knowledge, the first synthetic compound able to reproduce the activities of the recombinant IFN-γ, on both human and mouse cells regardless of their pinocytic behavior.[22]

The palmitoyl group may indirectly contribute to the biological activity of its associated functional cargo by different mechanisms: a transmembrane delivery of the peptide, a positive influence upon the helical organization, as recently described for epitopic[23] and mastoparan-derived peptides,[24]and/or a possible stabilization of the peptide−receptor or peptide−membrane associations throught van der Waals interactions.

We hypothesized that the relatively large size of this first compound (5000 Da) could limit the efficiency of the transmembrane delivery and decided to explore the possibility of reducing the length of the lipopeptide sequence while preserving its ability to induce expression of MHC class II molecules on murine and human cells. In this report, we describe new IFN-γ-derived lipopeptides, synthesized in order to investigate the role of the lipid chain and to determine the minimal active sequence, with reference to the first identified IFN-γR agonist (L-mIFN-γ 95−132), and to its derived scrambled lipopeptide (termed SL-mIFN-γ 95−132).

Results and Discussion

All the peptides shown in Table 1 were obtained by automated solid-phase synthesis and easily purified by reversed-phase HPLC. A rhodamyl-labeled analogue of the L-mIFN-γ 95−132 was also synthesized in order that its intracellular distribution in murine spleen cells might be studied. Following a 10-min incubation period, this fluorescent lipopeptide probe was found intracellularly, in the proximity of the inner surface of the cell membrane (Figure 1A), following the observed localization of clusters formed by its target receptor (IFN-γR α-chain) (Figure 1B), as confirmed by colocalization experiments (Figure 1C). The cluster-associated immunostaining of the IFN-γR α-chain was also observed on cells nonstimulated with the IFN-γ-derived lipopeptides

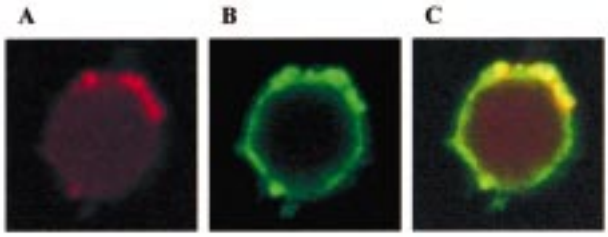

Figure 1. Colocalization of L-mIFN-γ 95−132 with the cytoplasmic domain of the IFN-γR α-chain. Spleen cells were incubated for 10 min with the rhodamine-labeled lipopeptide L-mIFN-γ 95−132 (A). The cytoplasmic domain of the IFN-γR α-chain was detected by indirect immunostainning and was visualized by fluorescein (B). Double exposure (C) (yellow) shows the overlapping fluorescence associated with the lipopeptide L-mIFN-γ 95−132 and the IFN-γR α-chain.

(not shown) and may represent spots of higher density of IFN-γR α-chain expression.

The mechanism involved in the transmembrane transfer was presumably passive, as it could be observed after incubation of the cells at 4 °C, and fast enough to avoid the complete degradation of the peptide by exopeptidases in the culture medium. This observation corroborated our previous reports,[22] where a species nonspecific activation of human cells upon stimulation with the murine-derived L-mIFN-γ 95−132 was described, suggesting the contribution of an intracellular stimulation of the IFN-γR.

We hypothesized that the relatively important size of this first peptide could be a limiting factor for its efficient intracellular delivery and hence for its biological activity. We thus defined shorter lipopeptides with reference to the structural requirements for agonist activity of the peptide: helical organization and the polycationic tail have been described as essential for binding.[19] The 108−132 peptide was previously described as the shortest peptide able to bind to the cytoplasmic domain of IFN-γR.[19] Its ability to induce MHC II expression upon murine cells was reduced by a factor of 2 when compared to the 95−133 peptide.[19]

The crystal structure of the homologous human cytokine[25] shows in this part of the molecule the presence of five turns of helix F, corresponding to 18 out of the 38 residues (47%) of the biologically active C-terminal peptide. To get clear-cut responses to our question, we decided to amputate large parts of our lead compound:

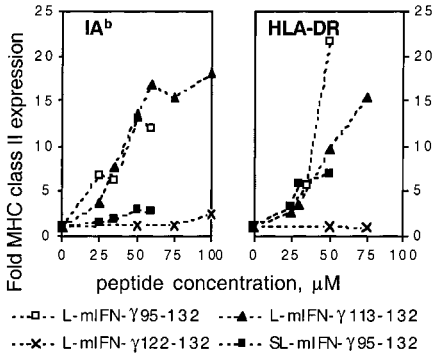

Figure 2. Induction of MHC II molecules on murine and human cells stimulated with IFN-γ-derived lipopeptides. Murine spleen cells (A) and human cell line COLO 205 (B) were incubated for 24 h with different concentrations of IFN-γ-derived lipopeptides. Cells were stained with monoclonal antibody against MHC II molecules and analyzed by flow cytometry. The fold corresponds to the ratio of the mean fluorescence intensity of the cells treated by lipopeptides divided by the mean fluorescence of untreated cells. Data shown are the results of one representative experiment from three independent ones performed.

the first 19 residues, including 3 out of the 5 turns of helix F were omitted in the L-mIFN-γ 113−132 construct, while only the 11 C-terminal residues were present in L-mIFN-γ 122−132. The cysteine found in position 133 (C-terminal extremity) of the cytokine was omitted in all lipopeptides in order to avoid their disulfide dimerization formation. A carboxamide group was introduced in the C-terminal extremity to re-inforce the stability toward carboxypeptidases.

The different lipopeptides were compared on the basis of their ability to induce MHC class II molecule expression upon murine spleen cells or the human COLO 205 cell line, incubated for 24 h with different concentrations of lipopeptides (the nonlipidic mIFN-γ 95−132 peptide is inactive under our experimental conditions, data not shown) (Figure 2). A clear dose-dependent increase of MHC class II expression was observed for both cell types upon stimulation with either L-mIFN-γ 95−132 or L-mIFN-γ 113−132, whereas neither L-mIFN-γ 122−132 nor SL-mIFN-γ 95−132 proved active. The weak induction of MHC class II molecule expression observed upon cells stimulated with the scrambled control lipopeptide (SL-mIFN-γ 95−132) can be considered as background for unspecific induction, as previously described.[22] The results presented in Figure 2 reveal that the biological activity of the truncated lipopeptide L-mIFN-γ 113−132 was equivalent to that of the longer analogue L-mIFN-γ 93−132 on spleen cells freshly recovered from mice, with a 13-fold increase of MHC class II expression upon cells stimulated with a 50 μM concentration of either lipopeptide (140 or 230 μg/mL, respectively). L-mIFN-γ 113−132 was less active than L-mIFN-γ 93−132 on human cells, with a 10-fold increase of HLA-DR expression versus 22-fold at 50 μM concentration. However, in contrast to the longer lipopeptide analogue, high concentrations of the truncated lipopeptide (L-mIFN-γ 113−132) were not cytotoxic: a 75 μM (210 μg/mL) concentration of this peptide induced a 15-fold expression of HLA-DR on human cell lines, while a concentration of 100 μM (280 μg/mL) increased the expression of IA^b on the murine cells by a factor of 18.

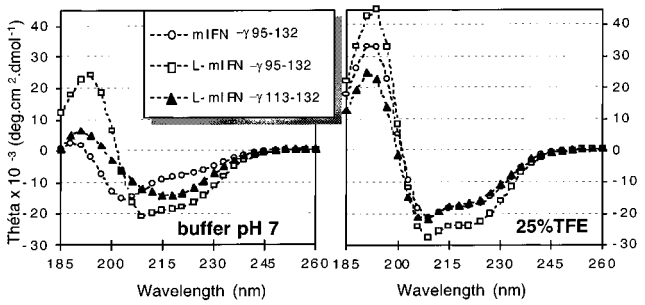

Figure 3. CD spectra of mIFN-γ 95−132, L-mIFN-γ 113−132, and L-mIFN-γ 95−132. Temperature: 298 K. Peptide concentration: 20 μM in 2 mM phosphate buffer, pH 7, without TFE (left panel) or with 25% TFE (right panel).

To further characterize the influence of lipid modification, circular dichroism studies of the peptide and lipopeptide were used to probe the conformational changes induced in the peptide by the lipidic tail. As previously observed by others,[19] the CD spectra of mIFN-γ 95−132 suggested that a low-populated state (22%) was present in aqueous buffer, reaching 53% in the presence of 25% trifluoroethanol. The spectrum of the lipopeptide L-mIFN-γ 95−132 in water (shown in Figure 3) was superposable to the spectrum of the nonlipidic peptide mIFN-γ 95−132 in 25% trifluoroethanol.

The CD spectra of truncated lipopeptide L-mIFN-γ 113−132 in buffer or in the presence of 25% trifluoroethanol are also presented in Figure 3: the low-populated helical or β-sheet populations observed in buffer changed to approximately 50−60% helical conformation upon addition of 25% trifluoroethanol. The scrambled lipopeptide SL-mIFN-γ 95−132 also adopted a partial helical conformation (17% in water, 60% in 25% TFE, not shown): this observation confirmed that the biological activity relies upon the helical conformation and specific sequence-dependent interactions.

The surprising ability of such a relatively large water-soluble compound to passively cross the cell transmembrane could be related to their spontaneous tendency to adopt α-helical organization in water, confirming the observations published by others.[23,24] Recently, Laczko et al.[23] have reported the positive influence of the palmitoylation of an epitopic peptide on its helical organization. Niidome et al.[24] have observed membrane insertion properties of peptides modified by a long acyl chain, resulting in conformational changes following interaction with liposomes, with enrichment of the population of folded conformers. If liposome−lipopeptide interaction can be considered as a model of cell−lipopeptide interaction, then one would predict a similar insertion of lipopeptides upon the cell surface, followed in our case by a rapid translocation of the functional cargo sequence into the cells and subsequent stimulation of its target receptor.

Our results indicate at least a double role for lipid modification, which contributes to the stabilization of helical organization of the associated peptide (even in the case of a short peptide devoid of helical propensity) and to its delivery into the cytoplasm. Since the shortest peptide previously reported as being able to bind IFN-γR and displaying weak biological activity was the 108−132,[19] the persistence of biological activity in a lipopeptide 5 residues shorter suggests another role

for the lipid tail, which might be to contribute to the stabilization of the peptide−receptor binding through additional hydrophobic interactions with the receptor itself or its hydrophobic environment, as the cytoplasmic binding site for IFN-γ has been localized within the membrane-proximal portion of the cytoplasmic domain.[10,18]

Conclusion

This report illustrates the apparent ability of functional peptides to recognize selectively the biologically relevant sites of protein targets, a property which is the basis for the development of large peptide libraries as sources for the identification of ligands for highly diverse targets. In this study, the introduction of a lipid tail has dramatically improved the biological activity of the previously discovered peptide, suggesting a possible direct use of such lipopeptides as immunomodulators. Indeed, we have previously experienced the efficiency of physical associations between 6- and 8-lipopeptide antigens prepared as mixed micelles,[26] allowing copresentation of the distinct constituents of the experimental vaccine by antigen-presenting cells. Such a formulation could be easily adapted to the incorporation of lipopeptidic immunomodulators into a vaccinal preparation: their biologically active concentration (few hundreds μg/mL) and water solubility (above 5 mg/mL) are compatible with clinically acceptable volumes of injection. Another advantage of the synthetic lipopeptide over the recombinant cytokine is their stability upon storage and their compatibility with cold-chain disruption.

The possibility of intracytoplasmic delivery of biologically active peptides could permit a refinement of their structure−activity studies in a context that is probably more relevant that the reducing biochemical assays: our observations allowed a readjustment of the minimum requirements for IFN-γR stimulation to the 113−132 sequence being extended by acylation with a long hydrophobic chain. More generally, we suggest that lipidated analogues of biologically active peptides could be utilized for target validation in the drug discovery process.

Experimental Section

Peptide Synthesis and Characterization. The peptide and lipopeptides were elaborated on a Rink amide resin (Senn Chemicals AG, Dielsdorf, CH) using the Fmoc-tBu strategy[27,28] and activation with 0.45 M *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethaminium hexafluorophosphate *N*-oxide (HBTU)−hydroxybenzotriazole (HOBt) in *N*-methylpyrrolidinone (NMP). Systematic double coupling was performed using 4 equiv of protected amino acids, followed by a systematic acetylation step using acetic anhydride/diisopropylethylamine in NMP in the presence of 0.5 M HOBt, in an Applied Biosystems 430A peptide synthesizer (Foster City, CA). A Fmoc-Lys(pam)-OH (Bachem, Bubendorf, CH) was incorporated into the N-terminal extremity to obtain the lipopeptides. The peptide and lipopeptides were deprotected and cleaved from the resin by a treatment with trifluoroacetic acid (TFA) in the presence of phenol/ethanedithiol/thioanisole/H_2O (0.75 g:250 μL:250 μL:500 μL). The peptides were isolated from the TFA solution by precipitation in diethyl ether and were then lyophilized. Purifications were performed by several parallel runs of reversed-phase HPLC on a 12.5-mm × 250-mm column filled with Nucleosil C18 (300 Å, 5 μm) as stationary phase, using an acetonitrile/H_2O/0.05% TFA solvent system. Homogeneity was confirmed in two different reversed-phase HPLC systems: all peptides and lipopeptides were more than 90% pure. Identity was confirmed by determination of amino acid composition following total acid hydrolysis and molecular mass determination by time-of-flight plasma desorption mass spectrometry (TOF-PDMS) (Bio-Ion 20 plasma desorption mass spectrometer, Uppsala, Sweden). All compounds were water-soluble.

The antepenultimate lysine 94 was introduced with a 4-methyltrityl (Mtt) protecting group for the synthesis of the fluorescent analogue of the L-mIFN-γ 95−132, final step being the acetylation of the N-terminal palmitoyl-lysine. After selective deprotection of the Mtt group by 1% TFA in dichloromethane, an aliquot of resin was cleaved as above; the quality of the synthesis was evaluated by analysis of the resulting crude lipopeptide: this analysis verified that the majority of contaminants were intermediates capped before introduction of the Ac-K(Pam)-AK terminal sequence. Introduction of 5(6)-carboxytetramethylrhodamine was achieved on the remaining resin by HBTU/HOBt activation. Following final TFA deprotection and cleavage as above, the rhodamyl-labeled lipopeptide L-mIFN-γ 95−132 was precipitated by diethyl oxide from the TFA scavengers solution and used without further purification.

Circular Dichroism Studies. Circular dichroism measurements of peptides were determined at 25 °C using a temperature-controlled Jobin-Yvon CD-6. Scans were conducted with a 0.1-cm path length cell with a 5-s averaging time. The wavelengths measured ranged from 185 to 260 nm with a scan step rate of 0.5 nm/step. Scans were conducted upon peptides at neutral pH in 2 mM phosphate buffer with or without the helix-stabilizing reagent trifluoroethanol (TFE). The peptide concentration was adjusted to 20 μM following determination of the exact concentration of an approximate 100 μM stock solution by quantitative amino acid analysis. The mean values of four scans were expressed as mean molar ellipticity per residue (deg·cm^2·dmol).

Cell Culture and Stimulation. Spleen cells were freshly recovered from 7-week-old female 129 Sv mice (provided by Dr. Zeiner, CDTA, Orléans, France). COLO 205 human colon carcinoma lines were purchased from the American Type Culture Collection (ATCC). Both spleen cells and COLO 205 were maintained in RPMI 1640 (Gibco BRL, Courbevoie, France) supplemented with 10% FCS (Gibco BRL), 5 mM sodium pyruvate (Sigma, St. Louis, MO) and incubated at 37 °C, 5% CO_2. Cells were stimulated for 24 h with different concentrations of either L-mIFN-γ 95−132, L-mIFN-γ 113−132, L-mIFN-γ 122−132, or SL-mIFN-γ 95−132. Murine spleen cells and COLO 205 were stained for MHC II expression, with 1 μg of FITC anti-mouse IA^b mAb (Pharmingen, San Diego, CA) or 10 μL of FITC mouse anti-HLA-DR mAb clone TAL.1B5 (Cymbus Biotechnology Ltd., Hants, U.K.), respectively. As isotype control we used a FITC mouse IgG1 negative control (DAKO S.A. trappes, France). Cells were incubated for 1 h at 4 °C in PBS, 10% FCS and then washed, and MHC II expression was analyzed by flow cytometry on a Coulter EPICS II cytometer (Coulter, Hialeah, FL) at 10000 events/sample.

Immunostaining and Fluorescence Microscopy. The intracellular delivery of lipopeptides was performed with 10^6 spleen cells freshly recovered from mice and incubated for 10 min at 37 or 4 °C with 1 μM rhodamine-labeled lipopeptide L-mIFN-γ 95−132. Cells were washed twice with cold PBS and fixed with 4% paraformaldehyde in PBS for 15 min at 4 °C. Fixed cells were permeabilized with 0.05% NP-40, 1% BSA in PBS for 10 min at 4 °C, and unspecific sites were blocked with 2% BSA in PBS. The cells were incubated with rabbit IgG directed against the cytoplasmic domain of IFN-γR α-chain (1:100 dilution) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). As secondary antibody sheep anti-rabbit IgG was used coupled to fluorescein (Santa Cruz Biotechnology, Inc.) at a 1:200 dilution. The cells were then washed four times with PBS, mounted on glass coverslips in Vectashield, and photographed in a Leica laser scanning confocal microscope.

Acknowledgment. This work was supported by grants from the Centre National de la Recherche Scientifique and Institut Pasteur de Lille. Kader Thiam was supported by the Ministère de l'Enseignement Supérieur et de la Recherche and Estelle Loing by the Agence Nationale de Recherche sur le SIDA. The skillful technical assistance of Anne Delanoye, Cédric Cheminay, Eric Diesis, and Marc Bossus is gratefully acknowledged. We thank also Dr. Steve Brooks for proofreading the manuscript and Hervé Drobecq for electrospray-mass spectrometry analysis.

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JM991025F