Generation and Expression of Recombinant Glutathione-S-Transferase
Full-length Importin α-3 (GST-imp α-3) Fusion Protein and
Study of Its Role on the Nuclear Transport of Protein

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Abstract

Nuclear import of protein from cytoplasm into the nucleus occurs through nuclear pore complex (NPC) and typically requires a specific nuclear localization signal (NLS). Protein import mediated by transport receptor molecules importin α and β. Importin α is composed of a large central domain that consists of 10 tandemly repeated armadillo (arm) motif, which are organized in a superhelix flanked by small N and C terminus domain. The N terminus importin β binding (BBB) domain of importin α mediates binding to importin β. Importin α-3 is one of six importin a family member which has been identified in human.

In the present study, to investigate the role of importin α-3 on nuclear transport of protein, it has been generated and expressed a recombinant glutathione-S-transferase full-length importin α-3 (GST-importin α-3) as fusion protein from expression plasmid pHIM 17970. By co-immunoprecipitation the involvement of importin α-3 on the nuclear import of wild type recombinant hepatitis B core protein (WT rHbc) and simian virus 40 large T antigen (SV40 Tag) was determined. Then expression analysis of GST-importin α-3 fusion protein was determined by immunoblotting.

Keywords: NPC, NLS, importin α, importin β, GST-importin α-3 fusion protein, WT rHbc, SV40 Tag, co-immunoprecipitation, immunoblotting

Introduction

Transport of protein between cytoplasm and nucleus is highly regulated and it occurs through a protein-lined aqueous channels called nuclear pore complex (NPC). This transport is facilitated by soluble transport receptors importin α and β. Importin α is the nuclear import receptor which recognized cargo protein bearing conventional basic monopartite and bipartite nuclear localization signals (NLSs) and mediated their import from cytoplasm into the nucleus. Importin α which contains the NLS-binding site together with importin β is responsible for the translocation of importin-substrate complex through the NPC. Importin α consists of two structural and functional domains, a short basic N-terminus importin β-binding domain (Görlich et al., 1996; Weis et al., 1996; Moroi et al., 1996) and a large NLS-binding domain built of armadillo (Arm) repeats (Peifer et al., 1996). The structural basis of monopartite and bipartite NLS recognized by importin α has been studied crystallographically in yeast and mouse importin α protein. The two basic clusters of bipartite NLSs bind to two separate binding sites on importin α involving Arm repeat 1-4 and 4-8, respectively. Monopartite NLSs can bind in both site but primary use the binding site corresponding to the C-terminus basic cluster of bipartite NLS, referred to as the major site (Conti et al., 1998; Conti and Kuriyan, 2000; Fontes et al., 2000). Currently, six importin a family members have been identified in human, namely importin α-1 (Rch1, hSRP1a), importin α-2 (Rch2), importin α-3 (Chp1), importin α-4 (hSRP1g), importin α-5 (hSRP1, NP11), importin α-6 and importin α-7 (Cuomo et al., 1994; Cortes and Baltimore 1994; Köhler et al., 1997; Seki et al. 1997; Nachury et al., 1998; Köhler et al., 1999).
Importin a molecules contain a large central domain that consists of 10 tandemly repeated armadillo (arm) motifs, which mediated the interaction with the NLS-containing cargo protein. Each importin α has two potential NLS binding sites that directly interact with the NLS of the cargo (Conti et al., 1998, Fontes et al., 2000; Melen et al., 2003).

Gölchi and Kutay (1999) have been reviewed that classical pathway of nuclear import mediated importin α/β was started when importin α binds to the NLS of a karyophilic cargo followed by importin β binding to the complex. Importin β mediates contact with NPC and the import of cargo/importin α/β complex from the cytoplasm to the nuclear side of NPC. Nuclear import is terminated by binding of RanGTP to importin β, which releases the complex from the NPC. The importin β-RanGTP complex can directly exit from the nucleus. Importin α releases karyophilic cargo and binds to CAS and RanGTP for its re-export. Importin α/CAS/RanGTP complex is exported to the cytoplasm through the NPC. In the cytoplasm, importin α/CAS/RanGTP complex is disassembled by hydrolysis of GTP to GDP. This reaction is catalysed by RanGTPase and supported by RanBP1. Importin α is released from importin α/CAS/RanGTP complex. Similarly, importin β is disassembled. Importin α and β can recombine for the next round of another cargo protein.

In this study, we generated and expressed recombinant glutathione-S-transferase full-length importin α-3 (GST-importin α-3) fusion protein from expression plasmid pHM 1970. Then by co-immunoprecipitation we investigated the role of importin α-3 in the nuclear import of wild type recombinant hepatitis B core protein (rHbc) as well as simian virus 40 large T antigen (SV40 Tag).

Materials and Methods

1. Samples Preparation and Transformation

Recombinant DNA plasmid pHM 1970 derived from pGex4T1, which encodes full length of importin α-3, was transformed into competent E. coli. Transformation was performed by adding 10 µl plasmid DNA into 50 l competent E. coli in 1.5 ml E-cup. After gently shaking to mix the content, E-cup was stored on ice for 10 minutes. A Heat shock was performed by preheated the E-cup at 42°C for 2 minutes on the heat block. Then into the E-cup was added 1 ml LB liquid medium and incubated in shaking incubator (INFORS-Bottmingen) at 37°C for 1 hour. The transformed competent cells were plated onto agar plate LB medium containing 30 µg/ml ampicillin. The plate was left at 37°C until the liquid has been absorbed. Then the plate was inverted and incubated over night at 37°C. The E. coli culture was grown at 37°C over night. After 16 hours the bacterial colonies were picked and grown into 5 ml LB medium containing ampicillin. The E. coli culture were incubated at 37°C over night in shaking incubator.

2. Expression of GST-importin α-3 Fusion Protein

Next day 4 ml E. coli culture was grown into 200 ml LB medium containing ampicillin (100 µg/L). The culture was incubated at 37°C for 12-16 hours in shaking incubator until optical density (OD) reaches 0.9 at fixed wavelength 600 nm. The expression of GST-importin α-3 fusion protein was induced by adding isopropyl-B-D-thiogalactoside (IPTG from ROTH) in a final concentration of 2 mM. The bacteria were allowed to grow for an additional 3 hours at 37°C in shaking incubator. To stop the bacterial growing, the bacteria cultures were cooled on ice for 10 minutes. Then the cells were centrifuged at 4,500 rpm for 10 minutes (BECKMANN). Supernatant was removed and the pellet was resuspended with 3,75 ml lysis buffer (75 µl leucoprotepin (SIGMA) 20 µg/ml; 37,5 µl chymostatin (SIGMA) 10 µg/ml; 1,275 µl β-mercaptoethanol (ROTH) 5 mM; 3,2 ml Tris-HCl 200 mM / NaCl 500 mM). To destroy the cell wall, the resuspended pellet was frozen in liquid nitrogen and thawed directly at 37°C in water bath (KOTTERMANN). This step was repeated three times, then the cells were homogenized using ultrasonic sonicator (BANDELIN ELECT. UW 70) by 70% maximal capacity for 60 seconds three times. The cells were kept on ice at all times. After that the cells were centrifuged at 15,000 rpm at 4°C for 20 minutes (BIOFUGE 15 R HERAUS). The supernatant was collected into 50 ml conical tube (FALCON) and added by PBS ad 25 ml. The 25 ml supernatant was applied to the glutathione sepharose beads containing column (AMERSHAM PHARMACIA) and allowed by gravity flow. The supernatant were collected again and repeated this step three times. The columns were washed four times with cooled PBS. The proteins were eluted from beads using elution buffer (20 mM reduced glutathione in 50 mM tris pH 8,0). The supernatant were collected in 5 fractions, each fraction contains 2.5 ml. The column was regenerated with 12,5 ml 3 M NaCl/PBS two times, 12,5 ml PBS five times, 25 ml ethanol 70%, 10 ml ethanol 20%/PBS. Then the column was covered with parafilin (AME. NAT. CAN. Tm) and stored at 4°C.

3. Concentrating of GST-importin α-3 Fusion Protein

Nanosep 10 K centrifugal concentrator (PALL FILTRON) was used to concentrate the GST-importin α fusion protein. In 2.5 ml of sample volume was added by 50 µl dithiothreitol (DTT) 1 M and concentrated in the less amount of sample volume. The 500 µl sample was pipetted into sample reservoir of Nanosep 10 K. Afterwards the Nanosep 10 K was centrifuged at 10,000 rpm for 10 minutes at 4°C (BIOFUGE 15R HERAUS) until achieved the sample volume in 100 µl. The concentrated samples were transferred from Nanosep 10 K and collected in new E-cup.

4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Electrophoresis of proteins is carried out usually in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits which minimize aggregation. The strongly anionic detergent SDS is used in combination of reducing agent and heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind SDS and become negatively charged. Because the amount of SDS bound is almost always proportional to the molecular weight of the polypeptide and it is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide. By using markers of known molecular weight, therefore it is possible to estimate the molecular weight of the polypeptide chains.

Each sample consist of 15 µl supernatant from each fraction, 3.5 µl Nupage loading buffer and 1.5 µl DTT 1 mM. All of samples were preheated at 75°C for 10 minutes before loading. 20 µl from each sample was loaded up into the bottom of the wells in 4-12% SDS PAGE Nupage gel (INVITROGEN). The Nupage electrophoresis apparatus (INVITROGEN) was attached to an electric power supply, a voltage was applied of 8V/cm to the gel. Then the voltage was increased to 15 V/cm and the gel was run until the bromophenol blue reaches the bottom of the resolving gel and the power supply was turned off. The plastic plates was removed from electrophoresis apparatus and placed them on paper towel. Using spatula, the plates were pried apart. The orientation of the gel was marked by cutting a corner from the bottom of the gel that is closest to the leftmost well. Afterwards the gel was stained by Sypro red dye (INVITROGEN).

5. Co-immunoprecipitation of GST-importin α-3 Fusion Protein.

Firstly, the GST-importin α-3 fusion protein was bound to glutathione sepharose beads. 360 µl glutathione sepharose beads was centrifuged at 14.000 rpm for 1 minute (BIOFUGE 15 R HERAUS). The supernatant was through out and pellet was washed three times with 1 ml PBS. The pellet was
resuspended with 1 ml PBS and divided into two reaction tubes. First 500 µl reaction was added with 5 µl BSA 10% and the other was added with 5 µl and 24 µl GST-importin α-3. Then both of the samples were incubated over night at 4°C on rotating roller. Next day, both of samples were washed twice with PBS/0.1% BSA and resuspended each in 240 µl. From each 240 µl sample was divided in three reaction tubes, each is 80 µl. The first 80 µl was added with 20 µl phosphorylation wild type recombinant HBV core protein (WT HBc), the second was added with 1.2 µl recombinant HBV-Core (fHBc) and the third was added with 16 µl SV40 Tag FTIC-BSA, as well as the 3 others. After that, all of the six reaction tubes were incubated at 37°C on rotating roller. In the length time 1 hour, 2 hours, 4 hours, and over night, the reaction of each of samples was stopped by adding 5 µl loading buffer and 1 µl DTT 1 M. Then 20 µl of each sample was preheated at 70°C for 10 minutes, run in reducing SDS-PAGE and stained with sypro red dye.

6. Sypro Red Staining
A working stain solution of sypro red was prepared by diluting the stain stock solution 1 to 5,000 in a 7.5% acetic acid solution. The gel was stained in plastic box with gentle agitation at room temperature for 30 minutes. The plastic box was covered by aluminium foil to prevent the photobleaching of the staining. Then the gel was destained in a 7.5% acetic acid solution at room temperature for 15 minutes. For Imaging, the wet gel was placed directly onto the Typhoon 9200 machine (AMERSHAM PHARMACIA) in small amount of water. The air bubbles between the gel and the glass was removed to avoid trapping. In the scanner control setup windows, the appropriate laser and emission filter combination were chosen and typhoon imaging was adjusted for focal depth setting.

7. Western Blott (Immunoblotting)
After separation of protein by SDS-PAGE, gel was blotted on PVDF membrane (MILLIFORE) using protein blotting apparatus (INVITROGEN). Western analysis was performed by rabbit antibody anti importin a (BD-CLONETECH) which diluted 1:1000 in the blocking solution (10% BSA in PBS and 0.1% Tween 20) for 3 hours at room temperature. As secondary antibody used peroxidase (POD) anti rabbit antibody which diluted 1:5000 in blocking solution for 1 hour at room temperature. The proteins were visualized by enhanced chemiluminescence detection method (ECL, AMERSHAM PHARMACIA).

Results and Discussion
1. Expression and Isolation of GST-importin α-3 fusion protein
To express the GST-importin α-3 fusion protein, the recombinant DNA plasmid pHM 1970 containing E. coli was grown into liquid LB medium in presence of ampicillin (10 µg/µl). Then to induce the protein expression IPTG was added to final concentration of 2 mM. Soluble expressed protein was harvested by destruction the cell wall of E. coli then homogenized using ultrasonic sonicator in presence of protease inhibitor. GST-importin α-3 fusion protein was isolated by protein affinity chromatography in glutathione sepharose beads containing column. The protein was collected in several fractions and concentrated in 1 ml volume using Nanosep 10 K, by this concentration all soluble protein with molecular weight under 10 kDa was removed out by centrifugation. The sypro red staining of SDS-PAGE gel shown that GST-importin α-3 fusion protein with molecular weight 58 kDa found in fraction 1,2,3,4. More detail see fig. 1. In comparison the serial dilution of BSA standards (MW 66 kDa) were run in lane 6, 7 and 8.

2. Co-immunoprecipitation
Co-immunoprecipitation is a classical method to detect protein-protein interaction. The basic procedure of co-immunoprecipitation consists of generation of cell lysate, antibody adding, precipitation using antigen and washing. Then the bound protein are eluted and analysed by SDS-PAGE (Phizicky and Fields, 1995).

To study the role of importin α-3 in the nuclear import of recombinant HBV core protein (HBc) in compare with Simian Virus 40 (SV40) large T antigen, firstly the GST-importin α-3 containing lysate was allowed to bind to the glutathione sepharose beads. To analysis binding of GST-importin α-3 to the glutathione sepharose beads, the complex was run in the reducing SDS-PAGE and stained using sypro red. The sypro red staining showed that GST bound to the matrix of glutathione sepharose (fig. 2). Then ready to perform the Co-immunoprecipitation to find out the involvement of importin α-3 to the nuclear import of protein. Importin a is adapter protein that mediate import by binding to its cargo protein in the cytoplasm via recognition of NLS. The importin a cargo complex translocates through the NPC via interaction with NPC protein.

![Figure 1: Sypro red staining of GST-importin α-3 as fusion protein. The importin α-3 shown as protein bands with molecular weight 58 kDa fraction 1,2,3 and 4 in lane 1 to 4. Lane 5-8 are serial dilution of BSA, (66 kDa) and M is rainbow marker 12.5% SDS-PAGE at molecular weight marker of protein.]

![Figure 2: Co-immunoprecipitation of GST protein. Sypro red staining shown that lane no. 1 is sample without GST and lane no. 2 is sample with GST. M indicated the molecular weight markers protein 12.5%. The GST shown as band of protein in size 25 kDa.]

3. Role of importin a to nuclear import of wild type recombinant Hepatitis B core protein.
To perform the co-immunoprecipitation, complex of glutathione sepharose beads/BSA 10% with and without GST-importin α-3 was bound to wild type recombinant Hepatitis B core protein (WT rHBc) then incubated at different length time 1 hour, 2 hours, 4 hours, and over night (on). The Co-immunoprecipitation indicated that importin
α-3 play an important role for the nuclear import of wild type HBV capsid. Involvement of importin α-3 in the nuclear import appears after 2 hours and tend to increase along with incubation time (see fig. 3). It could be speculated that binding of importin α-3 to WT rHBV capsid increase affinity to NPC and it can associate with NPC of a non-natural host without disassembly. The increasing of affinity to NPC implied that the viral capsid translocated through the NPC, in accordance with Kann et al. (1999) which stated that phosphorylation of cytosolic HBV capsid induces conformational changes that expose the hidden NLS within C-terminus on the surface protein and enable capsid interaction with importin α in order to be imported in the nucleus.

Figure 3. Co-immunoprecipitation of GST with recombinant WT rHBV core protein. Sypro red staining shown in different incubation times the GST protein with MW 26 kDa appear after 2 hours incubation time. By longer incubation time GST bands shown clearer and stronger. Lane 1,2,3,4 are samples without GST and lane 4,7,8 are samples with GST. M indicates the molecular weight markers protein RP756.

It has been reported that the NPC can accommodate human HBV capsid up to about 39 nm in diameter in the Xenopus oocytes (Pante and Kann, 2002). The rHBc devoid of DNA were found within the nuclear baskets, suggesting that the translocation process of these capsids was slow. In permeabilized cells, the association of HBV capsid with NPC required capsid phosphorylation, apparently to make NLS accessible for importin α and β binding (Kann et al., 1999), and a classical NLS was required for the association of the duck HBV core with NLS (Mabit et al., 2001).

4. Role of importin α to nuclear import of SV40 large T antigen

Co-immunoprecipitation using SV40 large Tag FITC-BSA also indicated that GST-importin α-3 interacts with SV40 large T Antigen. The interaction of GST-Importin α-3 to the SV40 large Tag FITC-BSA shown that it involves in the nuclear import by binding to the NLS of SV40 Tag. Involvement of importin α-3 in the nuclear import was indicated by appearing GST protein (26 kDa) in the samples after incubation 2 hours, 4 hours and over night. Intensity of GST protein tends to increase along with incubation time (fig. 4). As reported by Laird et al. (1984 a, b), that SV40 large Tag identified as first NLS. This NLS to be identified was a short stretch of basic amino acids (PKKKRK)-K. The SV40 T antigen together with nucleoplasmin has been determined as a classical NLS, which consists of one and two clusters of basic amino acids separated by linker (Görlich and Kutay, 1999). SV40 large Tag is bound to the N-terminus NLS binding site (Melen et al., 2003).

Figure 4. Co-immunoprecipitation of GST with SV40 Tag-FITC-BSA. Sypro red staining shown in different incubation times the GST protein (26 kDa) interacts with SV40 Tag after 2,4 hours and over night incubation times. Samples no. 1,2,3,4 are without GST and no 5,6,7,8 contain GST. M indicated the molecular weight markers protein RP756.

The nucleocytoplasmic transport of protein occurs through the NPC and is mediated by an active and selective mechanism which is regulated by saturable transport receptors and the corresponding cis acting transport signals that are termed NLS and NES (Görlich and Kutay, 1999). To NLS the best characterized transport sequences comprise one or two short stretches of basic amino acids. These basic, generally lysine rich signals are typified by SV40 large Tag (PKKKRK) or the cellular nucleoplasmic protein NLS (KRPAATKKAGQAKKKK) and are frequently referred to as classical NLSs. These sequences are recognized in the cytoplasm by heterodimeric import receptor composed of importin α and β. Importin α, from which six isoforms have been identified in humans (Köhler et al., 1999).

5. Expression Analysis

Analysis expression of GST-importin α-3 fusion protein was performed by Westernblot. As shown in fig. 5. The GST-importin α-3 fusion protein was expressed as protein bands in size of 58 kDa. By serial dilution 1:2 shown that the GST-importin α-3 fusion protein still appear in dilution 1:4 and are located between 46 and 66 kDa of marker protein RP756. In the nuclear import of protein, importin α like other importin α isoforms has a function as an adaptor molecule by binding importin β via its amino terminally located importin β binding (IBB) domain (Görlich et al., 1996; Weis et al., 1996), and by binding NLS bearing proteins via its two NLS binding sites in the central area (Conti et al., 1998; Herold et al., 1998). Whereas Importin β is the transport receptor that carries the importin α-NLS complex from the cytoplasm into the nuclear side of the NPC (Görlich et al., 1998).

Figure 5. Westernblot of importin α and β by mouse anti importin α. The importin α-3 has molecular weight 58 kDa. Lane 1, 2, 3 are serial dilution of importin α-3. M is rainbow marker RP756 which used as molecular weight marker of protein.

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688

A Potential Alkaloid Compound Isolated
from A Marine Sponge Collection Number MD-02 Agains Cancer

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Abstract

Majority of the currently available anticancer drugs are designed to have selective toxicity to rapidly dividing cells. Among these agents the focus of many studies are compounds obtained from natural products with high therapeutic index. In this study the cytotoxicity of a marine alkaloid compound isolated from sponge collection number MD-02 on cancer and normal cells was evaluated. The compound was obtained from chlorform fraction of sponges followed by Vacuum Liquid Chromatography and TLC-preparative. This compound in a concentration dependent manner inhibit the growth of human cancer cells and more toxic to Raji than HeLa cells with IC50 of 4.27 and 22.45 μg/ml, respectively. Cells treated with this compound showed cleavage of chromosomal DNA into fragments, suggesting the possibility of apoptotic cell death. This compound was less toxic to normal Vero cells showing its selectivity with IC50 of 100.36 μg/ml.

Key Words: Antitumor agents, sponges, alkaloid, HeLa, Raji, Vero

Intoduction

Until recently, cancer is still a problem and common cause of death around the world. Various therapeutic modalities have been employed in the fight against cancer. These include: alkylating agents, antimetabolites, radiomimetic drugs, hormones and antagonists, surgery and miscellaneous agents (Cram et al., 1992; Calabresi and Chabner, 1991; Hoppe et al., 1998; Lorigan et al., 1996). However new agents that produce satisfactory anticancer effect without relapse and most time, their therapeutic activity is accompanied by debilitating side effects (Green et al., 1982; Herzog et al., 1987). Despite the introduction of new drugs for the treatment of cancer, the overall survival of patients suffering from this malignancy is far from satisfactory.

A number of researches have been conducted to search anticancer with renewed vigor. Natural products are the major source of lead compounds for drugs against cancer. Marine invertebrates are known as rich sources of compounds with unique chemical structures and pronounced chemical activities, which suggests potential value as lead structures for the development of new pharmaceuticals.

A diverse range of bioactivities of these natural resources have been reported which included insectidal, antibacterial, antifungal and cytotoxic properties (Ang et al., 2001; El Sayed et al., 2001; Cafieri et al., 1995; Cafieri et al., 1996; Nakamura et al., 1984; Tsukamoto et al., 1999; Estada et al., 1996). Three isominalbaricaine triketones have been isolated from the marine sponge Rhodactria gibbosa and the results showed that these compounds were toxic to human colon tumor (Tasdemir et al., 2002). Manzamines isolated from Xestospongia rhodonea were found to be toxic to a mouse lymphoma cell line (Edra et al., 1996). This compound is reported to also found in other