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EXPRESSION OF SSX2 TUMOR ANTIGEN IN BACULOVIRUS EXPRESSION SYSTEM AND ITS APPLICATION FOR SCREENING OF BLOOD SERUM OF MELANOMA PATIENTS

*R.G. Kyyamova[1, *], V.S. Gryshkova[1], A. M. Zhyvoloup[1], S.I. Korovin[2], I.T. Gout[1, 3], V.V. Filonenko[1]*
[1]*Institute of Molecular Biology and Genetics, Kyiv, Ukraine*
[2]*Institute of Oncology, AMS of Ukraine, Kyiv, Ukraine*
[3]*University College London, Gower Street, London WC1E 6BT, United Kingdom*

Aim: **To clone, express in baculovirus expression system and purify SSX2 tumor antigen and to use it for screening of blood serum of melanoma patients.** ***Materials and Methods:*** **Cloning and expression of SSX2 antigen in Bac-to-Bac baculovirus expression system as His-tag fusion protein, expression of recombinant SSX2 in insect cells with following purification by affinity chromatography on Ni-NTA agarose, ELISA of blood serum of melanoma patients (n = 29) and healthy donors (n = 27) were used.** ***Results:*** **SSX2 was cloned in baculovirus expression system, expressed, purified using affinity chromatography and used in ELISA as antigen. Comparative analysis of blood serum of melanoma patients and healthy donors revealed higher level of SSX2-positivity of blood serum from the patients with cancer.** ***Conclusion:*** **SSX2 antigen expressed in baculovirus expression system can be used for serological analysis of blood serum of oncological patients.**

Key Words: **tumor antigen, SSX2, baculovirus, melanoma.**

The development of specific immunotherapy for human malignancies is based on explicit recognition by the immune system of tumor-specific or tumor-associated antigens. Therefore, the search for tumor-associated antigens has been the subject of intense research for many years. Recent advances in molecular biology and immunology have created the platform for the identification of novel tumor-associated antigens by various approaches, including serological identification of recombinantly expressed clones methodology (SEREX). To date more than 2,000 antigens have been listed in the SEREX database created by the Ludwig institute for Cancer Research. Detailed analysis of their expression profiles in normal and malignant tissues, extensive allogenic screening of patient's blood sera and testing T-cells response by ELISSPOT assay would allow selecting the most interesting candidates for the development of immunotherapeutic approaches and diagnostics. To carry out these studies large amount of recombinant proteins predominantly expressed in eukaryotic systems are needed. Bac-to-Bac® Baculovirus Expression System is an efficient site-specific transposition system to generate baculovirus for high-level expression and purification of large amounts of soluble and properly folded recombinant proteins in insect cells, especially for antigens carrying the antigenic determinant of conformational type, such as SSX2 protein. SSX2 was originally identified by SEREX screening of melanoma cDNA library [1]. SSX2 (HOM-Mel-40) is involved in the t (X; 18) translocation in synovial sarcoma and belongs to family SSX antigens [2]. SSX2 is expressed in tumors of different origins (lymphoma, malignant melanoma, glioma, synovial sarcoma, breast, colorectal, lung, prostate, bladder, head-neck, endometrial and renal cell cancer), but not in normal tissues except testis and thyroid gland that exhibits a weak SSX2 expression [3]. Significant levels of specific antibodies to SSX2 have been found in melanoma, colon and breast cancer patient's blood sera [4], indicating that spontaneous immune responses directed against SSX2 antigen can occur. The tumor specific expression profile of *SSX* genes together with the spontaneous immunogenicity of the corresponding gene products have encouraged researchers to use SSX2 for generic anticancer immunotherapy. Recently, it has been shown that $CD8^+$ T-cell response to $SSX2_{41-49}$ peptide frequently occurs in SSX2 expressing melanoma patients and suggest that $SSX2_{41-49}$ specific CTLs of high avidity and tumor reactivity are selectively expanded during immune response to SSX2-expressing tumors *in vivo* [5, 6]. At the same time accumulating evidence supports the requirement for both tumor-specific $CD8^+$ and $CD4^+$ T-cell responses for efficient tumor rejection to occur. The first $CD4^+$ T-cell epitope encoded by SSX2 mapped to the 19–34 region of the protein was identified using $CD4^+$ T-cells from an SSX2-expressing melanoma patient [7]. These findings confirm the previously observed immunogenicity of SSX2 in SSX2-expressing cancer patients and encourage the onset of clinical trials of vaccination with SSX2 immunogenic molecules. These data indicate that SSX2 antigen is a promising candidate for cancer immunotherapy.

It is essential for investigation of SSX2 antigen as a target for immunotherapy of cancer to perform high-scale analysis of SSX2 immunoreactivity with a panel of blood sera from cancer patients. For this purpose, a large amount of highly purified recombinant SSX2 protein is required. Expression of SSX2 antigen in bacterial system allowed the production of fully inso-

Received: April 14, 2006
*Correspondence: Fax +38 (044) 522 61 00
E-mail: kyyamova@imbg.org.ua

Abbreviation used: ELISA – enzyme-linked immunosorbent assay; SEREX – serological identification of recombinantly expressed clones; CT – cancer-testis.

luble protein. Therefore, the application of bacterially expressed SSX2 for serological analysis is limited [1, 9, 10]. The aim of this study was to clone, express and purify soluble and properly folded SSX2 antigen using Baculovirus Expression System and to use it for screening of blood serum of melanoma patients.

MATERIALS AND METHODS

SSX2 gene was cloned into pFasBac™HTb donor plasmid from pcDNA3/SSX2 plasmid using *BamH1* and *Hind III* restriction sites. The full length sequence of *SSX2* was cloned in frame with the sequence for Histag necessary for affinity purification of recombinant protein. Competent XL1 *E.coli* cells were transformed by recombinant pFastBac™ HTb/SSX2 plasmid using standard procedure. Recombinant plasmid DNA was isolated from ampicillin-resistant transformants using the Qiagen MiniPrep Kit (Qiagen, USA). The size of insert was evaluated by restriction analysis at *BamH1* and *Hind III* sites.

MAX Efficiency DH10 Bac™ chemically competent cells (Invitrogen, USA) have been transformed with 1 ng (5 µl) pFastBac™ HTb /SSX2 construct to produce recombinant bacmid. Selection of DH10 Bac™ transformants was carried out on LB agar plates containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracyclin, 100 µg/ml Blue-gal, and 40 µg/ml IPTG for 48 h at 37 °C. Recombinant bacmid DNA was purified from selected white colonies using Qiagen MiniPrep Kit (Qiagen, USA) with some modifications.

To generate recombinant virus, 9 x 10^5 Sf9 cells were transfected in incomplete IPL41 medium (Sigma, USA) with 1 µg (5 µl) of purified recombinant bacmid DNA as describe in Bac-to-Bac Baculovirus Expression System manual (Invitrogen, USA). We used Insectin Plus™ reagent (Invitrogen, USA) instead of Cellfectin Reagent for transfection. Tranfected cells were incubated in supplemented IPL 41 insect medium (10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin, yeast extract) for 72 h at 27 °C until the signs of viral infection become visible.

Sf9 insect cells have been removed from the medium by centrifugation at 500 x g for 5 min and clarified supernatant (P1 viral stock) was transferred to fresh tube and stored at +4 °C, protected from light. Amplified P2 viral stock was obtained by infection of 2 x 10^7 insect cells with 400 µl of P1 viral stock in 15 ml growth medium.

Purification of His-SSX2 antigen has been performed from the pellet of insect cells infected with P1 viral stock. Cells were lysed in 1.5 ml of cold lysis buffer (50 mM Tris HCl, pH 8.5, 100 mM KCl, 5 mM 2-ME, 1% NP40, 1 mM PMSF, 10 mM benzamidine, 1 µg/ml pepstatin), centrifuged at 10000 x g for 15 min and clarified supernatant was used for binding with 50 µl Ni-NTA agarose during 1 h at 4 °C. The beads were consequently washed with 10 ml of ice-cold buffer A (20 mM Tris HCl, pH 8.5, 500 mM KCl, 5 mM 2-ME, 10% glycerol, 10 mM imidazole), 2 ml buffer B (20 mM Tris HCl, pH 8.5, 1000 mM KCl, 5 mM 2-ME, 10% glycerol) and 2 ml buffer A again. The bound proteins were eluted with 1 ml buffer C (20 mM Tris HCl, pH 8.5, 100 mM KCl, 5 mM 2-ME, 10% glycerol, 300 mM imidazole). The purity of obtained protein was analyzed by SDS-PAGE electrophoresis.

In the study, the samples of blood serum from 29 patient with skin melanoma of stages II-III, cured in the Institute of Oncology, AMS of Ukraine (Kyiv, Ukraine) were used. Serum samples were obtained by routine procedure and stored in 50% glycerol until use at –20 °C. Blood serum samples from randomly selected healthy donors were used as the control.

For ELISA assay 1 µg/well of recombinant SSX2 protein in PBS buffer (150 mM NaCl, 2 mM KCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 1.5 mM KH_2PO_4, pH 7.4) was adsorbed to 96-well polystyrene plates (Sarsted, Germany) overnight at 4 °C. Plates were washed with PBS and blocked for 2 h at 37 °C with 200 µl/well of 0.1% Tween/PBS. After washing, 100 µl/well of diluted serum in PBS from cancer patients was added and incubated for 2 h at 37 °C. Plates were washed and 100 µl/well diluted secondary Goat anti–human IgG-AP (Life Technologies Inc, UK) antibody (0.05 % Tween/PBS) was added and incubated for 1 h at 37 °C. Sera were tested over a range of serial dilutions from 1 : 100 to 1 : 10,000. Plates were washed, incubated with 100 µl/well of substrate ABTS-solution (Sigma, USA) for 25 min at room temperature, and immediately developed on Anthos 2001 (Anthos Labtech Inc., Austria). A positive reaction is defined as an OD value of a 1 : 1000 diluted serum that exceeds the mean OD value of sera from normal donors (n = 27) by three standard deviations.

RESULTS AND DISCUSSION

Bac-to-Bac Baculovirus Expression System (Invitrogen, USA) has been employed for rapid and efficient generation of recombinant baculovirus and expressing of SSX2 protein in insect cells. Fig. 1 represents the most important steps of generation recombinant baculovirus and expression of gene of interest.

The full length *SSX2* gene was cloned into pFastBac™HT donor vector B as described in *Materials and Methods*. Generation of recombinant bacmid was performed in DH10Bac™ *E.coli* cells containing a baculovirus shuttle vector (bacmid) and a helper plasmid pMON7124. Then, the SSX2/pFastBac™HTb expression plasmid was transformed into DH10Bac™ cells. The transposition occurred between the mini-Tn7 element on the pFastBac™ vector and the mini-*att*Tn7 target site on the bacmid (see Fig. 1). This transposition reaction is facilitated by transposition proteins encoded by the helper plasmid. Insertion of the mini-Tn7 into the mini-*att*Tn7 attachment site on the bacmid disrupts the expression of the LacZα peptide and the colonies, containing the recombinant bacmid, become white on the background of blue colonies that harbours the unaltered bacmid. From the pool of colonies, we have selected three white colonies for the purification of recombinant bacmids. The quality of purified bacmids was tested by agarose gel electrophoresis. Successfully produced bacmids were transfected into Sf9 insect cells to produce recombinant baculovirus.

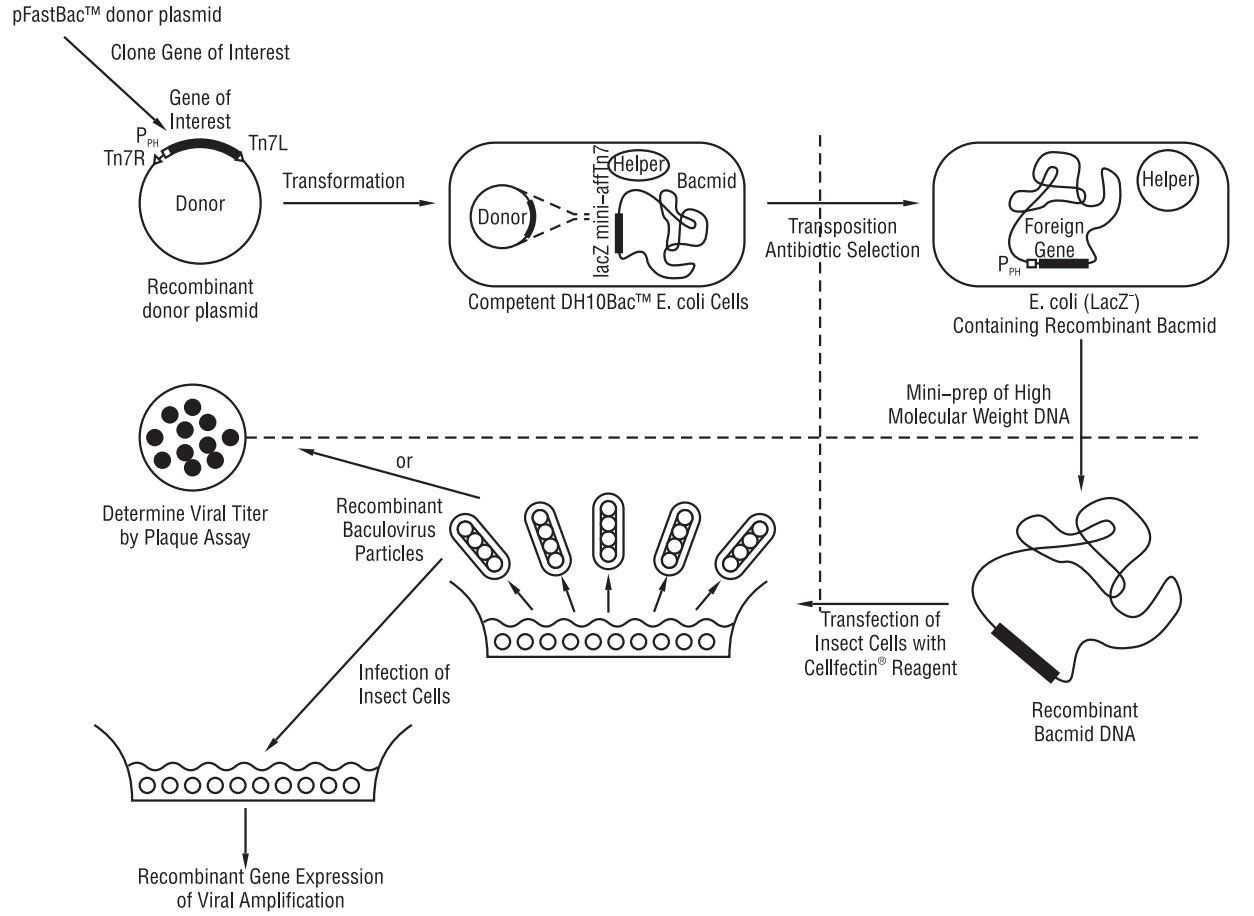

Fig. 1. Generation of recombinant baculovirus and the expression of gene of interest using Bac-to-Bac® Baculovirus Expression System

Starting from 72 h after transfection, we visually inspected the cells daily for infections. Virally infected insect cells usually exhibited increased cell diameter, large nuclei, granular appearance, and the detachment from plate at the final stage of infection followed by cell lysis. We have harvested supernatant from the cell culture medium that represented P1 viral stock (> 10^6 pfu/ml) and used this stock for viral amplification (P2 viral stock) and recombinant His-SSX2 expression (see Fig. 1). The P2 viral stock represents high-titer baculovirus stock (> 10^7 pfu/ml) that can be stored at –80 °C for a long time and used for viral amplification (P3 viral stock) and expression of recombinant protein.

Initially, we verified the expression and solubility of recombinant His-SSX2. For this purposes, we used the lysate of insect cells obtained after amplification of P1 viral stock. Clarified supernatant was used for affinity purification of His-SSX2 recombinant protein by the Ni-NTA agarose chromatography. As shown in Fig. 2, recombinant His-SSX2 antigen is expressed as a soluble protein in baculovirus-infected insect cells (lane 1 and 2). Moreover, it binds very efficiently to the Ni-NTA agarose. Imidazole-eluted proteins were collected, dialyzed in buffer containing 20 mM Tris HCl, pH 8.5, 100 mM KC., 5 mM 2-ME, 10% glycerol. The concentration of purified His-SSX2 was measured by Bradford assay (0.3 mg/ml). Recombinant protein was stored at –70 °C before usage.

The data presented above show that we have created recombinant SSX2 baculovirus and induced the expression of soluble His-SSX2 protein in infected Sf9 cells. Large-scale infection of insect cells with produced virus allowed us to purify in one step appropriate quantities of His-SSX2 protein, which could be used for biochemical, structural and immunological studies.

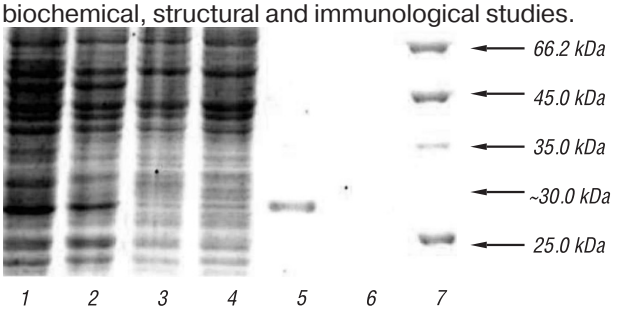

Fig. 2. Expression and purification of recombinant SSX2 protein (SDS-PAGE). Lane 1 — total cell lysate of Sf9 cells infected with generated SSX2 virus; Lane 2 — soluble fraction of cell lysate of Sf9 cells, infected with generated SSX2 virus; Lane 3 — total cell lysate of Sf9 cells infected with the wild type baculovirus (control); Lane 4 — soluble fraction of Sf9 cells infected with the wild type baculovirus (control); Lane 5 — immidazole-eluted fraction from Sf9 cells, infected with generated SSX2 virus; Lane 6 — immidazole-eluted fraction from Sf9 cells infected with the wild type virus; Lane 7 — protein markers

We have developed an ELISA technique using purified His-SSX2 for immunoscreening of blood serum from healthy donors (n = 27) and patients with melanoma (n = 29). We choose this type of human malignancy, since SSX2 antigen was initially identified in melanoma. Blood serum from patients with melanoma showed 1.7-fold higher immunoreactivity to SSX2 antigen in comparison with serum of healthy donor's (Fig. 3). Moreover, the percent of SSX2-posi-

tive cases was 27.5% in our study versus 11% to 18% in the study where phase expressed SSX2 antigen was used [1, 9, 11] or < 1% — if bacterially expressed SSX2 was used. Since different assays with different sensitivity were used in these studies, the observed differences should be matter of concern.

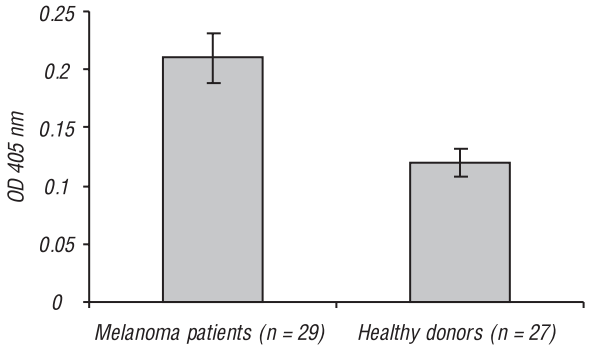

Fig. 3. Immunoreactivity of sera from patients with melanoma (1) and healthy donors (2) against recombinant His-SSX2 antigen (dilution of sera 1 : 1000)

The data presented above clearly indicate that baculovirally produced SSX2 could be used efficiently for immunological studies, especially for screening of blood serum from patients with various types of cancer. Moreover, the availability of highly purified and fully soluble recombinant SSX2 may be useful for development immunotherapy of SSX2-positive tumors.

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ЭКСПРЕССИЯ ОПУХОЛЕВОГО АНТИГЕНА SSX2 В БАКУЛОВИРУСНОЙ СИСТЕМЕ И ЕГО ПРИМЕНЕНИЕ ДЛЯ СКРИНИНГА СЫВОРОТКИ КРОВИ БОЛЬНЫХ МЕЛАНОМОЙ

Цель: **клонировать, экспрессировать в бакуловирусной системе и очистить опухолевый антиген SSX2, и использовать его для скрининга сыворотки крови здоровых доноров и больных меланомой.** ***Материалы и методы:*** **использовали бакуловирусную систему экспрессии, метод афинной хроматографии на Ni-NTA агарозе, иммуноферментный анализ для скрининга сыворотки крови больных меланомой (n = 29) и здоровых доноров (n = 27).** ***Результаты:*** **с использованием бакуловирусной системы экспрессии клонирован и экспрессирован рекомбинантный SSX2; SSX2 был очищен методом афинной *х*роматографии и *п*рименен в ELISA как антиген. Сравнительный анализ сывороток крови больных меланомой и здоровых доноров выявил более высокий уровень SSX2 в сыворотке крови больных меланомой по сравнению с таковым у здоровых доноров.** ***Выводы:*** **SSX2 антиген, экспрессированный в бакуловирусной системе, может быть успешно применен для серологического анализа сыворотки крови онкологических больных.**

Ключевые слова: **опухолевый антиген, SSX2, бакуловирус, меланома.**