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A safety-modified SV40 Tag developed for human cancer immunotherapy

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Abstract: Simian virus 40 (SV40)-like DNA sequences have been found in a variety of human tumors, raising the possibility that strategies targeting SV40 may provide a potential avenue for immunotherapy directed against SV40 large T Antigen (Tag)-expressing tumors. We generated a recombinant vaccinia (vac-mTag) expressing mTag and herein assessed the ability of mTag to transform cells and to interact with anti-oncoproteins, as well as screened for the presence of potential HLA-A2.1-restricted epitopes within mTag. We found that transfection of cells with mTag did not lead to their transformation. Also, we demonstrated that mTag protein is degraded rapidly in cells. In addition, our work revealed that mTag did not physically interact with certain anti-oncoproteins. Finally, two potential HLA-A2.1-restricted functional epitopes within mTag sequence were identified. Our results show that mTag lacks the oncogenecity of full-length Tag and harbors potential HLA-A2.1-restricted immunogenic epitopes, hence suggesting the safety of vac-mTag for use in cancer immunotherapy.

Keywords: modified SV-40 T antigen, recombinant vaccinia, cancer immunotherapy

Introduction

Simian virus 40 (SV40) is a polyomavirus with DNA capable of transforming human and rodent cells in vitro and in vivo. SV40-transformed human cells are able to produce tumors when administered to nude mice (Brooks et al 1988; Reddel et al 1993). SV40 large T antigen (Tag), a multifunctional protein that orchestrates virtually every aspect of SV40 infection, is necessary and often sufficient for tumorigenesis (Shah 2000; Jasani et al 2001; Klein et al 2002; Garcea and Imperiale 2003).

At least a part of Tag’s ability to induce tumors stems from its ability to bind specific cellular tumor suppressor proteins, such as p53, pRb (retinoblastoma protein), and other Rb-related proteins (p107, p130, and p300), all of which exhibit properties of negative regulators of cell proliferation (Vilchez and Butel 2003a; Ahuja et al 2005).

Compelling evidence shows that SV40 homologous DNA sequences are present in human osteosarcomas, ependymomas, choroid plexus tumors, mesotheliomas, and non-Hodgkin’s lymphoma (Jasani et al 2001; Klein et al 2002; Garcea and Imperiale 2003). This suggested a link between SV40 and carcinogenesis in humans. It has been strongly speculated that the failure to inactivate SV40 contamination in the poliovaccines and adenovaccines from 1955 to 1963 played a significant role in introducing SV40 to humans (Butel and Lednicky 1999).

Although the direct role that SV40 Tag plays in tumorigenesis in humans is still to be determined, the fact that it is expressed in human tumor cells makes it a potential target for immunotherapy targeting these tumors.

Vaccinia virus recombinants are being used as efficient tools for antigen delivery in cancer immunotherapy both in mice and humans (Shen and Nemunaitis 2005; Phelps et al 2007; Song et al 2007). We have constructed a vaccinia-based recombinant (vac-mTag), safety-modified version of SV40 Tag (mTag), devoid of pRb, p53 binding
sites, and the amino-terminal oncogenic CR1 and J domains to optimize potential clinical safety, but still preserve immunogenic domains. Our previous studies have shown that vac-mTag can induce tumor antigen-specific immunity in rodents (Xie et al 1999). In the present report, we describe the suitability of use of mTag in immunotherapy by evaluating both safety and immunogenicity of the protein.

**Materials and methods**

**Plasmids**

An approximately 2.2-Kb BamHI DNA fragment containing SV40 large Tag was cut out from pSP64-Tag (a kind gift from Dr J Butel, Baylor College of Medicine, Houston, TX), subcloned onto pcDNA3 (Invitrogen, Carlsbad, CA) at BamHI site, and named Tag/pcDNA3. The orientation of Tag was verified by PsI and XhoI digestions. pSC65-mTag plasmids (Xie et al 1999) were digested with BglIII and PacI to recover an about 1.1-Kb DNA fragment containing mTag and the mTag DNA fragments were then ligated to pcDNA3 plasmids digested with BamHI and EcoRV. The resulting product was a 6.5-Kb linear DNA fragment carrying ligated pcDNA3 and mTag through the BamHI and BglIII site, respectively. The linear DNA fragment was then run on a 0.8% agarose gel, recovered, and blunted-ended at the PacI end with Mung Bean Nuclease (Life Technologies, Inc., Gaithersburg, MD). The blunt-ended products were subjected to a ligation reaction to form a circular plasmid through the EcoRV of pcDNA3 and the blunt-ended PacI of mTag and named mTag-pcDNA3. Two independent clones were used for the experiments.

**Cell lines**

BALB/c 3T3 embryonic fibroblast cell line was obtained from ATCC (Manassas, VA). 209 R1B1, an SV40-infected cell line, has been described previously (Bender et al 1983). All cell lines in this study were maintained in complete Dulbecco’s Modified Eagle’s Medium (DMEM). Geneticin was used to select plasmid-carrying cells.

**Transfection and transformation assays**

10⁶ BALB/c 3T3 cells/well were transfected with 5 μg of empty pcDNA3, TagpcDNA3, and/or mTagpcDNA3 using the transfection kit ProFection (Promega, Madison, WI). Cells were then washed once and incubated in fresh medium for 24 hours. Approximately 6/10 of the cells from each well were transferred onto a 100 mm culture dish for examining focus-formation on monolayer cells. 3/10 of the cells were grown in media supplemented with a final concentration of 500 μg/ml of Geneticin to select for transfectants for soft agar assays. The remaining 1/10 cells were subjected to Geneticin selection to determine transfection efficiency for each plasmid.

For the focus-formation on monolayer cells, 100 mm culture dishes were incubated at 37 °C/5% CO₂ for four weeks. Cells were then stained with 0.5% Wright’s dye. Cells were washed and air-dried before the pictures were taken.

To set up soft agar assays, 10,000 transfectants in 1 ml of medium were mixed with 1 ml of medium containing 2% agarose LE. The mixture was plated on top of solidified bottom agar (7 ml of 2% agarose LE-containing medium) on 60 mm culture dishes and incubated at 37 °C/5% CO₂ for 6 weeks. A small amount (~0.5 ml) of fresh liquid media was added onto the plates occasionally during the incubation to prevent solid media from drying out. Colony numbers were then scored under a microscope.

**Immunoprecipitation**

Cells were collected after incubation in media containing 1 mM EGTA for about 16 hours and resuspended in 0.5 ml iced-cold cell lysis buffer (50 mM Tris-HCl [pH. 7.5], 120 mM NaCl, 5 mM EDTA, 0.05% IGEPAL CA-630, 1 μg/ml Aprotinin, 1 μg/ml Leupeptins, and 1 μg/ml Pepstatin A) and incubated on ice for 10 minutes. Cells in suspension were lysed in a glass dounce homogenizer (Knotes) on ice. The crude cell lysates were cleared by centrifugation and protein concentration was determined by the Bradford assay. Immunoprecipitation assays were performed using the Seize X Protein G kit (Pierce, Rockford, IL) following the manufacturer’s protocol. Immunoprecipitated proteins on each column were eluted with 190 μl of elution buffer after extensive washing. The eluted protein products were neutralized with 10 μl of 1 M Tris-HCl, pH. 9.5 and separated by SDS-PAGE.

**Western immunoblot**

Proteins were separated on SDS-PAGE and transferred to a Hybound-P membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was blocked with 5% non-fat dry milk in TBS-T. Antibodies against Pab204, pRb and p53 were added at appropriate dilutions in 2% nonfat milk/1X TBS-T and incubated at room temperature for 2 hours. The membrane was then washed and incubated with a suitable secondary antibody, and then washed, developed using the ECL+Plus chemiluminescence detection system (Amersham Pharmacia Biotech), and exposed to X-ray films.

**T2-loading assay**

Potential HLA-A2.1-restricted peptides were predicted using the algorithm BIMAS (see http://bimas.dct.nih.gov/
ELISPOT

HLA-A*0201/Ka-transgenic mice (generated by Dr Linda Sherman at The Scripps Research Institute, La Jolla, CA) were purchased from Harlan Sprague-Dawley (Indianapolis, IN) were immunized with 10⁷ pfu of vac-mTag through tail-vein injection. Three weeks later, spleens were harvested and CD8⁺ T cells were isolated from splenocytes using negative selection kits from Miltenyi Biotec Inc. (Auburn, CA) following the manufacturer’s protocol. DCs were derived from bone marrow using IL-4 and GM-CSF as described previously with some modifications (Inaba et al 1992). DCs were loaded overnight with individual peptides (20 μg/ml). The next day, peptide-loaded DCs were irradiated with 5,000 rads. For the ELISPOT assay, multiscreen 96-well plates were coated with capture anti-IFNγ Ab (4 μg/ml; PharMingen; San Diego, CA) overnight at 4 °C, washed and blocked with 1% BSA/1X PBS (150 μl/well). One million CD8⁺ splenocytes and 0.1 million of peptide-loaded DCs were seeded into each well and the plates were incubated at 37 °C/5% CO₂ for 24 hours. The plates were washed and 100 μl/well of biotinylated rat anti-mouse IFNγ Ab (2 μg/ml; PharMingen) were added and incubated overnight at 4 °C. On day 3, the plates were washed and 100 μl/well of anti-biotin Ab (1:1000 dilution; Vector; Burtingame, CA) were added and left for 90 min. The plates were washed and developed with NBT/BCIP. The number of spots was determined using an ELISPOT reader (Cell Technology Laboratory, Inc.; Cleveland, OH).

Statistical analysis

Statistical analysis was performed where appropriate using Student’s T-test. Differences were considered as significant when the p value was <0.05.

Results

mTag-bearing plasmids do not induce transformation in BALB/c 3T3 cells

We first assessed the ability of Tag- and mTag-bearing plasmids to transform transfected cells. To this aim, we used focus-formation on monolayer cells and colony formation on soft agar. BALB/c 3T3 cells were transfected with empty vectors, Tag-pcDNA3, and/or two independent clones of mTag-pcDNA3. Transfected cells were plated onto 100 mm culture dishes separately without geneticin selection to look for focus-formation. 209R1B1 cells, which are BALB/c 3T3 cells with integrated SV40 DNA and carry at least two copies of Tag (Bender et al 1983), were plated as a positive control. As expected, numerous foci were found on plates with 209R1B1 cells after four weeks of incubation. Approximately a hundred foci were observed on each plate with cells transfected with Tag-pcDNA3 as well. In contrast, only a few foci developed on the plates with cells transfected with empty vectors or mTag-pcDNA3 (Figure 1).

For soft agar assays, cells were subjected to geneticin treatment to select for transfectants. 10,000 transfectants were then plated onto each soft agar plate. Colonies growing on each plate were counted after six weeks of incubation. Approximately 20% of 209R1B1 cells and 2% of cells transfected with Tag-pcDNA3 were able to form colonies on soft agar, whereas less than 0.1% cells transfected with empty plasmids and/or the two independent clones of mTag-pcDNA3 exhibited growth (Figure 1).

mTag protein is expressed in transfected cells and subjected to a faster proteolytic degradation in vivo compared with SV40 Tag

We further sought to evaluate the expression of mTag protein in transfected cells. Cell extracts were prepared and subjected to Western blot analysis using Pab204, a monoclonal anti-Tag antibody which has been shown to be able to detect both full-length Tag and mTag proteins (Xie et al 1999). The 42-kDa band corresponding to mTag protein was not detectable by Western blot in cell extracts of cells transfected with two independent clones of mTag-pcDNA3 after long exposures (Figure 2). In contrast, RT-PCR and Northern blot analysis showed that there was at least equal amount of steady-state mTag mRNA compared to that of full-length Tag in cells (data not shown). These observations indicated that mTag was expressed and suggested that mTag protein might be unstable in cells. To investigate this possibility, cells were pre-treated with EGTA prior to cell lysis preparation to decrease protease activity. Interestingly, a robust expression of mTag proteins could be detected after the EGTA treatment (Figure 2). This result indicates that mTag protein was synthesized in mTag-pcDNA3-transfected cells and is highly sensitive to protease degradation.
Figure 1 Focus-formation on monolayer cells. (A) BALB/c 3T3 cells transfected with pcDNA3, Tag-pcDNA3, mTag-pcDNA3 clone 1 (mTag-pcDNA3-1), and mTag-pcDNA3 clone 2 (mTag-pcDNA3-2), and 209 R1B1 cells (BALB/c 3T3 cells infected with SV40) were plated. Four weeks later, cells were stained with 0.5% Wright's stain in methanol. Each dish is a representative of four plates in two independent transformation assays. (B) Numbers of colonies growing on soft agar (>50 cells). Statistical analysis showed that colony formation after transfection with Tag-pcDNA3 was greater (p = 0.001, T-test) than transfection with pcDNA3 vector (negative control). In contrast, mTag-pcDNA3 transfection did not show significant increase of colony numbers over pcDNA3 background.

Figure 2 Expression and stability of Tag and mTag proteins in transfected 3T3 cells. Protein extracts were prepared from transfected cells with or without 1 mM EGTA treatment. 100 μg of total proteins from each extract were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with Pab204 (a monoclonal anti-Tag (mTag) antibody), HRP-conjugated goat anti-mouse antibody, and developed with ECL-Plus reagent. Protein extracts of 209 R1B1 and BALB/c 3T3 cells were used as positive and negative control for SV40 Tag protein. vac-mTag-infected CV-1 cells (vac-mTag/CV-1) served as a positive control for mTag protein.
mTag proteins do not physically interact with pRb and/or p53
Because SV40 Tag exerts its oncogenecity mainly through its interaction with regulators of cell growth, such as pRb and p53, we designed a safety-modified version (mTag) that lacks the pRb- and p53-interacting domains, as well as the amino-terminal oncogenic CR1 and J domains (Xie et al 1999). To prove the loss of interaction of mTag with pRb and p53, cell extracts were prepared after EGTA treatment and subjected to immunoprecipitation using anti-pRb (Figure 3A) and/or anti-p53 antibodies (Figure 3B). To include approximately equal amounts of p53 protein in the immunoprecipitation assays using anti-p53 antibodies, five times lesser amount of total proteins of Tag-expressing lysates (Tag-pcDNA3 and COS-1) were used in the reactions (Figure 3B, top panel). As expected, both anti-pRb and anti-p53 antibodies co-precipitated full-length Tag (Figure 3A and B), but not mTag (Figure 3C). Immunoprecipitation assays were subsequently performed using Pab204 as well. Consistent with above results, immunoprecipitated-Tag protein, but not mTag protein, pulled down both pRb and p53. Taken together, these data suggest that mTag, in contrast to Tag, does not physically interact with pRb and/or p53.

Identification of two novel potential HLA-A2.1-restricted epitopes within mTag sequence
Having established the safety of mTag, we further evaluated its immunogenic potential by screening its sequence for potential HLA-A2.1-restricted epitopes. We tested two peptides (Tag 8 and Tag 27) in addition to a previously identified A2.1-restricted epitope (Tag 37) (Schell et al 2001), which also localizes within mTag sequence. Both peptides (Figure 4A; Tag 8: lower left; Tag 27: lower right) as well as Tag 37 (upper right), but not a negative control peptide (Tag II/III; H2-Db) (light grey) (Tanaka et al 1988), exhibited binding to HLA-A2.1 molecules on T2 cells, as compared with unloaded T2 cells (upper left; dark grey).

To determine if the binding peptides could be functional HLA-A2.1 epitopes, we used the IFN-γ ELISPOT assay to examine whether elicited CD8+ splenocytes from vac-mTag immunized HLA-A2.1 transgenic mice were capable of responding to peptide-specific stimulation. Peptides were loaded onto A2.1 Dendritic Cells (DCs) to serve as stimulators for the ELISPOT assay. vac-mTag immunization elicited CD8+ splenocytes that responded to Tag 23 stimulation, compared to that of unloaded DCs. This response was
mTag-specific as the CD8+ splenocytes from A2.1 mice immunized with a control vaccinia (vac-PSA) did not respond to Tag 23 stimulation. Compared to Tag 23, Tag 8 and 37 peptides induced significantly lower reactions (Figure 4B).

**Discussion**

We have previously characterized vac-mTag, a recombinant vaccinia virus expressing a safety-modified version of SV40 Tag, that confers protection against tumors and provides therapeutic efficacy against pre-established SV40 Tag-expressing tumors in rodents (Xie et al 1999). Since SV40 Tag protein is a potent oncoprotein, it is therefore crucial to address the safety of mTag as a therapeutic.

It is well established that expression of SV40 Tag in rodent cells, such as BALB/c 3T3 cells, leads to cell transformation, including focus-formation on monolayer cells and ability to grow on soft agar. These characteristics of transformed cells were therefore adopted to examine if...
mTag protein retains the oncogenic ability of its full-length version. As expected, SV40 Tag-transfected cells formed hundreds of foci on monolayer cells and acquired the ability to grow on soft agar. In contrast, mTag-transfected cells neither formed foci on monolayer cells nor grew on soft agar when expressed in BALB/c 3T3 cells. This indicates that mTag-transfected cells were not transformed. The expression of mTag was demonstrated by RT-PCR and Northern analyses (data not shown). Additionally, the transfection efficiency of Tag- and/or mTag-carrying plasmids was nearly identical (10%–15%; data not shown).

To test the stability of mTag in transfected cells, mTag-transfected cells were treated with EGTA to decrease cellular protease activity before cell lysate preparation. Under these conditions, the presence of mTag protein was readily detected, suggesting that mTag protein was indeed unstable. The labile nature of mTag protein could be attributed to the fact that it is a truncated form of protein and is hence unable to fold into a correct and stable structure. In addition, mTag protein lacks the Nuclear Localization Signal (NLS) of full-length Tag and is localized into the cytoplasm (data not shown), which is not a native environment for SV40 Tag protein. Accordingly, the mis-localization of mTag protein may also contribute to its unstable behavior. The short life span of mTag protein, which further reduces its capability to interact with anti-oncoproteins, provides an additional safety feature for mTag. The fact that mTag protein does not transform rodent cells, a preferred system for the oncogeneity of SV40 Tag compared to human cells, suggests that mTag protein is unlikely to be able to transform mammalian cells in general.

mTag was constructed to exclude the oncogenic motifs of full-length Tag, mostly the domains interacting with anti-oncoproteins. To confirm the loss of oncogenicity, immunoprecipitation assays using an anti-pRb, an anti-p53 antibody, and/or an anti-Tag (mTag) antibody (Pab204) were employed. In contrast to the full-length Tag protein, mTag protein neither co-immunoprecipitated with pRb nor with p53 under a condition where mTag protein was stable due to EGTA treatment. Previous studies have shown that the expression of Tag protein leads to an over-expression of p53 (Deppert et al 1987; Kohli and Jorgensen 1999). Western blot using a monoclonal anti-p53 antibody indeed revealed that there was a robust expression of p53 in cells transfected with Tag-pcDNA3, but not in cells transfected with either clone of mTag-pcDNA3 clone (data not shown). Immunoprecipitation assays using an anti-p53 antibody was designed in such a way to include approximately equal amounts of p53. Consequently, the failure of anti-p53 antibody to co-immunoprecipitate mTag protein was not due to the fact that there was less p53 protein in the starting lysates. Additionally, it is thought that the reason for an over-expression of p53 in SV40 Tag-expressing cells is caused, in part, by the binding of Tag to p53 that leads to its stabilization (Deppert et al 1987). These results were further supported by the fact that p53 levels were not elevated in cells transfected with mTag-pcDNA3, showing that there is no physical interaction between mTag protein and p53. Interestingly, we also observed an over-expression of pRb in CV-1 cells infected with vaccinia virus (Figure 3A, top panel). When cell lysates prepared from CV-1 cells infected with vac-mTag were used in immunoprecipitation assays, anti-pRb failed to co-immunoprecipitate mTag. These data showed that mTag does not interact with pRb even in a condition when pRb is unusually abundant. Therefore, our results conclusively demonstrated that mTag, unlike full-length Tag, does not interact with pRb and/or p53.

Schell and colleagues (2001) have previously identified a human HLA-A2.1-restricted epitope (Tag 37) from Tag. Here we identified two new potential epitopes within mTag sequence capable of binding to HLA-A2.1 molecules. It is worthy of noting that both our peptides caused greater stabilization of HLA-A2.1 compared with Tag 37, as judged by the shift in HLA-A2.1 expression intensity on T2 cells following peptide loading (MFI units 19.1, 25.7 and 18.8 for Tag 8, Tag 27 and Tag 37, respectively), suggesting that Tag 8 and Tag 27 are stronger binders to HLA-A2.1. Consistent with the binding profile of these peptides, Tag 23 elicited stronger CTL activity than did Tag 8 or Tag 37. Although Tag 23 was able to induce detectable CTL activity, the number of Tag 23-specific population was relatively low (10–80 per million CD8+ splenocytes) compared with the well-studied H2-Kb-restricted Tag epitope (epitope IV (Mylin et al 1995); 1200–1500 per million CD8+ splenocytes; data not shown) in a similar setting. The low expression of HLA-A2.1 in A2.1 mice may account for the relatively low reaction we observed in this study. Also, in the present work, we did not test the ability of the CTL elicited by these mTag-derived peptides to recognize tumor cells expressing full length Tag. This is due to the fact that we have previously demonstrated that immunization of mice with mTag included an SV40 Tag-specific cytolytic T-lymphocyte activity against syngeneic (identical genetic background) SV40 Tag-expressing tumor targets, and immunization of mice with a single dose of mTag-expressing vaccinia (vac-mTag) resulted in potent protection against
subsequent challenge with a lethal mouse cancer expressing SV40 Tag (Xie et al 1999).

Research utilizing HLA-A2.1 transgenic mice lacking the mouse MHC I (HHD mice) will be able to provide us with more insight into the strength of the epitopes. Additionally, the algorithm we used to predict binding epitopes has generated many other potential HLA2-A2.1 epitopes within mTag sequence, which could be evaluated in future studies. Although involvement of SV40 Tag in neoplastic cell transformation in humans remains controversial, its presence in a variety of human tumors is well established (Carbone et al 1997; Butel and Lednicky 1999; Shah 2000; Jasani et al 2001; Klein et al 2002; Garcea and Imperiale 2003; Vilchez and Butel 2003b). SV40 Tag protein is therefore a potential target for immunotherapy. In our previous (Xie et al 1999) and present report, we have shown that mTag protein is immunogenic but deprived of its oncogenic potential. Our work provides an initial safety characterization of mTag protein and a rationale for using vac-mTag as a tool in immunotherapy in humans.

Disclosure
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