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Invited Review

The Interaction of HIV-1 with the Host Factors

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CONTENTS:

SUMMARY: Human immunodeficiency virus type 1 (HIV-1) is a causative agent of acquired immunodeficiency syndrome (AIDS) in humans. In the last decade, the functions of HIV-1-encoded genes have been intensively studied. These studies have contributed to the development of the effective anti-AIDS drugs directing against the HIV-1-encoded enzymes, namely reverse transcriptase and protease. However, even the combination of these drugs is not sufficient enough to stop the progression of AIDS partly due to the emergence of drug-resistant HIV-1 mutants as well as the severe side effects. Understanding the molecular mechanisms by which cellular factors support the efficient replication of HIV-1 should contribute to develop means to control the progression of AIDS. This field is now expanding rapidly. Here we review the host factors involved in the replication of HIV-1 and highlight some findings that have a substantial impact on the retroviral research.

1. Introduction

After the discovery of human immunodeficiency virus type 1 (HIV-1) as the causative pathogen of acquired immunodeficiency syndrome (AIDS) in the early 1980s, thanks to the current molecular biological and virologic techniques, the development of the laboratory diagnosis, the anti-AIDS drugs, and vaccine have progressed in a substantial speed. However, the development of the effective therapeutic/preventive vaccine has been still on the struggle. Despite our efforts, the number of AIDS patients is increasing, which raises a worldwide socio-economic concern.

The hunting for the cellular factor that regulates the HIV-1 replication started soon after the characterization of the virus as summarized in Fig. 1. The molecular interaction between the host and HIV-1 is the key to understand the pathogenesis of HIV and to develop means to control HIV-1 replication. However, we still do not have the perfect picture of the precise molecular mechanisms of the life cycle of HIV-1, even it has a small genome of approximately 10 kbp encoding less than a dozen genes.

The field of the interaction between HIV-1 with host factors is currently expanding enormously (Table 1). In this review, we cast a light on some of the recently found host factors that directly or indirectly interact with HIV-1 to influence its replication. These factors called the attention of the general life scientists because they have deciphered the complex nature of the replication of HIV-1 as well as opened up the new research fields. To easy-to-update, it is helpful to visit the web resource NIAID HIV Protein Interaction Project that shows the comprehensive list of the cellular proteins which interact with HIV-1 (online; available from http://www.ncbi.nlm.nih.gov/RefSeq/HIVInteractions/index.html). We recommend to refer the textbooks and reviews for the further reading (1-4).

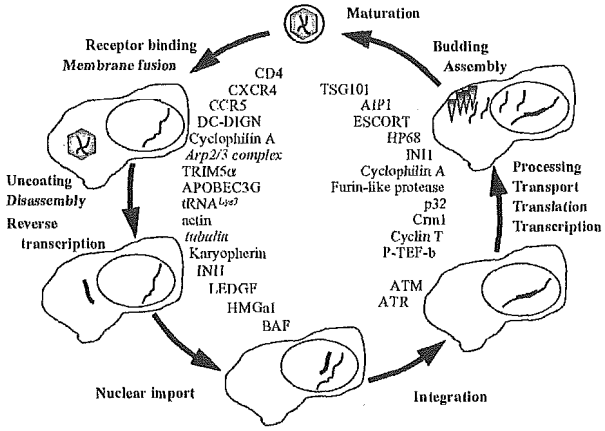

Fig. 1. The schematic representation of the replication cycle of HIV-1. Each step of the viral life cycle is indicated out of the replication circle in bold. Inside the circle, the major cellular factors involved in each step are shown. Factors affecting the HIV-1 replication via an indirect fashion are indicated in italic.

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Table 1. The major interaction between viral gene products and cellular factors

Viral protein	Cellular protein
Gag	
MA	Karyopherins
	Histidyl-tRNA synthetase-like (HO3)
	Calmodulin
	VAN/NAF1
CA	Cyclophilin A
	TRIM5α
NC	HP68/RNase L inhibitor
	Actin
p6	TSG101
	AIP1
	Nedd4
	Ubiquitin
Pol	
IN	INI1/hSNF5
	LEDGF/p75
	ATR
	ATM
	Karyopherins
	BAF
	XRCC5 (Ku autoantigen)
Env	CD4
	CXCR4
	CCR5
	DC-SIGN
Nef	PACS-1
	ASK1
	PAK
	PI3-kinase
	lck
	VAN/NAF1
Rev	Crm1
	p32
Tat	Cyclin T1
Vpr	Karyopherins
	Uracil-DNA glycosylase
	Wee1
Vif	APOBEC3G

2. Cellular entry molecules

Until recently, the cellular entry molecules for HIV-1 were known to be the T cell surface marker CD4, one of the immunoglobulin superfamily, and the chemokine receptor CXCR4 or CCR5. More recently, a new member jointed, a dendritic cell-specific ICAM-3 DC-SIGN (5,6). Not only through a cell free virus-mediated infection, HIV-1 propagates in vivo through a cell-to-cell transmission namely between the dendritic cell (DC) and CD4-positive T cell (7). HIV-1 seems to be internalized into the DC through interacting with DC-SIGN. The DC "holds" the infectious viruses in the intracellular compartment stably and, upon in contact with T cells, "passes" the viruses to T cells so that T cells are effectively infected with HIV-1. The viral transmission from DCs to T cells may partly explain the efficient and rapid establishment of HIV-1 infection upon the viral exposure. Many vaccine developers believe it unlikely that the neutralizing antibody against HIV-1 is able to perfectly protect individuals from the HIV-1 infection partly because the HIV-1's quick hide in DCs where the antibodies can not reach the target. Recently, the site of cell-to-cell contact, or the immunological synapse (even called the virological synapse), was visualized by the high resolution laser scanning microscopy. The detailed analysis revealed that the site of cell contact provides a unique environment where the viral receptors cluster such that the viral transmission takes place at a high efficiency (8,9). HIV-1 seems to take advantage of the immunological synapse formation between the DC and the T cell, which is crucial to maintain the immunological integrity. These findings further point the substantial significance of macrophages and DCs in the pathogenesis of AIDS (reviewed in [10]).

3. From the epidemiologic study

The epidemiologic observations disclosed the presence of the exposed-uninfected individuals or HIV-1-infected long-term non-progressors or slow progressors. The genetic analysis revealed that a certain genetic background seemed to confer these phenotypes (11). Through the survey of the genetic determinant that confers the resistance to the HIV-1 infection, a gene variant of CCR5 was found (12). The CCR5Δ32 was a truncated form of CCR5 that does not traffic to the cell surface, therefore does not serve as the viral receptor. The homozygote of CCR5Δ32 displays a strong resistance to HIV-1 suggesting that the functions of CCR5 are dispensable for the human well-being and, therefore, the anti-CCR5 drug is quite feasible. The presence of the HIV-1 infection-resistant individuals that are positive for the auto-antibody against CCR5 supports this idea.

The V64I polymorphism of CCR2 expresses the destabilized CCR2A isoform. It also protects cells from HIV-1 infection by interacting with and therefore downregulating expression of CCR5 at the cell surface (12,13). The V64I CCR2 variant is one of the good examples that affects the life cycle of HIV-1 negatively without binding to HIV-1 gene products directly. In contrast to the CCR5Δ32, the frequency of V64I CCR2 allele in Asian population is higher than Caucasians. The cohort study also revealed that the HLA A2/A28 confers the resistance to HIV-1 partly because of the efficient presentation of HIV-1 peptides on the HLA molecule to potentiate the immune response towards HIV-1 infected cells (14). On the other hand, there are some genetic backgrounds that promote the AIDS disease progression such as CCR5P1 (15). On the age of the highly active anti-retroviral therapy (HAART), it is quite helpful to conduct similar genetic analyses to provide data on the genetic variants that influence the effectiveness of anti-AIDS drugs (e.g., cytochrome P450 and P-glycoprotein). These clinico-genetic studies are very powerful, yet, need to be evaluated by independent cohort studies or laboratory studies (reviewed in [16]).

4. Restriction factors

Certain cell lines are hardly infected with HIV-1 even the receptors were introduced into them. This is the restriction of viral infection in the broad sense. The restriction occurs at any levels of the viral life cycle. In the field of retrovirology, the inhibition of viral entry is historically termed as the restriction of viral infection in the narrow sense (reviewed in [17,18]). It suggests either the presence of the factor that block the viral replication or the absence of the one that supports the viral replication.

Tat, an essential viral transcriptional activator from the viral promoter LTR, binds to the TAR element of the viral RNA and recruits the basal transcriptional machinery close to the transcription start site in order to enhance the transcription of viral RNA from LTR. The viral RNA undergoes the splicing to become mRNA for the most of the genes. However, the viral intact RNA genome has to be present in the cytoplasm to assemble a replication-competent progeny virus. HIV-1 encodes *rev* that is also essential for the viral replication, which transports the unspliced viral RNA from the nucleus to the cytoplasm by binding to the *rev*-responsive element (RRE) on the viral RNA as well as RRE-baring singly-spliced transcripts. These systems work only when a help from the host cell is provided. The murine cells do not support HIV-1 replication because both the murine cyclin T1 and Crm1 that are unable to support *Tat* and *Rev*, respectively, to function (19,20). Surprisingly, the murine cyclin T1 differs from human's by a single amino acid. More recently, another mechanism that inhibits HIV-1 replication in the murine cells is reported in which a member of the murine spliceosome complex p32, different from the human's by a single amino acid similar to cyclin T1, is shown to be unable to support the function of *Rev* (21). However, being these factors provided, HIV-1 does not replicate fully in the transgenic mouse, suggesting that there are other points of restriction in mouse cells.

Some primate cells (i.e., cells from the rhesus macaque) are known to be resistant to the HIV-1 infection. The protection occurs mostly at the level of reverse transcription. Recently, a cellular protein TRIM5α was identified as the responsible factor for this restriction (22). The human counterpart of TRIM5 α does not have the restriction activity towards HIV-1. The amino acid homology between human and monkey TRIM5 α was approximately 90%, not as high between rat and human p32. Similarly, cyclophilin A is able to modulate the cellular sensitivity toward being infected with HIV-1, suggesting that the cyclophilin A is also involved in the restriction of retroviral infection (23). Not only in the entry process, cyclophilin A also plays a role in the late phase of HIV-1's life cycle, namely the assembly.

On the virus side, the capsid (CA) is the major determinant of the susceptibility to these restriction factors although direct interaction between TRIM5α and CA is not evidenced. The molecular mechanism by which these factors inhibit the reverse transcription through interacting with CA remains largely unclear. Characterizing the restriction factors and the mechanism of their actions holds the key to understand the uncoating/disassembly processes. The inter-species variation of these factors may shed lights on the theory behind the co-evolution of retrovirus and the host. In addition, it will give us some clue to build a small animal model of AIDS.

5. Binding partners

The hunting for the cellular protein binding to the viral gene products associates with the historically-known peculiar behaviors of HIV-1. The p6 domain within *gag* is called the late domain because the lack of p6 leads to the failure of the late step of viral life cycle – budding (Fig. 2, reviewed in [24]). A p6 binding protein, TSG-101, was found and shown to play a critical role in the budding of HIV-1 (25,26). The TSG-101 recognizes the PTAP motif within the p6 and recruits the pinch-off machinery endosome-associated complex required for transport, ESCORT, to the site of budding.

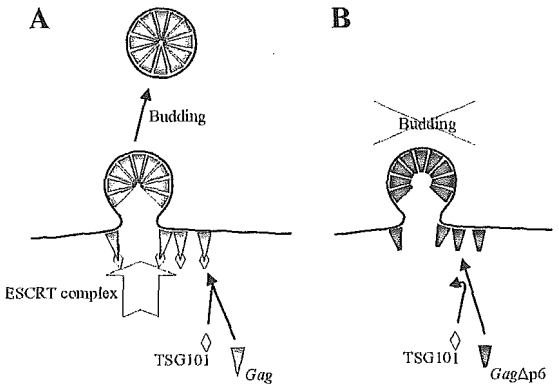

Fig. 2. The role of TSG101 in the viral budding. (A) The TSG101 (diamond) binds to the PTAP motif within the p6 domain of *Gag* (triangle) and recruits ESCRT complex (big arrow) to drive the budding process. (B) The *Gag* lacking the late domain is able to assemble at the cell surface. However, the viral particle attaches to the cell surface via a stalk-like structure and hardly buds. Expressing the wild-type *Gag* in conjunction with the downregulation of TSG101 by the siRNA technology or the expression of the dominant-negative TSG101 displays the similar phenotype.

The HIV-1 lacking the functional late domain shows the dramatic decrease in the viral production. The defective virus accumulates at the cell surface but seems unable to pinch-off. The isolation of TSG-101 as the p6 binder beautifully deciphers how the late domain works. Recent data further demonstrated that the multifunctional protein AIP1/ALIX also binds to p6 and plays a similar role to TSG101, recruiting the ESCORTIII complex at the cell surface to promote the pinch-off process (27,28). The ESCORT complex has been identified as the vesicular sorting machinery. The endosomal vesicle buds and fuses to the membrane organelles like the enveloped virus buds and infects cells. Since many retroviruses share the similar motifs in the late domain of *gag*, the involvement of the vesicular sorting pathway in the *Gag* trafficking and budding of retroviruses shall be emphasized (reviewed in [29]).

The *Vif*, a viral accessory protein, was long known to be required for virus to replicate in certain cells. The *vif*-deficient virus has a poor infectivity when the virus is produced by a "non-permissive" cell. The *Vif*-binding factor apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) now explains how it happens (Fig. 3, reviewed in [30]). APOBEC3G was originally identified as CEM15 that was able to convert the "permissive" cells to "non-permissive" (31). APOBEC3G has an ability to deaminate the C to yield the U on the minus-strand reverse-transcribed viral genome. It ends up with the G-to-A conversion on the plus-strand of the genome. The G-to-A hypermutation damages the genomic integrity and subsequently the virus may fail to replicate. Also it may lead to the degradation of the genome by unknown mechanisms in the infected cells. The *Vif* seems to protect the viral genome by binding to APOBEC3G to induce the proteasome-dependent degradation and, therefore, blocking it from being incorporated into the viral particles in the virus producing cells. This revealed another class of the innate immunity that also works on the hepadna virus (e.g., hepatitis B virus) in which the replication depends on the reverse transcription (32).

The lentivirus can infect non-dividing cells whereas the oncoretrovirus cannot. It involves the active transport of

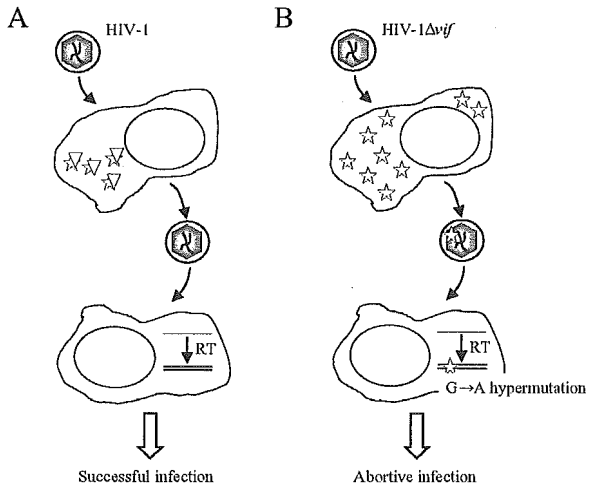

Fig. 3. The *Vif*-APOBEC3G interaction. (A) The *Vif* (triangle) binds to APOBEC3G (star) and induces the degradation of APOBEC3G that preventing APOBEC3G from being incorporated into the viral particles. (B) The *vif*-deficient virus does not block the APOBEC3G incorporation into the viral particles, which allows the C-to-U conversion on the minus-strand cDNA synthesis leading to the G-to-A hypermutation upon the plus-strand DNA synthesis by the viral reverse transcriptase. This results in the abortive infection. However, the *vif*-deficient virus is able to replicate in "permissive cells" lacking the APOBEC3G.

the genetic material from the cytoplasm to the nucleus. Viral proteins including matrix (MA), *Vpr*, and integrase play the major role in the active nuclear import of the preintegration complex (PIC). Among the binding partners to these viral proteins, the integrase interactor 1 INI1/hSNF5, a component of SNF-SWI complex, calls attention lately. INI1 was originally found by the yeast two-hybrid system (33) and was shown to facilitate the nuclear import of the PIC as well as the integration reaction *per se*. Not only the early phase of the viral life cycle, the INI1's binding to the integrase is shown to be required for late events in the viral life cycle via yet unknown mechanisms (34). It is also intriguing that a single host factor plays multiple roles in the life cycle of HIV-1. Such a protein should be the premier target for the anti-AIDS drug discovery. The human lens epithelium-derived growth factor/transcription co-activator p75 (LEDGF/p75) also binds to integrase and contributes to the nuclear import of the PIC (35). The fact that INI1 and LEDGF specifically interact with lentiviral integrases, not with oncoretroviral integrases, suggests their functional significance in the lentiviral active nuclear transport (36,37).

6. Other cellular factors

A cellular microenvironment seems to play a role in the life cycle of HIV-1. The detergent-insoluble membrane fraction, so called the lipid raft, is a membrane microdomain rich in cholesterol. The efficient budding of HIV-1 seems to depend on the lipid raft because the removal of cholesterol resulted in the marked reduction of viral production (38). The myristoylated proteins such as *Gag* preferentially accumulate at the lipid raft. This may help the viral assembly and budding at the lipid raft. Indeed, the lipid content of the viral envelope is reported to be similar to that of the lipid raft. Also, the lipid raft may play a role in the viral infection process. The CD4 molecule tends to localize on the lipid raft. This may provide a microenvironment where the multiple virus-cell interaction takes place rapidly when HIV-1 encounters the target cell. It is interesting if a specific membrane lipid constituent can play an active role in the life cycle of HIV-1.

Not only the cellular proteins directly bind to HIV-1-encoded proteins, proteins incorporated into the viral particles potentially play roles in the HIV-1 replication (Table 2, reviewed in [39]). The cyclophilin A is one of the examples that was originally isolated as a *Gag* binder but later found in the virion. Some of the described factors (e.g., TSG101 and INI1) are also found in the purified viral preparations, which strengthen the idea that they are indeed the binders to the viral proteins. It is however difficult to determine the specificity and the functional significance because many of the factors (e.g., actin and EF-1a) are abundant in the cytoplasm.

The immunological interaction between HIV-1 and the host

Table 2. The major cellular proteins incorporated into the virion

In the viral particle
Actin
Cofilin
Cyclophilin A
Elongation factor 1α (EF-1α)
Ezrin
GAPDH
Heat shock protein 70 (HSP70)
HS-1
INI1/hSNF5
Lck
Moesin
Phosphatidylethanolamine-binding protein
TSG-101
Ubiquitin
VAN/NAF1
On the envelope
β2-microglobulin
CD molecules
HLA class I/II molecules
ICAM-1,2,3
LFA-1,2

Table 3. The major soluble factors affecting the replication of HIV-1

α-1-antitrypsin
α-defensins 1,2,3
CD8-positive cell product modifying anti-thrombin III
D Lactoalbumin
Granulocyte-macrophage colony-stimulating factor (GM-CSF)
Interferon α, β, γ (IFN α, β, γ)
Interleukins (IL)
Leukemia inhibitory factor (LIF)
Lymphotactin
Macrophage colony-stimulating factor (M-CSF)
Macrophage-derived chemokine (MDC)
Monocyte chemotactic protein-2 (MCP-2)
Natural killer cell enhancing factor A, B (NFEF)
RANTES, macrophage inflammatory protein-α, β (MIP-1α, β)
RNase
Secretory leukocyte protease inhibitor (SLPI)
Stromal cell-derived factor-1 (SDF-1)
Transforming growth factor-β (TGF-β)
Tumor necrosis factor-α, β (TNF-α, β)

is another viewpoint. HIV-1 is able to escape from the host immunity because of its high self-mutagenicity. Beside that, HIV-1 targets cellular factors to protect the infected cells from the host immune system. *Nef* down-modulates expression of MHC class I molecule through interacting with PACS-1, the regulatory protein that controls the membrane protein trafficking, thereby the cellular immunity can not eliminate the HIV-1-infected cells effectively (40,41). Some cellular soluble factors are known to either positively or negatively affect the HIV-1 replication (Table 3, reviewed in [42-44]). For example, the tumor necrosis factor-alpha (TNF-α) induces the HIV-1 replication. It does so via activating the nuclear factor-kappa B (NF-κB) that translocates into the nucleus and stimulates the transcription from HIV-1's LTR. Identifying the soluble factors and understanding how they function are also attractive subjects because the study links directly to the understanding of HIV-1 pathogenesis and the chemotherapy against AIDS.

7. Concluding remarks

The HAART appears successful in controlling the AIDS disease progression. However, we can not be optimistic because the drug-resistant viruses have already emerged making the HAART being ineffective and the adverse effects of anti-AIDS drugs are considerable. Because the mutation rate of cellular genome is far lower than the viral genome, developing a new class of anti-retroviral drugs that target a cellular factor is one of the options. It is no doubt that charactering cellular factors that interact with HIV-1 contributes to not only the understanding of HIV-1 virology but also the advancement of cell biology. It ultimately provides novel targets for the antiviral intervention.

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Mycobacterial Codon Optimization Enhances Antigen Expression and Virus-Specific Immune Responses in Recombinant *Mycobacterium bovis* Bacille Calmette-Guérin Expressing Human Immunodeficiency Virus Type 1 Gag†

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Although its potential for vaccine development is already known, the introduction of recombinant human immunodeficiency virus (HIV) genes to *Mycobacterium bovis* bacille Calmette-Guérin (BCG) has thus far elicited only limited responses. In order to improve the expression levels, we optimized the codon usage of the HIV type 1 (HIV-1) p24 antigen gene of *gag* (p24 *gag*) and established a codon-optimized recombinant BCG (rBCG)-p24 Gag which expressed a 40-fold-higher level of p24 Gag than did that of nonoptimized rBCG-p24 Gag. Inoculation of mice with the codon-optimized rBCG-p24 Gag elicited effective immunity, as evidenced by virus-specific lymphocyte proliferation, gamma interferon ELISPOT cell induction, and antibody production. In contrast, inoculation of animals with the nonoptimized rBCG-p24 Gag induced only low levels of immune responses. Furthermore, a dose as small as 0.01 mg of the codon-optimized rBCG per animal proved capable of eliciting immune responses, suggesting that even low doses of a codon-optimized rBCG-based vaccine could effectively elicit HIV-1-specific immune responses.

The *Mycobacterium bovis* bacille Calmette-Guérin (BCG) has been widely used as a live bacterial vaccine against *Mycobacterium tuberculosis* infection. Its recombinant form, rBCG, which has been used successfully to express foreign antigens and to induce immune responses, has been proposed as a vaccine candidate against a number of diseases (26, 32, 33), especially human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) (11, 13, 30). Moreover, mucosal immunization of rBCG has been found to elicit a long-term virus-specific immunity in animals (10, 14, 15), even in Th1- and Th2-deficient conditions (10). In short, an rBCG-based vaccine offers several clear advantages over other types of recombinant vector-based approaches in that it (i) induces cellular immune responses that are maintained for at least 1 to 2 years; (ii) is easy to administer, usually requiring only one or two immunizations; (iii) and is affordable because it can be easily and cheaply produced. These findings suggest that rBCG could be a potent vaccine against HIV-1 infection, one that is likewise capable of inducing safe, virus-specific immunity.

However, the results described above were obtained with high doses of rBCG, doses 10- to 100-fold larger than that needed for a practical BCG vaccination dose against tuberculosis in humans (7, 11). Therefore, the low immunogenicity seen in rBCG-inoculated animals is likely due to their inoculation with only a "normal," not a high, vaccination dose (15). Moreover, high doses of BCG administration in vivo may also act as the driving force for the replication of the immunodeficiency virus and its dissemination by hyperactivating T cells (6, 41).

We sought here to produce an rBCG vaccine that would be efficacious even in the low doses required for human vaccination. Because low-dose immunization of rBCG has been suggested to act as a prophylactic vaccination against HIV-1 (15, 28), we adopted the preferred codon of BCG to enhance the expression of the foreign HIV gene. In recombinant protein production, the potency of codon-optimized gene expression systems was demonstrated in *Escherichia coli* (39) and in mammalian cells (42). These results clearly show that codon-optimized recombinant genes induce vigorous expression by foreign genes in the host. Since 1998, many groups have reported that a sequence-modified DNA vaccine confers high immunogenicity against various foreign antigens, e.g., listeriolysin O of *Listeria monocytogenes* (37), HIV-1 Gag (43), Env (3), tetanus toxin (34), L1 protein of human papillomavirus (18), and merozoite surface protein 1 of *Plasmodium falciparum* (25). Most of these studies focused on demonstrating how mammalian codon usage bias efficiently enhanced the expression and immunogenicity of foreign antigens in DNA vaccination. However, although the effect of codon optimization in mammalian cells has been well documented, its effect in recombinant BCG vector-based vaccines has never been fully elucidated.

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MATERIALS AND METHODS

Animals. Female BALB/c (*H-2*^{d}) mice aged 6 to 8 weeks were purchased from Charles River Japan, Inc. Mice were maintained in the experimental animal facility under pathogen-free conditions and in a manner consistent with the institutional animal care and use guidelines of the National Institute of Infectious Diseases of Japan. The study was conducted in a biosafety level 2 facility with the approval of an institutional committee for biosafety and in accordance with the requirements of the World Health Organization.

Construction of an HIV antigen expression vector and transformation of BCG. We used *E. coli* HB101-competent cells (Takara Bio, Inc.) for gene manipulation and the $BCG_{Tokyo172}$ as a mycobacterial strain which does not accelerate disease progression in HIV-infected children (9). Middlebrook 7H9 broth containing albumin-dextrose complex (7H9-ADC; BBL Microbiology Systems) was used as the culture medium for rBCG. A DNA fragment encoding the *hsp60* gene of BCG (36) was cloned into SmaI-SalI sites of pUC18 (pUC-hsp60). A synthetic DNA fragment corresponding to the multicloning site and terminator region of the *hsp60* gene was cloned into the MunI-KpnI sites of pUC-hsp60. A KpnI linker was then inserted at the EcoRI site, giving rise to the pUC-hspK vector. The *gag* p24 gene of the subtype B NL4-3 virus was amplified by PCR from pNL4-3 plasmid using the primers AATggatccTATAGTGCAGAACCTC (forward, with lowercase letters indicating the BamHI site) and AATgggcccTT ACAAAACTCTTGCTTTATGG (reverse, with lowercase letters indicating the ApaI site). The PCR product was cloned into BamHI-ApaI sites of pUC-hspK in frame (pUC-hspK-p24Wt). The whole p24 gene was also chemically synthesized with the preferred codons in BCG and then cloned into the same sites of the pUC-hspK vector (pUC-hspK-p24Mu). These vectors were digested with KpnI, and then small fragments containing p24 expression units were subcloned into a KpnI site of the stable *E. coli*-mycobacteria shuttle vector pSO246 (pSO-p24Wt and -p24Mu) (19). These plasmids and pSO246 were transformed into BCG by using a Gene-Pulser (Bio-Rad Laboratories, Inc.), and transformants were selected on Middlebrook 7H10 agar containing 20 μg of kanamycin/ml and supplemented with an OADC enrichment (BBL Microbiology Systems).

Western blot analysis. Transformants of rBCG were grown in 7H9-ADC broth for 2 weeks. A portion of the culture medium was periodically collected, sonicated, and subjected to immunoblot analysis with V107 monoclonal antibody (20) as described previously (11).

Lymphocyte proliferative assays. Single-cell suspensions from spleens of immunized animals were cultured with or without 25 μg of HIV-HXB2 Gag-overlapping peptide (NIH AIDS Research and Reference Reagent Program)/ml or 2.5 μg of tuberculin purified protein derivative (PPD)/ml. In the present study, the overlapping peptides p11 (LERFAVNPGLLETSE) through p35 (NIQGQ MVHQAISPRT) covering the Gag p24 region were used for stimulation, either as a whole or in pools of 5. Proliferation was measured by determining the level of [^{3}H]thymidine uptake (31).

Antigen-specific IFN-γ ELISPOT assay. P24- and PPD-specific IFN-γ-secreting cells were assessed by using the mouse gamma interferon (IFN-γ) development module and the enzyme-linked immunospot assay (ELISPOT) blue color module (R&D Systems, Inc.). Briefly, single-cell suspensions were cultured in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 55 μM β-mercaptoethanol, 50 U of penicillin/ml, and 50 μg of streptomycin/ml) with or without 25 μg of pooled Gag-overlapping peptide (p11-35)/ml, 5 μg of recombinant p24 protein (rp24; $HIV\text{-}1_{IIIB}$ p24; ImmunoDiagnostics, Inc.)/ml, or 2.5 μg of PPD/ml for 48 h at 37°C in a humidified 5% CO_2 environment. After incubation, cells were transferred to anti-IFN-γ antibody-coated 96-well nitrocellulose plates (Millititer HA; Millipore Co.) at various concentrations and incubated for 16 h at 37°C in a humidified 5% CO_2 environment before being developed according to manufacturer's instructions. Spot-forming cells (SFCs) were then quantified by using the KS ELISPOT compact system (Carl Zeiss) (23).

Assay for assessment on major histocompatibility complex class I-restricted $CD8^+$-T-cell response. *H-2*^{d}-restricted $CD8^+$-T-cell responses were measured by ELISPOT assay using A9I (AMQMLKETI) peptide (27, 38). Single-cell suspensions were labeled with microbead-conjugated anti-CD8a monoclonal antibody (53-6.7; Miltenyi Biotec GmbH) and depleted labeled cells by using Auto MACS (Miltenyi Biotec GmbH). Whole splenocytes and CD8-depleted splenocytes from each mouse were used in an A9I-specific IFN-γ ELISPOT assay. The cells were incubated with or without A9I peptide at 50 μM for 24 h at 37°C in a humidified 5% CO_2 environment, and the subsequent steps were as described above.

Serum antibody titration by HIV-1 Gag p24- and PPD-based ELISA. P24- and PPD-specific immunoglobulin G titers in plasma were determined by an end-point enzyme-linked immunosorbent assay (ELISA) (10).

Statistical analyses. Statistical analyses were carried out by using the StatView program (version 3.0; SAS Institute). The lymphocyte proliferative activities and IFN-γ SFC counts of each group were compared by using the two-sided Student *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Mycobacterial codon usage optimization of HIV-1 *gag* p24 gene and construction of an rBCG encoding the codon-optimized gene. In order to determine whether mycobacterial codon optimization could enhance the expression of the HIV gene in vitro, we first targeted the HIV-1 subtype B NL4-3 *gag* p24 gene for our research. Once we had designed the mycobacterial codon-optimized p24 gene, aligned it with the wild-type gene, and deduced the amino acid sequence (Fig. 1), we determined that the total G+C content of the coding region in the synthetic p24 gene was higher (67.4%) than that of the wild-type p24 gene from pNL4-3 (43.4%). (A translation table showing all 20 amino acids used in the present study is available [Table S1 in the supplemental material]). These two genes were initially cloned into the pUC-hspK vector (Fig. 2a) and subcloned into the pSO246 vector (Fig. 2b). Once these expression vectors were transformed into the $BCG_{Tokyo172}$ strain, rBCG-p24Mu (with optimal codon usage of the p24 gene) and rBCG-p24Wt (with wild-type codon usage) were selected for further experimentation.

Effects of codon usage modification on the expression levels of HIV-1 Gag p24 in vitro. We next sought to compare the expression levels of the p24 gene in the two types of BCG-HIV recombinants by studying the kinetics of the growth curve of the cultured rBCG cells and by measuring the levels of p24 protein to assess the production ability of the HIV antigen (Fig. 3). Using Western blot analysis at 2-week intervals, we observed that recombinant p24 protein in each of the lysates of rBCG-p24Wt and -p24Mu consistently appeared as a single band measuring ca. 24 kDa (lanes 1 and 2 of Fig. 3a, respectively). The p24 antigen expression level of rBCG-p24Mu was 37-fold higher (175.0 ± 25.1 ng/5 × 10^7 CFU of bacilli) than that of rBCG-p24Wt (4.7 ± 0.3 ng/5 × 10^7 CFU of bacilli) (Fig. 3b). Both rBCG-p24Mu and -p24Wt showed a more normal BCG growth curve than did the rBCG-pSO246 control transformant, and both peaked 21 days after cell culture (Fig. 3c), suggesting a correlation between p24 antigen generation and the growth rate of cultured rBCG-p24Mu. Thus, the codon-optimized BCG recombinant was successfully generated and found to express remarkable levels of p24 antigen, i.e., almost 200 ng of p24 antigen/5 × 10^7 CFU or 1 mg of bacilli.

Codon optimization of the HIV-1 Gag p24 antigen in rBCG generates strong HIV-specific immune responses in mice after intradermal immunization. We then analyzed how the modification of codon usage affected the immunogenicity of BCG vector-based vaccines encoding the HIV-1 *gag* p24 antigen gene. 35 BALB/c mice were divided into three experimental groups of 10 mice each, with the remaining five mice administered saline alone and used as normal healthy controls. Five mice from each experimental group were intradermally immunized with 0.01 mg, and five mice from each group were immunized with 0.1 mg of rBCG-p24Mu, -p24Wt, and -pSO246. At 10 weeks postinoculation (p.i.), we examined lymphocyte proliferation, IFN-γ ELISPOT cell generation, and antibody

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pNL4-3[a]      1 GGATCCtatagtgcagaacctccaggggcaaatggtacatcaggccatatcacctagaactttaaatgcatgggtaaaagtagtagaaga
optimized      1 .....g..c..c.....g.....c.g.....c..c.....c.g.gc.c..cc.g..c..c.....c.g..c..c.g..
A.A. seq.          P  I  V  Q  N  L  Q  G  Q  M  V  H  Q  A  I  S  P  R  T  L  N  A  W  V  K  V  V  E  E

pNL4-3        91 gaaggctttcagcccagaagtaatacccatgttttcagcattatcagaaggagccaccccacaagatttaaataccatgctaaacacagt
optimized     91 .....c...tcg.g..g..c..c..g.....c..g..cc.g..g..g..c.........g..g..cc.g..c.........g.....c..
A.A. seq.          K  A  F  S  P  E  V  I  P  M  F  S  A  L  S  E  G  A  T  P  Q  D  L  N  T  M  L  N  T  V

pNL4-3       181 gggggacatcaagcagccatgcaaatgttaaaagagaccatcaatgaggaagctgcagaatgggatagattgcatccagtgcatgcagg
optimized    181 c..c..c..c..g..c.........g...c.g..g.....c.....c...g..c..c..g.....cc.cc.....c..g..c..c..c..
A.A. seq.          G  G  H  Q  A  A  M  Q  M  L  K  E  T  I  N  E  E  A  A  E  W  D  R  L  H  P  V  H  A  G

pNL4-3       271 gcctattgcaccaggccagatgagagaaccaaggggaagtgacatagcaggaactactagtacccttcaggaacaaataggatggatgac
optimized    271 c..c..c.....g.........c.c..g..tc.c..ctcg.....c..c..c..c..ctcg.....c.....g..g..c..c.......
A.A. seq.          P  I  A  P  G  Q  M  R  E  P  R  G  S  D  I  A  G  T  T  S  T  L  Q  E  Q  I  G  W  M  T

pNL4-3       361 acataatccacctatcccagtaggagaaatctatagaaagatggataatcctgggattaaatagaaatagtaagaatgtatagccctaccag
optimized    361 c..c..c..g..g.....g..c..c..g.....c..gc.c.....c.........cc.g..c..g..c..cc.c.....ctcg..g...tc
A.A. seq.          H  N  P  P  I  P  V  G  E  I  Y  K  R  W  I  I  L  G  L  N  K  I  V  R  M  Y  S  P  T  S

pNL4-3       451 cattctggacataagacaaggaccaaaggaaccctttagagactatgtagaccgattctataaaactctaagagccgagcaagcttcaca
optimized    451 g..c.....cc.c..g.t..g.....g..g..cc.c.....c..c.....c.....c..g..c..cc.c.....g..g..g...
A.A. seq.          I  L  D  I  R  Q  G  P  K  E  P  F  R  D  Y  V  D  R  F  Y  K  T  L  R  A  E  Q  A  S  Q

pNL4-3       541 agaggtaaaaaattggatgacagaaaccttgttggtccaaaatgcgaacccagattgtaagactattttaaaagcattgggaccaggagc
optimized    541 g.....c..g..c.....c..g...c..c.....g..c..c.....g..c..c..c.....c..cc.g..g..cc.....t..g..c..
A.A. seq.          E  V  K  N  W  M  T  E  T  L  L  V  Q  N  A  N  P  D  C  K  T  I  L  K  A  L  G  P  G  A

pNL4-3       631 gacactagaagaaatgatgacagcatgtcagggagtgggggacccggccataaagcaagagttttgtaaGGGCCC
optimized    631 c..c..g..g..g.....c..c..c...c..c..c..c..g.....c..g..gc.c..cc..........
A.A. seq.          T  L  E  E  M  M  T  A  C  Q  G  V  G  G  P  G  H  K  A  R  V  L  *
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FIG. 1. Nucleotide sequences and deduced amino acid sequence alignments of the p24 gene from pNL4-3 and the synthetic p24 gene with mycobacterial optimal codons. For cloning to the pUC-hspK vector, the BamHI and ApaI restriction sites were attached at both the 5′ terminus and the 3′ terminus of each DNA fragment (shown as boldface uppercase letters). Dots indicate sequences identical to those of the pNL4-3 p24 gene. The asterisk indicates the termination codon. The superscript *a* indicates sequence data that are available from GenBank under accession no. AF324493.

production in immunized animals. The same study was repeated three times, and all three results were summarized.

Significant lymphocyte proliferative responses (stimulation indices of 5.04 ± 1.09 and 4.02 ± 0.44) were obtained with pooled peptides p16-20 (pool 2) and pooled total p24 peptides p11-35 (pool 1-5) in mice immunized with 0.01 mg of rBCG-p24Mu. When this dosage was increased to 0.1 mg, the lymphocyte proliferative responses to pool 2 and pool 1-5 increased to 10.08 ± 2.40 and 8.05 ± 1.16, respectively (data not shown). In contrast, we could not detect any significant virus-specific proliferation in mice immunized with 0.01 or 0.1 mg of rBCG-p24Wt (Fig. 4). These in vivo differences in proliferative

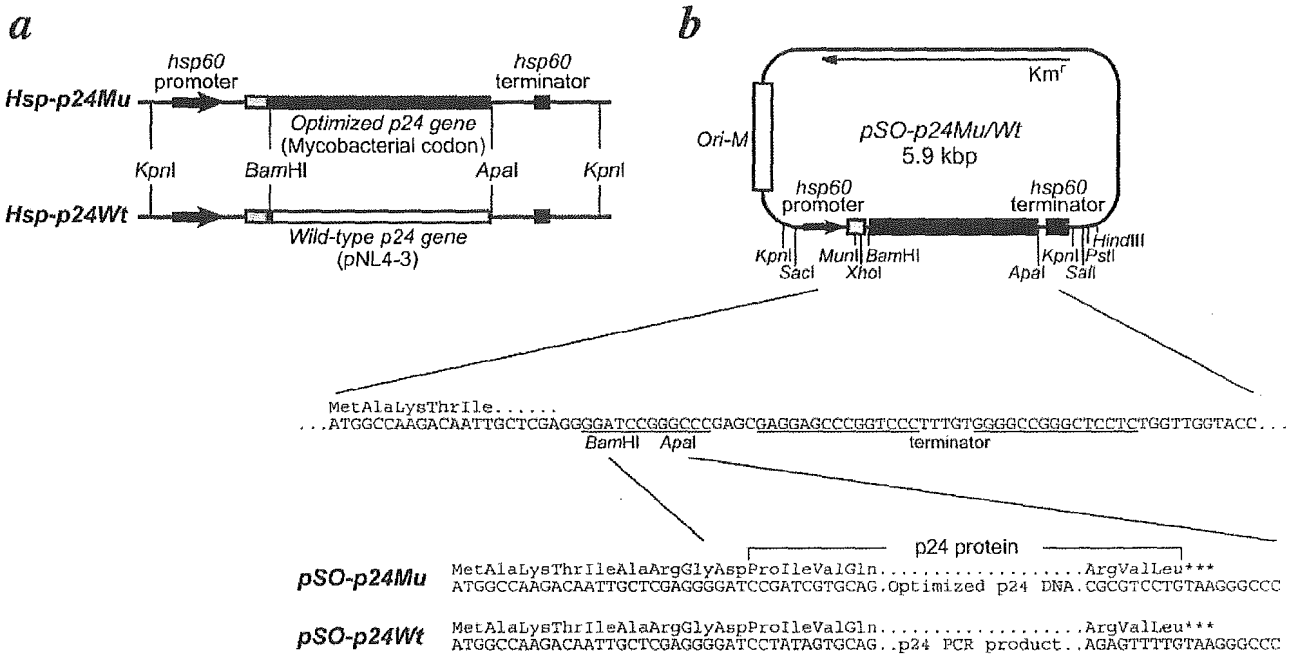

FIG. 2. Structure of expression vectors pSO-p24Mu and -p24Wt. (a) Schematic representation of the expression units of p24. Solid and open bars indicate the synthetic p24 gene and the PCR fragment of the p24 gene, respectively. The arrows and solid squares indicate the transcriptional direction of the *hsp60* promoters and terminators. Gray bars show the DNA fragment of the mycobacteria. (b) Details of expression vectors pSO-p24Mu and -p24Wt. *Ori-M* indicates the origin of mycobacterial replication, and Km^r denotes the kanamycin resistance gene. Asterisks indicate the termination codon for each gene.

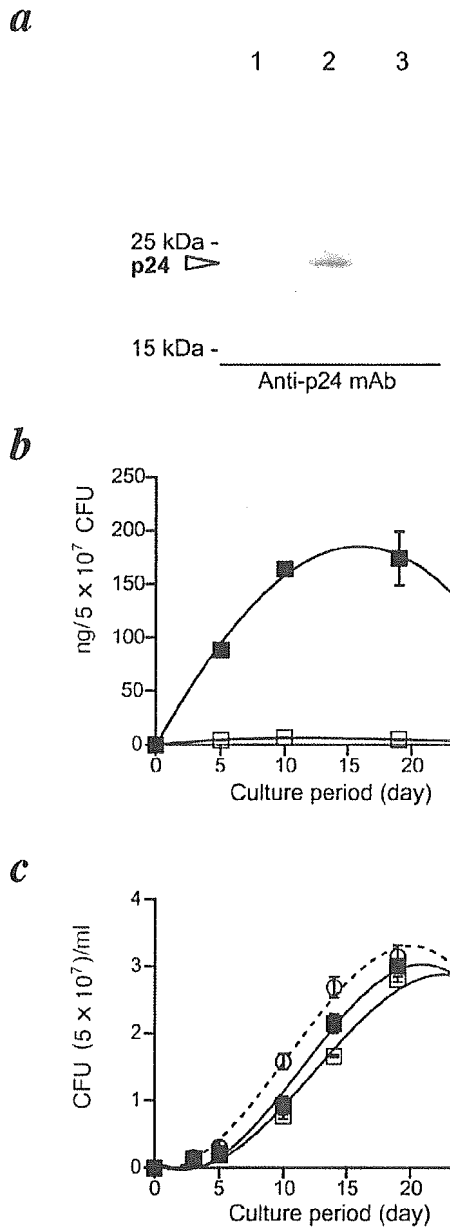

FIG. 3. Comparison of p24 expression levels and growth rates for rBCG-p24Mu and -p24Wt. (a) Anti-p24 monoclonal antibody (V107)-reacted proteins were visualized by Western blotting. Lane 1, lysate of rBCG-p24Wt; lane 2, lysate of rBCG-p24Mu; lane 3, lysate of rBCG-pSO246 (negative control). (b) Comparison of p24 concentration in whole-cell lysates of rBCG-p24Mu and -p24Wt. rBCG cells were harvested from each culture periodically, sonicated, and subjected to a commercial p24 antigen enzyme immunoassay (HIVAG-1MC; Abbott Laboratories). Expression of the p24 protein is represented as p24 concentrations (in nanograms per milligram) or 5×10^7 CFU of bacilli. Solid and open squares indicate rBCG-p24Mu and -p24Wt, respectively. Data are presented as means ± the standard deviations. (c) Kinetics of growth rates in recombinant clones. After periodic collection of each culture, the optical density at 470 nm was measured. The cell densities were calculated based upon the rate of absorbance, using the following formula: density (μg/ml) = absorbance at 470 nm × 1,412.3 + 73.063. The CFU were translated from densities and plotted. The ■, □, and ○ symbols indicate rBCG-p24Mu, -p24Wt, and -pSO246, respectively. The data represent means ± the standard deviations.

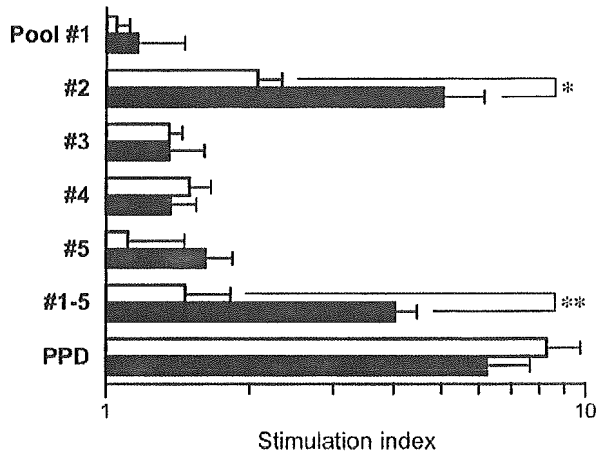

FIG. 4. Lymphocyte proliferation against Gag p24 overlapping peptides and PPD. Splenocytes from immunized animals were cultured with or without antigen for 48 h at 37°C in a humidified 5% CO_2 environment. In the final 6 h before harvesting, [^{3}H]thymidine was added. The level of [^{3}H]thymidine uptake was then measured. Proliferative activity is measured by using the stimulation index. Solid and open columns indicate stimulation index values of rBCG-p24Mu- and -p24Wt-immunized mice, respectively. The data represent the mean stimulation index plus one standard deviation. Asterisks indicate statistical significance (*, $P < 0.02$; **, $P < 0.002$).

responses between rBCG-p24Mu and -p24Wt were statistically significant comparing pool 2 ($P = 0.010$) and pool 1-5 ($P = 0.001$). No p24-specific proliferation was detected in either rBCG-pSO246-immunized mice or normal healthy controls (data not shown). PPD-specific lymphocyte proliferations were obtained in all immunized animals similarly (stimulation indices were ca. 7).

In addition, p24-specific IFN-γ-secreting cells were determined by ELISPOT assay. Both pooled p24 peptides (pool 1-5) and rp24-specific SFCs were detected in mice immunized with 0.1 mg of rBCG-p24Mu and -p24Wt but not in those immunized with the same dosage of rBCG-pSO246 (Fig. 5). In rBCG-

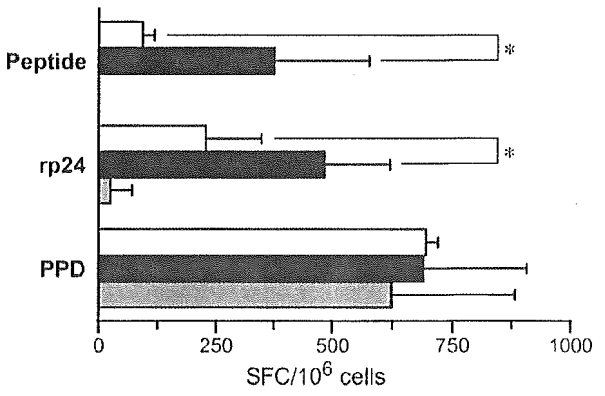

FIG. 5. Determination of antigen-specific IFN-γ-secreting cells by ELISPOT assay. Solid, open, and shaded columns indicate the numbers of SFCs of rBCG-p24Mu-, -p24Wt-, and -pSO246-immunized mice, respectively. The data represent the mean numbers of SFCs/10^6 cells plus one standard deviation. Asterisks indicate statistical significance ($P < 0.05$ against rBCG-p24Wt-immunized mice).

p24Mu-immunized mice, stimulation with peptides resulted in 375 ± 202 SFC/10^6 splenocytes and stimulation with rp24 resulted in 483 ± 138 SFC/10^6 splenocytes—rates much higher than those observed for rBCG-p24Wt (93 ± 25 and 227 ± 120 SFC/10^6 splenocytes, respectively). These differences in response by groups immunized with rBCG-p24Mu and -p24Wt to peptides and to rp24 were also statistically significant (peptides, $P = 0.033$; rp24, $P = 0.031$). PPD-specific SFCs were strongly expressed in all mice receiving rBCGs (670 ± 180 SFC/10^6 splenocytes). Furthermore, similar levels of rp24-specific IFN-γ SFC activity were observed in splenocytes of rBCG-p24Mu-immunized animals even 6 months p.i. (402 ± 198 SFC/10^6 splenocytes, data not shown).

Furthermore, we studied whether these IFN-γ ELISPOT activities were attributed to major histocompatibility complex class I-restricted $CD8^+$-T-cell response with 12 BALB/c mice immunized with 0.1 mg of rBCG-p24Mu ($n = 4$), -p24Wt ($n = 4$), or -pSO246 ($n = 4$). After 2 weeks p.i., the mice were sacrificed, and their spleens were used for the study. By peptide-antigen-specific IFN-γ ELISPOT assay, $H\text{-}2^d$-restricted $CD8^+$-T-cell responses specific for the $CD8^+$-T-cell epitope A9I were detected in the two animal groups immunized with rBCG-p24Mu and -p24Wt (Fig. 6b). In rBCG-p24Mu-immunized mice, stimulation with 50 μM A9I resulted in 130 ± 16 SFC/10^6 splenocytes, activities significantly higher than that obtained by immunization with rBCG-p24Wt (70 ± 21 SFC/10^6 splenocytes, $P = 0.011$). Furthermore, by using magnetic cell sorting, the $CD8^+$-T-cell-depleted cell fractions were purified to be >97% and >99% viable (Fig. 6a). The $CD8^+$-T-cell response of immunized animal groups decreased significantly upon stimulation with A9I peptide compared to nonseparated splenocytes (rBCG-p24Mu immunized, 15 ± 12 SFC/10^6 cells, $P = 0.001$; rBCG-p24Wt immunized, 3 ± 3 SFC/10^6 cells, $P = 0.006$) ("CD8-depleted" in Fig. 6b). No A9I-specific IFN-γ responses were detected in rBCG-pSO246-immunized mice either whole or CD8-depleted splenocytes were used (data not shown).

Finally, sera from all animals immunized with 0.1 mg of rBCG-p24Mu, -p24Wt, and -pSO246 were assessed for specific antibody generation at 10 weeks p.i. by endpoint antibody-ELISA against rp24 and PPD (Fig. 7). Again, only low levels of antibodies against rp24 were generally elicited in animals immunized with rBCG-p24Mu and -p24Wt (antibody titers in sera of $10^{2.41}$ and $10^{2.03}$, respectively). Moreover, PPD-specific antibodies were similarly detected in all immunized animals at titers of ca. 10^3. In summary, virus-specific cell-mediated immunity was significantly induced during the initial immune response, but its antibody response was low.

DISCUSSION

In this study, we have clearly demonstrated that codon optimization is a useful strategy for enhancing foreign antigen expression in rBCG and for obtaining significant levels of foreign antigen-specific immune responses. This strategy is key to rBCG-HIV vaccine development, since low-dose immunization and/or intradermal immunization with 0.1 mg of codon-optimized rBCG has proven effective for induction of HIV-specific cellular immunity by (i) allowing for a smaller dosage of rBCG, one that is far more practicable for use in human tuberculosis vaccination than the 1 to 10-mg dose otherwise

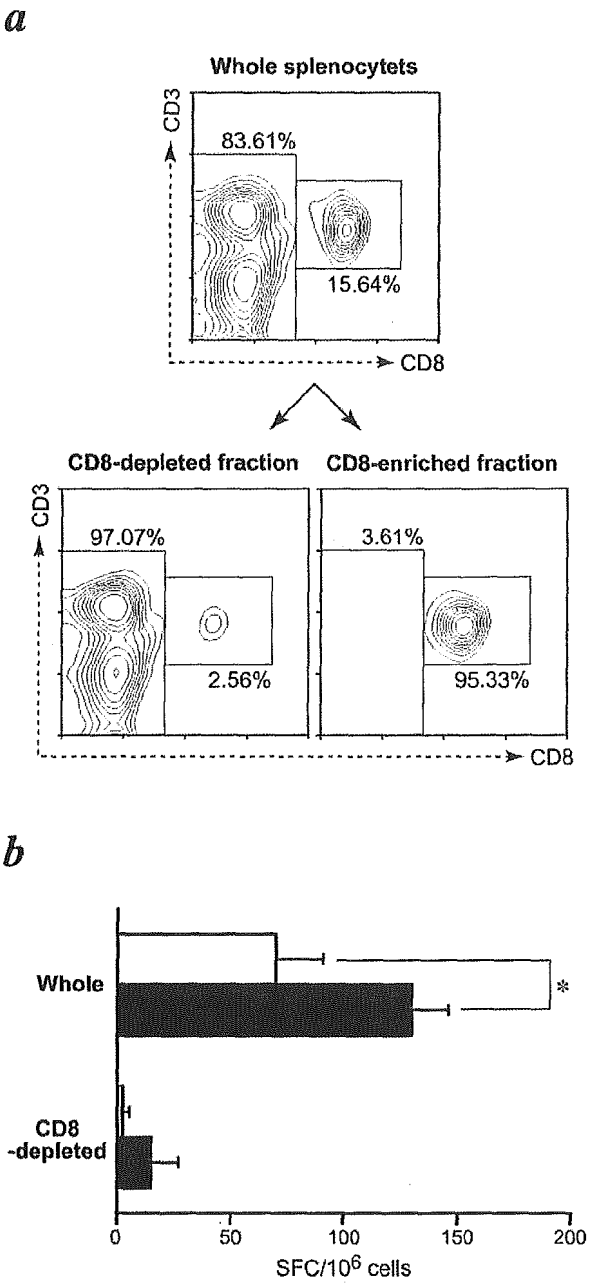

FIG. 6. Decrease of p24-specific cellular immune responses by the depletion of $CD8^+$ T cells. (a) Depletion of $CD8^+$ T cells from splenocytes of immunized animals by magnetic cell sorting of $CD8^+$ T cells by a specific antibody. Splenocytes from mice immunized with rBCGs were sorted and analyzed by flow cytometry. Whole splenocytes (upper row) and CD8-depleted fraction (lower left panel) were used for subsequent study. (b) Assessment of A9I-specific $CD8^+$-T-cell responses by peptide-specific IFN-γ ELISPOT assay. Effect of $CD8^+$-T-cell depletion from splenocytes from immunized animals was studied by measuring the A9I peptide-specific IFN-γ ELISPOT response of whole or CD8-depleted splenocytes. Solid and open columns indicate the numbers of SFCs of rBCG-p24Mu and -p24Wt-immunized mice, respectively. The data represent the mean numbers of SFCs/10^6 cells plus one standard deviation. The asterisk indicates statistical significance ($P < 0.02$ against rBCG-p24Wt-immunized mice).

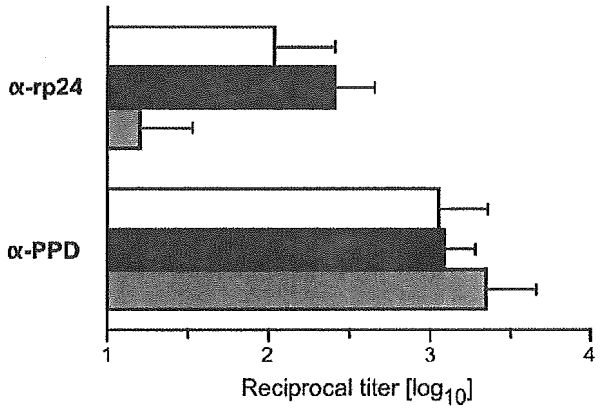

FIG. 7. Anti-p24-specific and anti-PPD-specific IgG antibodies in the plasma of mice immunized with rBCGs. Solid, open, and gray columns indicate reciprocal $\log_{10}$ titers of rBCG-p24Mu, -p24Wt, and -pSO246-immunized mice, respectively. The titers were determined by using an endpoint ELISA. The data represent mean titers plus one standard deviation.

required, and by (ii) thereby reducing the risks associated with high-dosage cutaneous administration, including adverse local skin reactions, possible association with Th2-type immune responses, or exacerbation of retroviral infections. Given these results, rBCG is clearly poised to play a key role in the development of an HIV/AIDS vaccine.

When the mycobacterial codon usage of the p24 antigen gene of the HIV-1 *gag* was optimized, the codon-optimized rBCG expressed nearly 40-fold more antigen than did the wild-type rBCG. This enhancement of the Gag p24 expression level in rBCG is on a par with the 10- to 50-fold increase seen when DNA vaccine is codon optimized (3). Why was the mycobacterial codon optimization so effective? BCG is a high G+C gram-positive bacteria, with a genomic G+C content ca. 64.8%, and so has a strong bias toward C- and G-ending codons for every amino acid. Overall, the G+C content at the third position of codons is 81.0% (2). From the accumulated information on BCG genes (24), it should be noted that the AGA codon for Arg and the TTA codon for Leu make up only 0.9 and 1.6% of the total codons for Arg and Leu, respectively. In contrast, HIV-1 prefers the adenine or the thymidine at the third position of the codon (60.9%). In the coding sequence of the p24 gene of HIV-1 *gag*, 9 out of 11 Arg codons used AGA and 6 out of 18 Leu codons used TTA. Because it is generally accepted that codon preference correlates with the amount of aminoacyl tRNA in unicellular organisms (12), only low levels of aminoacyl tRNA for AGA and TTA codons would be expected in the BCG cell. These low levels of aminoacyl tRNA for AGA and TTA codons might help explain why the codon-optimized p24 gene was highly expressed in BCG.

Recombinant HIV-1 Gag p24 antigen expression in codon-optimized rBCG is 175 ng/mg of bacilli of $BCG_{Tokyo172}$ or ca. 5.3% of the total cytoplasmic rBCG protein, when calculated using the method of Langermann et al. (17). The previously reported production levels of recombinant HIV protein were all for non-codon-optimized BCG using a different expression system and a different BCG strain. The levels varied from 1% of cellular protein (HIV-1 Nef [40] and SIV_{mac251} Gag [22]) to 0.1% of the HIV-1 Gag protein (1), suggesting that codon-optimized recombinant HIV-1 protein induced responses 5- to 50-fold higher than those previously reported for non-codon-optimized rBCG. The codon-optimization of HIV Gag p24 is also effective in elicitation of antigen-specific $CD8^+$-T-cell responses in animals. Since there is no difference in the growth/persistence in the various BCG (S. Yamamoto et al., unpublished data), the enhanced expression of the HIV protein by the recombinant construct suggests that it is responsible for the enhanced immunogenicity of the codon-optimized rBCG vaccine.

A successful preventive HIV vaccine must not only effectively protects against HIV-1 or SIV, a goal already achieved in nonhuman primate AIDS models using different vaccine modalities, but also will prove safe for use in humans. Instead of seeking to elicit sterilizing protection from the HIV infection, current vaccine research on HIV/AIDS is focused mainly on the induction of efficient cellular immune responses that may play a critical role in protective immunity.

One of the prospective measures is to evoke host immunity by delivering recombinant vector-based vaccines expressing recombinant antigens, e.g., modified vaccinia virus Ankara (4, 21), adenovirus type 5 (29), fowlpox virus (16), canarypox virus (8), and NYVAC (5). In combination with boosting or priming antigens, most of these recombinant vector-based vaccines effectively induce antiviral immunity. We also showed that rBCG could induce long-lasting anti-HIV-1 or -SIV specific immunity in small animals (14). In the present study, we have demonstrated the promise of a codon-optimized rBCG-HIV vaccine, one which could, even at low doses, elicit long-lasting cell-mediated immune responses without triggering humoral immunity.

Previous reports have demonstrated that a high-dose intravenous inoculation of BCG can induce disease progression, as it did, for example, with BCG-specific $CD4^+$-T-cell activation in monkeys infected with SIV (6). Others have reported a correlation between the magnitude of T-cell activation of CDR3-restricted cells and the disease progression to AIDS in monkeys (41). These results suggest that these $CD4^+$ T cells, once activated by a high dose of any live vaccine, may become infectious and even lead to the replication of the immunodeficiency virus at the coinfection stage. In this regard, our previous study indicated that high doses of BCG did indeed induce a remarkable expansion of I^a-positive activated T cells in guinea pigs but that intradermal inoculation with 0.1 mg of BCG, the common dose and route of BCG vaccination in humans, did not (35).

In showing that a low-dose vaccination with rBCG-HIV is both possible and practicable with the mycobacterial codon optimization of the foreign HIV gene, we offer here a way around this problem. Collectively, these results suggest that a novel vaccination strategy using a low dose of codon-optimized rBCG-HIV, one comparable to the common dosage used for BCG vaccination in humans, might promote stable cell-mediated immune responses and thereby help establish positive immunity against subsequent immunodeficiency virus infection.

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Production and Characterization of a Monoclonal Antibody Specific to Nef-Associated Factor 1 (Naf1)/A20-Binding Inhibitor of NF-κB Activation (ABIN-1)

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ABSTRACT

Cellular protein Naf1 (Nef-associated factor 1) or ABIN-1 (A20-binding inhibitor of NF-κB activation) is an important cellular protein, expressed in various human tissues and T-cell lines. Naf1 protein has two isoforms (Naf1α and Naf1β) with different C-termini, produced by alternative splicing. Naf1α and Naf1β have approximately 2800 and 2600 nucleotides, with an open reading frame of 1941 and 1781 nucleotides, encoding the 72-kDa Naf1α and 68-kDa Naf1β proteins, respectively. In the present study, we generated a monoclonal antibody (MAb) against human Naf1, which recognizes full-length, endogenous Naf1 of both isotypes. For this purpose, recombinant 6×His and myc-tagged N-terminal Naf1$^{38-135}$, Naf1(N) protein was produced by using the baculovirus expression system. Recombinant Naf1(N) protein was used to immunize Balb/c mice, and a hybridoma cell line producing stable and highly specific MAb with strong affinity to Naf1 was established. We further characterized this antibody by immunofluorescent assay and Western blot analysis to confirm effectiveness in detecting recombinant and endogenous Naf1. By Western blot analysis of recombinant Naf1-N fusion proteins with overlapping N-terminal sequences, the epitope targeted by anti-Naf1 MAb was determined as the 81–88–amino acid region of human Naf1.

INTRODUCTION

NAF1 (NEF-ASSOCIATED FACTOR-1) or ABIN-1 (A20-binding inhibitor of NF-κB activation) is a cellular protein with four putative leucine zippers and four predicted regions of coiled coil structures. The gene encoding Naf1, which consists of 18 exons, is located on human chromosome 5q 32-33.1.[1] Naf1 mRNA is ubiquitously expressed in several human tissues, with strong expression in peripheral blood lymphocytes, spleen, and skeleton muscles. Naf1 is also detected in various human hematopoietic cell lines, such as Jurkat, Molt-4, H-9, and HL60.[1,2] According to the previous reports, Naf1 associates with human immunodeficiency virus type-1 (HIV-1) viral proteins Nef and matrix in the yeast two-hybrid system and pull-down assay using transfected human cell lysates.[1,2]

HIV-1 Nef not only enhances the viral infectivity, but also plays an important role in viral replication and pathogenesis.[3–10] Nef-defective HIV-1 virions are isolated from some cases of long-term non-progressors.[11] Nef induces down-regulation of cell surface expression levels of CD4 and major histocompatibility complex (MHC) class I molecules in HIV infection.[12–14] Thereby, Nef helps the virus to evade host defense and to increase viral infectivity.[15–17] CD4 is the primary receptor for HIV-1 and interferes with the infectivity of HIV-1 particles released from T cells.[18] Down-regulation of CD4 reduces the formation of complexes between CD4 and newly synthesized HIV-1 envelope protein on the infected cell surface, facilitating the release of HIV-1 virions. Nef-induced degradation of CD4 is also reported to result in the release of the normally CD4-bound tyrosine kinase, lck, and this could have a marked effect on signaling pathway in cellular activation. Because MHC class I is required to present viral peptide epitopes to cytotoxic T lymphocytes (CTL), down-regulation of cell surface MHC class I could inhibit the CTL-mediated lysis of HIV-

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1–infected cells. Naf1 overexpression increases cell surface CD4 levels, but overexpression of Nef, in turn, inhibits Naf1-induced CD4 augmentation.[1]

Naf1 (ABIN-1) also interacts with A20 zinc finger protein in yeast two-hybrid screening. Zinc finger protein A20 has been characterized as a dual inhibitor of nuclear factor–kappa B (NF-κB) activation and tumor necrosis factor (TNF)–induced apoptosis.[19–24] NF-κB plays a pivotal role in immune and inflammatory responses through the regulation of the expression of several proteins, including pro-inflammatory cytokines, chemokines, and adhesion molecules. Uncontrolled activation of the NF-κB pathway is involved in the pathogenesis of several chronic inflammatory diseases and autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, and asthma. A20 binding protein Naf1 (ABIN-1) has been reported to inhibit NF-κB–dependent gene expression induced by TNF-α, and IL-1. Overexpression of Naf1 blocks NF-κB activation by TNF-α, and it is also thought that the expression of Naf1 is NF-κB dependent. It has been suggested that Naf1 takes a role in negative feedback regulation of NF-κB expression by competing with IKK-γ and also acts as an endogenous brake for the expression of some TNF-α–driven genes.[24–27] Moreover, Naf1 appeared to attenuate the EGF/ERK2 nuclear signaling, which is important for cell growth, differentiation, and cell death.[28] Nevertheless, intriguing questions regarding the mechanism of functions and regulation of Naf1 as well as the importance of physical associations between Naf1 and Naf1 interacting proteins, to carry out their functions in the molecular signaling pathways, remain to be answered.

For further understanding of the molecular mechanisms of the functions of Naf1, the most important tasks are to investigate intracellular localization, to investigate the relationship between the nucleocytoplasmic shuttling of Naf1 and its functions, and to discover Naf1-interacting proteins along with their functions. To perform these tasks, anti-Naf1 MAb is an essential tool, and in this report, we describe the production, characterization, and epitope mapping of an MAb specific to human cellular protein Naf1/ABIN-1. We also report the expression and subcellular localization of endogenous Naf1 in primary and various cell lines such as human PBL, Jurkat, MT-4, Molt-4, 293, and U-937 cell lines by using this MAb.

MATERIALS AND METHODS

Cell cultures

Spodoptera frugiperda (Sf9) cells were grown and maintained in complete Grace's insect medium (Gibco) supplemented with 10% fetal bovine serum (FBS), and *Trichoplusia ni* (High Five) cells in serum-free Sf-900II medium (Gibco). Both cell lines were cultured in monolayers at 27°C. SP2/0 murine myeloma cells, human PBL from a healthy adult donor, Jurkat, MT-4, Molt-4, 293, and U-937 cells were maintained in RPMI-1640 medium (Sigma) supplemented with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin.

Construction of Naf1(N)-fusion protein cDNAs

cDNA encoding 6×His and myc-tagged Naf1(N) (amino acid residues 38–135) was constructed by polymerase chain re-

TABLE 1. OLIGONUCLEOTIDE PRIMERS USED FOR POLYMERASE CHAIN REACTION

Gene	*Primer*	*Sequences (5′ → 3′)*
Naf1(N)	His-Naf1(N)/F	CGCGGATCCGCGCATCATCATCATCATCATCAAGGGAT AAAGATGTTAGGGGAGC
	myc-Naf1(N)/R	CCGCTCGAGCGGCTAATTCAAGTCCTCTTCAGAAATG AGCTTTTGCTCCATTGAATTCTGCTCCTCAGGAGTGA
Naf1(N)-1	His-Naf1(N)-1/F	CGCGGATCCGCGCATCATCATCATCATCATATGCAAGG GATAAAGATGTTAGGG
	myc-Naf1(N)-1/R	CCCAGCTTGCTCTAATTCAAGTCCTCTTCAGAAATGA GCTTTTGCTCCAT CTCAGCCAGGGGGTCG
Naf1(N)-2	His-Naf1(N)-2/F	CGCGGATCCGCGCATCATCATCATCATCATGAGGAGCT AGTGAAGGACAACGA
	myc-Naf1(N)-2/R	CCCAAGCTTGCTCTAATTCAAGTCCTCTTCAGAAATGA GCTTTTGCTCCATGACTGGTGCTGGCTTGTCAC
Naf1(N)-3	His-Naf1(N)-3/F	CGCGGATCCGCGCATCATCATCATCATCATCTCACAGG AAAGGACTCAAATGTC
	His-Naf1(N)-3/R	CCCAAGCTTGCTCTAATTCAAGTCCTCTTCAGAAATGA GCTTTTGCTCCATTGAATTCTGCTCCTCAGGAGTG
Naf1(N)-A	His-Naf1(N)-A/F	CGCGGATCCGCGCATCATCATCATCATCAT GAGGAGCTAGTGAAGG ACAACGAGCTGCTCC
	myc-Naf1(N)-A/R	CCCAAGCTTCCATGGGCTCTAATTCAAGTCCTCTTCAG AAATGAGCTTTTGCTCCATAGGTGGTGGGAGCAGCT
Naf1(N)-B	His-Naf1(N)-B/F	CGCGGATCCGCGCATCATCATCATCATCATAACGAGCT GCTCCCACCACCTTCTCCCT
	myc-Naf1(N)-B/R	CCCAAGCTTCCATGGGCTCTAATTCAAGTCCTCTTCAG AAATGAGCTTTTGCTCCATGCCCAAGGAGGGAGAAG
Naf1(N)-C	His-Naf1(N)-C/F	CGCGGATCCGCGCATCATCATCATCATCAT
	myc-Naf1(N)-C/R	CCCAAGCTTCCATGGGCTCTAATTCAAGTCCTCTTCAG AAATGAGCTTTTGCTCCATCTCAGCCAGGGGGTCGA

action (PCR) technology, and the plasmid containing cDNA of full-length Naf1 was used as the template. The primers used in PCR reaction consisted of sense and antisense oligonucleotides containing *Bam*HI and *Xho*I sites respectively. The amplified cDNA was restricted by *Bam*HI/*Xho*I digestion, and subcloned into *Bam*HI/*Xho*I–digested pMelBacA baculovirus transfer vector (Invitrogen). cDNAs encoding 6 × His and myc-tagged Naf1(N) fusion protein fragments 1, 2, and 3, and A, B, and C were prepared by PCR amplification using specific primers with *Bam*HI and *Hind*III restriction sites, and ligated into pMAL-C2 vector containing MBP (maltose binding protein) sequence (Table 1). All constructs were confirmed by sequencing.

Generation of recombinant baculovirus in Sf9 cells

Recombinant baculovirus expressing Naf1(N) was produced by homologous recombination using Bac-N-Blue transfection kit (Invitrogen) according to procedures described previously.[29] Single viral clones were isolated by plaque assay and amplified by infecting Sf9 cells at a multiplicity of infection (MOI) of less than one. Infectivity was determined by titration and immunofluorescence staining of infected cells with mouse anti-myc MAb, followed by fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse second antibody. Virus stock was stored in aliquots at −80°C.

Production of recombinant Naf1(N) protein in High Five cells

Exponentially growing High Five cells in 75-cm^2 tissue culture flasks were infected with the recombinant baculovirus at an MOI of 10–20 plaque-forming units (pfu)/cell and incubated at 27°C for 3–5 days. High Five cells were then sedimented by centrifugation, and supernatant was collected for purification using Ni-NTA columns (polyhistidine tag at the amino terminus of the recombinant Naf1(N) protein binds to Ni-NTA resin).[30–31] Purification of recombinant Naf1(N) protein was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining.

Mice and immunization

Immunization, hybridoma preparation, and purification of MAb were done according to the procedures previously described.[32] In summary, three 6-week-old adult female Balb/c mice were immunized with three subcutaneous (s.c) injections of purified Naf1(N) protein at 2-week intervals. 100 μL of purified Naf1(N) protein was emulsified in the same amount of Freund's complete adjuvant (Wako) for the first injection and in Freund's incomplete adjuvant for the following two booster injections. After the third injection, immune response was assessed by screening blood samples from the immunized mice with Western blot analysis of purified Naf1(N) protein. The mouse showing highest immune response was given a final intraperitoneal booster injection with 100 μL of antigen, 2-weeks after the third injection and 3 days before fusion with myeloma cells.

Hybridoma preparation

Three days after the last immunization, the mice were killed and the spleens removed aseptically. Spleen cells were mixed with SP 2/0 myeloma cells at a ratio of 5:1 and fused in the presence of polyethylene glycol (PEG 1500; Roche) at 37°C. The cells were then pelleted and resuspended in RPMI 1640 medium (Sigma) supplemented with 20% fetal bovine serum (FBS; Cansera International Inc), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2% HAT (100 μM hypoxanthine, 400 nM aminopterin, and 16 μM thymidine) for hybrid selection. Aliquots of the cell suspension were plated in 96-well plates and cultured at 37°C in 5% CO_2. After 24 h, 100 μL of HAT-selective medium was added, and every 2 or 3 days half of the medium from each well was replaced with fresh HAT medium. Between the 12th and 15th days, cell growth appeared in the majority of wells, and the supernatants were screened by immunofluorescence staining for the specific antibody-secreting clones. Cells from the positive hybridomas were transferred to a 24-well plate and cultured in 1 mL of 1% HT medium. As hybridoma cells grew well, aminopterin was omitted from the medium. At days 2 and 4, 0.5 mL of HT medium was added to each well, and supernatants were screened again by immunofluorescence staining. Among five positive hybridomas, one strongly positive hybridoma (Hybridoma-4) was selected and subcloned by limiting dilution in HT medium (GIBCO), followed by screening and selection of the strongest clone (C-3). Hybridoma clone (C-3) was cultured in large scale for inoculation into mice.

Purification and isotyping of MAb

Hybridoma cells (2×10^6) were inoculated into the peritoneal cavity of three BALB/c mice treated 1 week before with pristane (Sigma) to generate ascitic fluids containing anti-Naf1 MAb. After 10 days, ascitic fluid was collected and purified using HiTrap-Protein G Sepharose columns (Pharmacia Biotech). Immuno-globulin isotyping was performed using mouse MAb isotyping kit (Amersham) according to the manufacturer's instructions.

Immunofluorescence staining

Immunofluorescence staining of Sf9 cells infected with recombinant baculovirus was done as described in the previous report.[33] In summary, cells were fixed in methanol for 5 min and incubated for 1 h at 37°C with anti-myc or anti-Naf1 MAb, followed by washing three times with PBS. Cells were then incubated with FITC-conjugated goat anti-mouse second antibody (American Qualex) for 1 h. As for control, the second antibody only was used. After thorough washings, cells were evaluated under fluorescence microscope (BX50F, Olympus Optical Co., Ltd.).

Silver staining and Western blot analysis

The extent of the purification of recombinant Naf1(N) protein was analyzed by silver staining and Western blot analysis. Aliquots of elusion, flow-through, and wash fractions were subjected to 12% SDS-PAGE, followed by silver staining.[34]

Cell lysates of 293 cells transfected with full-length Naf1 and Naf1(N) cDNA were subjected to Western blot analysis. Cell lysates were separated on a 12% SDS gel and blotted on an Immobilon-P (Millipore) transfer membrane. After incubation with anti-Naf1 MAb (1:1000 dilutions) and second anti-mouse antibody conjugated with horseradish peroxidase,

antibody binding was detected by chemiluminescence reagent.

Epitope determination of anti-Naf1 MAb

For the epitope mapping of the anti-Naf1 MAb, truncated forms of Naf1(N) fusion proteins were expressed in *Escherichia coli*, as described previously.[35] cDNAs encoding 6×His and myc-tagged Naf1(N)-1,2,3 and Naf1(N)-A,B,C fusion protein fragments, ligated in pMAL-C2 vector after digestion with *Bam*H1 and *Hind*III were transformed into *E. coli* (XL 2-Blue). Single colonies of transformed *E. coli* were grown in Luria-Bertani (LB) medium with kanamycin (25 μg/mL). Protein expression was induced with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) and incubated at 37°C for 3 h. The cells were then pelleted by centrifugation and resuspended in 100 μL of lysis buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA; 100 mM NaCl) followed by sonication. Cell lysates were applied to SDS-PAGE, followed by Coomassie Brilliant Blue staining or immunoblotting with anti-Naf1 MAb.

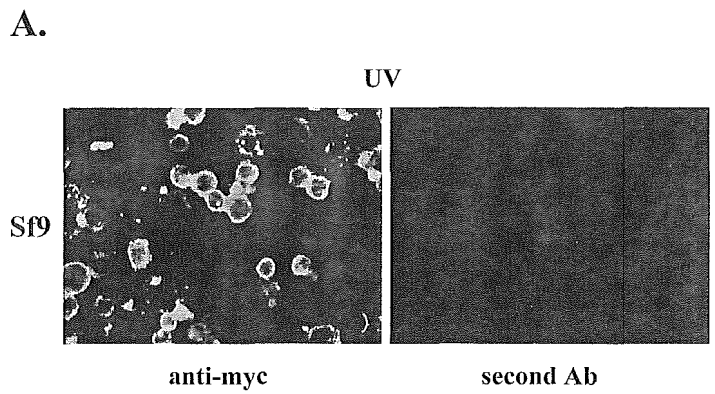
Immunofluorescence staining and confocal microscopy

Unstimulated peripheral blood lymphocytes (PBL), Jurkat, MT-4, and Molt-4 cells were washed with PBS and fixed in 2% paraformaldehyde for 20 min on ice. Cells were washed again with PBS containing 0.1% BSA, treated with 2% normal goat serum for 30 min to block non-specific binding, and then incubated with anti-Naf1 MAb (1:500 dilution) or control mouse IgG for 1 h at room temperature. After washing, cells were incubated with FITC-conjugated goat anti-mouse IgG antibody for 1 h at room temperature. Cells were then washed and mounted with Fluorescent Mounting Medium (Dako). Fluorescent images of endogenous Naf1 were evaluated by confocal microscopy using a Zeiss (LSM-510, V-2.5) Axioplan-2, laser-imaging confocal microscope.

RESULTS

Expression and purification of recombinant Naf1(N) protein

To generate MAb against Naf1 cellular protein, we first constructed the recombinant baculovirus encoding Naf1(N) cDNA by homologous recombination of pMelBac A-Naf1(N) cDNA with the replication-deficient baculovirus DNA in Sf9 cells using Bac-N-Blue transfection kit. Recombinant virus encoding Naf1(N) was then used to infect High Five cells for large-scale protein production. Expression of the recombinant Naf1(N) protein was examined by immunofluorescence staining of bac-



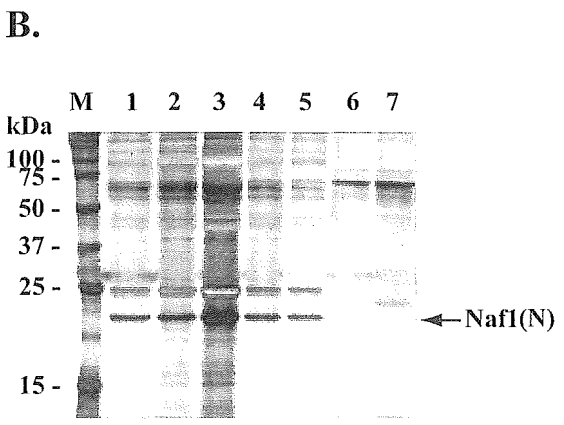


FIG. 1. (**A**) Immunofluorescence staining of Sf9 cells with anti-myc monoclonal antibody (MAb). Sf9 cells infected with baculovirus expressing 6×His and myc-tagged Naf1(N) were fixed in methanol and immunostained with anti-myc MAb, followed by fluorescein isothiocyanate (FITC)–conjugated second antibody (left panel) or with second antibody (Ab) only (right panel). (**B**) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Silver staining of purified recombinant Naf1(N) protein. M, unstained precision plus protein marker; lane 1–5, elusion fraction E1 to E5; lane 6, wash; lane 7, flow-through. The arrowhead at the right indicates the purified target of Naf1(N) protein.

ulovirus-infected Sf9 cells with anti-myc MAb (Fig. 1A). After protein purification, the purity of recombinant Naf1(N) protein was analyzed by SDS-PAGE and Silver staining, showing recombinant Naf1(N) protein as a major band as shown in Figure 1B. Silver staining of purified Naf1(N) protein denoted the presence of minor contaminants; however, none of these bands reacted with purified MAb in Western blot analysis (Fig. 2B), indicating that the contaminants are not the components of recombinant Naf1(N) protein.

Production and characterization of anti-Naf1 MAb

Female Balb/c mice, subcutaneously immunized with purified recombinant Naf1(N) protein, revealed antibody responses in Western blot analysis using mice sera, and we selected a mouse with highest antibody response for hybridoma preparation. We obtained five positive hybridomas, and from them, one strongly positive hybridoma (Hybridoma number-4) was selected by immunofluorescent assay. We then subcloned the hybridoma by limiting dilution, and the strongest clone (C-3) was selected and cultured in large scale to be inoculated into the peritoneal cavity of BALB/c mice. Ascitic fluid, containing anti-Naf1 MAb, was collected and purified by using Protein G Sepharose columns. Immunoglobulin isotyping showed the isotype of the purified anti-Naf1 MAb as IgG1 with a lambda (λ) light chain. The specificity of MAb was examined by immunofluorescence staining of Sf9 cells infected with recombinant baculovirus (Fig. 2A) and Western blot analysis using purified anti-Naf1 MAb (Fig. 2B, C). Anti-Naf1 MAb recognized recombinant Naf1(N) protein, but not control BSA (Fig. 2B), as well as full-length Naf1 and Naf1(N) in transfected 293 cell lysates (Fig. 2C). These results clearly show that anti-Naf1 MAb is specific to Naf1 protein, not to histidine or myc, tagged to the recombinant Naf1(N) antigen. According to previous reports, it has already been shown that hexahistidine tagging takes advantage of a high-affinity to Ni-NTA resin in the purification procedure without interfering with the protein function.

Epitope analysis of MAb

To complete the characterization of MAb, we investigated the Naf1 epitope recognized by this MAb. Polyhistidine and myc-tagged Naf1(N) fusion protein fragments (1, 2, and 3) with overlapping regions between amino acid residues 38 and 135, were generated (Fig. 3A). Protein expressions in induced *E. coli*

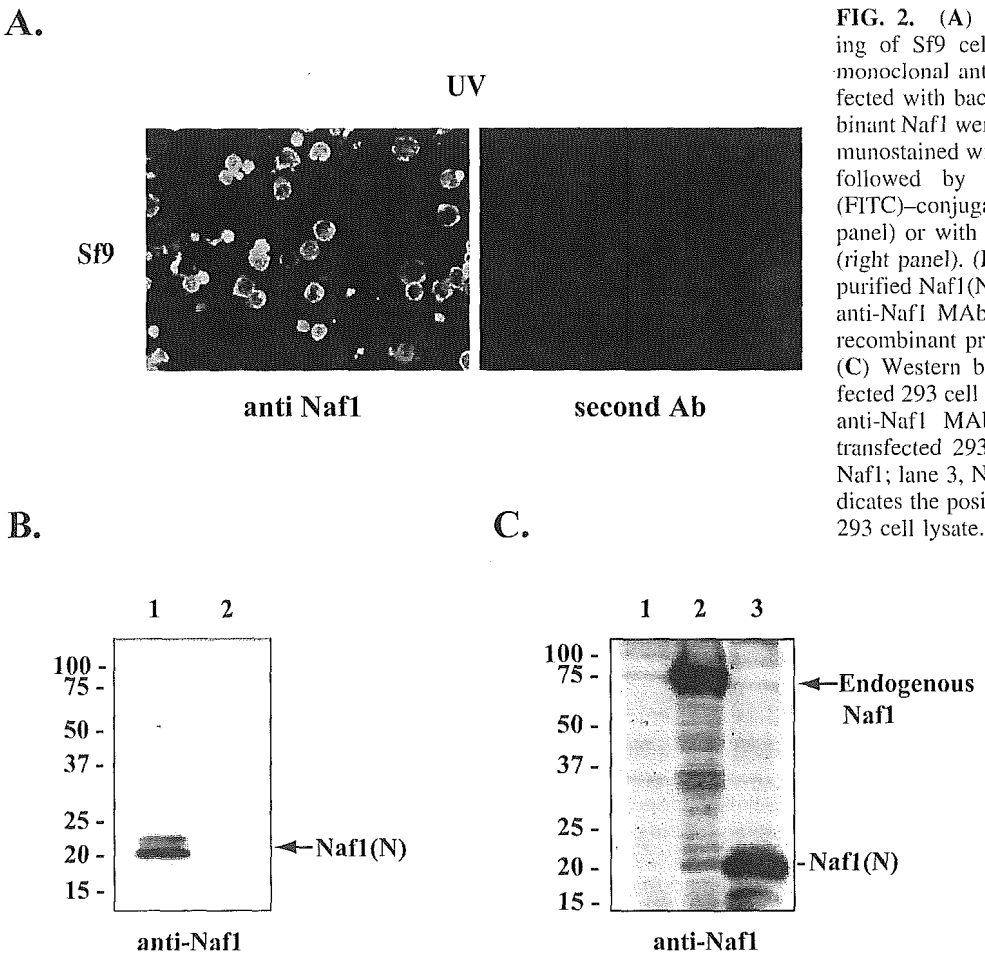


FIG. 2. (**A**) Immunofluorescence staining of Sf9 cells with purified anti-Naf1 monoclonal antibody (MAb). Sf9 cells infected with baculovirus expressing recombinant Naf1 were fixed in methanol and immunostained with purified anti-Naf1 MAb, followed by fluorescein isothiocyanate (FITC)–conjugated second antibody (left panel) or with second antibody (Ab) only (right panel). (**B**) Western blot analysis of purified Naf1(N), showing the reactivity of anti-Naf1 MAb. Lane 1, purified Naf1(N) recombinant protein; lane 2, control BSA. (**C**) Western blot analysis of Naf1-transfected 293 cell lysates for the specificity of anti-Naf1 MAb. Lane 1, control vector transfected 293 lysate; lane 2, full-length Naf1; lane 3, Naf1 (N). The arrowhead indicates the position of endogenous Naf1 in 293 cell lysate.