Expression Methods and Preliminary Purification of HIV-1 fusion protein GST-vif

Alena Janda, 2007

Advised by Dr. Joseph Wedekind, Ph.D.

Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry

The HIV-1 protein, vif, is essential for HIV-1 replication in T lymphocytes and macrophages. Recently, it was discovered that retroviral hypermutation of first strand viral DNA by the human protein hA3G occurs upon infection by HIV-1 virions. hA3G is a virion-encapsidated host protein able to deaminate minus-strand reverse transcript deoxycytosine bases to deoxyuracil. These C to U mutants are copied as G to A mutations by reverse transcription of the coding or + strand, thereby introducing catastrophic mutations into proviral DNA that blocks its infectivity. However, the interaction between vif and hA3G results in a dramatic reduction in the amount of hA3G encapsidated into virions. In fact, hA3G association with vif has been well documented, and the ensuing lack of hA3G incorporation into HIV-1 progeny virions has been ascribed to vif-mediated polyubiquitination of hA3G, leading to its rapid degradation. Specifically, vif targets hA3G through formation of an SCF-like E3 ubiquitin ligase complex that includes Cullin5, Elongin C and Elongin B. The structure of the vif-BC-Cul5 complex has been demonstrated to form a novel SOCS-box-like motif, which acts as an E3 ligase for hA3G in vitro. The new SOCS-box motif (Figure 1) varies from the originally identified SOCS-box by a cysteine residue replacement of alanine in the consensus sequence, as well as two identified cysteine residues outside the SOCS-box that are required to interact specifically with Cul5, suggesting that other protein interfaces between vif and the SOCS-box Elongin C interaction exist. Furthermore, recent studies have shown a complementary mechanism for vif-mediated inactivation of hA3G, involving direct vif-hA3G binding. D128K substitution in hA3G impaired protein-protein interaction between the C-terminal of vif and hA3G. In addition, the vif-BC-Cul5 complex interaction with wild type hA3G was inhibited by the D128K mutant, which does not interact with vif at all. Other vif mutants found to be defective were SLQ144/146AAA, which is a mutation in the vif-SOCS-box motif and C114S, C133S vif mutants that inhibit E3 ligase activity of the vif-BC-Cul5 complex. In experiments involving BC-box vif mutants, it has been shown that vif fails to recruit Cul5-E3 ligase; thus hA3G is not suppressed, yet vif-hA3G interactions are maintained. These results suggest that E3 ubiquitin ligase activity of the vif-BC-Cul5 complex is necessary for the inhibition but not for the binding of hA3G by vif.

The molecular characterization of HIV-1 proteins as well as those of their host partners such as hA3G is fundamental to elucidating the viral mechanism of action. In order to design rational therapeutic solutions that target the HIV-1 virus, the complete three-dimensional structures of viral and host proteins must be understood at the molecular level. This information is carefully collected through a multi-step process involving protein expression, protein purification, and ultimately, X-ray diffraction methods. With crystallization data, the active site structure(s) of a protein can be modeled in detail and can provide insight into the effects of mutants on the emergence of drug resistance. For example, the structure of HIV-1 protein protease double mutant dimer C95M/C1095F was determined by induced structural mutations. Modification of proteins to create therapeutics that target a wide variety of HIV-1 serotypes as well as drug resistant strains.

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**Figure 1.** Vif of HIV-1 and closely related SIV cpz viruses contain a SOCS-box-like motif (10).
transformed with GST-vif cloned by PCR into a commercially obtained pGEX (lactose inducible) vector from Amersham Biosciences. Kanamycin (Kan) and Chloramphenicol (Cam) resistance genes were used for antibiotic selection. Plates were streaked overnight using Cam and Kan-containing LB agar. Plates were then stored at 4°C until needed. The first inoculation was performed using 5 ml LB, 12 µl Kan (30 mg/ml) and 5 µl Cam (34 mg/ml). These were placed in a shaker at 37°C and 225 rpm overnight. The culture was expanded to 500 ml LB, and final Kan and Cam concentrations of 72 µM and 34 µM, respectively. The 5 ml culture was spun down at 12,000xg, resuspended in 0.5 ml of fresh LB, and then added directly to the 500 ml LB. The flask was placed into the shaker at the original setting until it reached OD$_{600}$ nm of 0.6. The culture was then induced with 1 mM IPTG (artificial lactose-mimic substrate) and grown at 30°C for 2 hours. Subsequently, the cells were collected by centrifugation at 1,789xg for 10 min and the pellet was frozen in liquid nitrogen and stored at -90°C.

A few modifications were made to the above procedure: 1) LB was modified to contain 1 mM betaine and 0.5 M NaCl; 2) Induction was done at OD$_{600}$ nm of 0.8 with 0.5 mM IPTG; and 3) the culture was heated to 47°C immediately following induction for 20 min and then grown overnight at 20°C to slow protein expression process.

Expression was done according to the modified method but without heat shocking the cells after induction.

Bacterial Cell Lysis

Frozen cell pellets were weighed and incubated in 4 ml Lysis Buffer/1 g of cells (Lysis Buffer contained: 0.5% Triton-X-100, 50 mM Tris pH 8.0, 10 mM BME, 1 mg/ml lysozyme, 5 µg/ml aprotinin, leupeptin, pepstatin A, 2 mM benzamidine, and 1 mM PMSF) that were then diluted 1:10 with 1xPBS and left for 30 min on ice. The cells were then sonicated and incubated.
in 1 ml Nucleotide Digestion buffer/1g of cells (Nucleotide Digestion Buffer contained: 0.5% Triton-X-100, 2 mM ATP, 10 mM MgSO$_4$, 33 µg/ml DNase 1, and 33 µg/ml RNase A) for 10 min at room temperature. Finally, the lysate was clarified at 8,000xg for 15 min and the supernatant was pooled. After lysis, samples were collected and run on an SDS-PAGE gel using an XCell SureLock system (Invitrogen).

Expression Solubility Profile and Vif Western Blotting

Time points during expression were taken immediately after heat shock at 10 min, 20 min, 50 min, 80 min, 110 min, 140 min, 170 min, 210 min, 8h, and 21h. Cell pellets were then analyzed by SDS-PAGE using an uninduced sample as a control. Another gel was run, transferred in an XCell II Blot Module (Invitrogen) onto a PVDF membrane using the manufacturer’s protocol, and a western blot was performed. A monoclonal primary antibody (#6459 from the AIDS Reagents Repository) reactive with vif was used in a 1:1000 dilution into 1% powdered milk, followed by a 1:2500 dilution of goat anti-mouse secondary antibody containing 1% BSA. The membranes were then scanned using Enzyme Chemifluorescence on a Storm 860 imaging system (GE Healthcare). Peaks were then analyzed by Image Quant software.

Purification by Glutathione Sepharose 4B

Purification of the GST-vif fusion protein was conducted as described by the manufacturer using glutathione Sepharose 4B (GE Healthcare). A 5 ml column was filled with 1 ml of 75% Glutathione Sepharose 4B slurry. The beads were then washed with 25 ml 1xPBS pH 7.3. Bacterial lysate was added and nucitated for 1 h at 4°C. Flowthrough was collected and the column was washed with 3x5 ml 1xPBS. After washing, 10 mM reduced glutathione at pH 8.0 was passed over the column as 5 bed volumes. The eluate was analyzed by SDS-PAGE.

GST-vif Concentration

After elution, an SDS-PAGE gel was run and a Bradford assay was performed to quantify the concentration of protein in the elutions. The most concentrated elutions were then pooled and dialyzed overnight in dialysis buffer. Pooled and dialyzed material was subjected to further concentration by use of a stirred cell (Amicon) pressurized with Argon gas using a 5,000 NMWL cutoff membrane. The sample was brought to a volume of 0.75 ml.

Modified Glutathione Sepharose 4B Elution

To elute the GST-vif fusion protein, 10 mM, 50 mM and 100 mM concentrations of reduced glutathione were used in 1xPBS buffer pH 8.0. Elutions were 0.75 ml (1 bed volume each) of 10 mM, 2x50 mM, and 2x100 mM reduced glutathione respectively and were allowed to nucitate at 4°C for 10 min each. Aliquots of 5 µl were removed from each elution, as well as a resin sample for running on an SDS gel.

Results

Standard Expression of GST-vif

The initial expression of GST-vif resulted in high concentrations of GST-vif in the pelleted material prior to purification. As seen in Figure 3, the growth lanes 2-4 indicate that GST-vif is in fact being expressed (GST-vif runs just below the 39 kDa mark). However, after lysis, the majority of expressed protein is in the insoluble phase (Lane 7) rather than in the supernatant (Lane 8). Furthermore, GST-column elution, as seen in Figure 4, indicates that very little protein is binding to
the column. Vif western blot of stirred-cell concentrated GST-vif confirms that GST-vif has been expressed (Figure 5).

**Modified Expression of GST-vif**

Heat shock improved quantities of soluble GST-vif by inducing expression of specific chaperones involved in protein folding and stability. After heat shock, betaine and NaCl addition acted to ionically stabilize the different charges on the protein's surface upon expression and during folding. Figure 6 demonstrates good bacterial growth under the modified conditions. Furthermore, to prevent protein aggregation, IPTG concentration was reduced from the standard 1 mM to 0.5 mM along with growth temperature and expression time, slowing down GST-vif expression. These methods produced more soluble GST-vif, as seen in Figure 7, represented by lanes 1 and 2 (the other lanes will be alluded to upon discussion of purification), illustrating respectively the insoluble and soluble phases after lysis.

Expression of GST-vif without heat shock immediately following induction results in GST-vif partitioning in the insoluble phase. In Figure 8, Lanes 1 and 2 show GST-vif expression in uninduced and induced samples. Lanes 4 and 5 are the insoluble and soluble phases following lysis, respectively.

Furthermore, the sample was loaded onto a GST column and GST-vif elutions were undetectable by Commassie stain. Thus, without heat shock, GST-vif does not fold into its soluble, native structure. This observation supports the hypothesis that specific chaperones from the Hsp family of proteins aid GST-vif in its three-dimensional folding process. The Hsp chaperones are only expressed at certain high temperatures and stabilize newly expressed proteins by providing folding microenvironments and by binding hydrophobic domains.

**Expression Solubility Profile**

According to the modified method of GST-vif expression, time points were taken at induction, 10 min, 20 min, 50 min, 80 min, 110 min, 140 min, 170 min, 210 min, 8h, and 21h. Figure 9, Lanes 3-23 show increasing concentrations of soluble GST-vif over time. The insoluble phase is followed by the soluble phase for each time point beginning with Lane 3. Lane 2 is a sample of cells before induction. A western blot for vif expression was performed on the samples from the kinetic analysis (Figure 10). Figure 10 better quantifies the amount of soluble protein at each time point, which is greatest after 8 hours of expression (Lane 20).

![Figure 7. Modified GST column elution](image1.png)

**Figure 7. Modified GST column elution.** Lane 1 is the insoluble phase after lysis. Lane 2 is the soluble phase after lysis. Lane 4 is flow through and Lanes 5-6 are washes. Lanes 8-11 are elutions of 10 mM, 50 mM, 100 mM and 100 mM glutathione respectively. The last lane is GST-vif remaining on the resin after modified elution. When compared to Figure 10, much more GST-vif is eluted off the resin with higher reduced glutathione concentrations. The arrow represents GST-vif.

![Figure 8. Modified expression protocol without heat shock](image2.png)

**Figure 8. Modified expression protocol without heat shock.** The first lane is a sample of cells before induction and the second is after induction. Lanes 4-5 show the insoluble and soluble phases after lysis respectively. GST-vif is most prominent in Lane 4, the insoluble phase. The arrow indicates GST-vif.
Following the manufacturer’s procedure for Glutathione 4B elution (GE Healthcare), 10 mM reduced glutathione was used to compete with GST-vif. As seen in Figure 11, 5 elutions were resolved in Lanes 2-6. Lane 7 shows the amount of GST-vif left on the bead resin after elution. Though Lanes 2 and 3 show a larger amount of protein than the rest, the bead resin contains much more GST-vif than any other lane. Thus, the elution buffer is a poor competitor at 10 mM concentration to GST-vif. Consequently, higher concentrations of reduced glutathione were used to elute GST-vif. Figure 7 illustrates the use of 10 mM, 2x 50 mM and 2x 100 mM column elutions (Lanes 7-11). Lane 12 in Figure 7 reveals the GST-vif remaining on the bead resin. In comparison to the amount of protein left on the resin in Figure 11, higher concentrations of reduced glutathione in the elution buffer were more effective in eluting GST-vif from the column.

**Glutathione Sepharose 4B Column Elution**

**GST-vif Concentration**

After concentration to 750 µl of GST-vif elutions in a stirred cell from lanes 3-5 in Figure 11 (Lane 3 of Figure 12), a vif western blot was run to confirm the presence of vif (Figure 13). Bradford Assays performed on the pooled elutions before and after concentration show more than a threefold increase in concentration (Figures 15 and 16).

**Discussion and Conclusions**

Further understanding of the HIV-1 vif protein requires successful expression methods that yield sufficient amounts of GST-vif to be obtained. The modified expression procedure employed in this paper addresses the issue of insoluble protein in three ways. First, it has been suggested that higher solute concentrations help stabilize native proteins in solution by interacting with their charged surfaces. Secondly, heat shock...
stimulates the expression of proteins known as chaperones, such as DnaK, DnaJ, GrpE, GroEL and GroES. Chaperones and other heat shock proteins aid protein folding in the following ways: by providing specialized folding microenvironments, by binding to hydrophobic domains of unfolded proteins in order to stabilize them and by aiding in protein transport. Third, extension of the expression time by lowering the growth temperature reduces protein incorporation into insoluble inclusion bodies by reducing the rate of protein expression and thus lowering the probability of aggregate formation between nascent proteins. The addition of 1 mM betaine and 0.5 M sodium chloride alone did not improve protein solubility (Figure 8), but in combination with heat shock and expression at lowered temperatures over a longer period of time, these additives increased expression (Figure 7). In the future, this method will be adapted and recommended to others with similar expression issues.

Following successful expression of GST-vif, purification ensues. Competitive binding with respect to affinity chromatography is often unpredictable when dealing with proteins whose native structures are not known. Many possibilities involving the folding of GST-vif may explain its unusually high affinity for the GST column (see Figure 11 Lane 7). Because of the varying nature of protein folding, standard procedures such as those given in the GE Healthcare GST manual are unlikely to be representative of all possible native protein structures and their interactions with glutathione sepharose 4B. The problems encountered in eluting protein from the GST column suggest that the concentration of reduced glutathione is not sufficient to successfully compete with the GST-vif bound to the column. Through inference, elution buffer concentrations of reduced glutathione were increased and resulted in higher concentrations of eluted GST-vif. A comparison of Figures 7 and 11 illustrates that higher amounts of reduced glutathione have a greater ability to elute GST-vif. Future methods will include preliminary elutions of reduced concentrations of reduced glutathione (i.e. 10 mM and 30 mM) in order to exploit purification steps by washing off proteins unbound to the column. Examination of Figure 7 shows that both high and low molecular weight contaminants exist on the column after washing. The first 10 mM reduced glutathione elution actively rids the beads from contaminants with a minimal loss of GST-vif. Thus, a few elutions of low reduced glutathione concentrations will be helpful steps to purify GST-vif, and will be followed by higher concentrations to elute GST-vif itself.

This study focused on the vif protein from HIV-1 since it appears to defeat the innate anti-viral protein from hA3G. Rescuing hA3G represents an exciting new therapeutic strategy since all HIV-1 therapeutics currently being used only target traditional viral proteins. In 2004, the WHO estimated about 40 million people worldwide were infected with HIV-1 and about 3.1 million with HIV-1 died. At present, multi-drug therapies have decreased the HIV-1 death rate by reducing viral expression in the host. However, these therapies cost about $15,000 per person per year, making them primarily accessible to medically insured patients in developed countries. It is estimated that the US economy alone spends about $3-6 billion dollars a year to control the virus, and about $514 billion is spent globally. Until an inexpensive, effective therapeutic is developed, the HIV-1 virus will relentlessly spread worldwide. It is believed that before long, many countries in Africa, Asia, and Eastern Europe, which cannot afford the current medications, may become completely devastated before a solution is found. Significantly, in the year 2005, 16-40% of new HIV-1 infections will be resistant to one or more existing forms of viral therapy. Hence it is of paramount importance that a new class of therapeutics be developed.

Results from the expression and binding experiments enabled an understanding that GST-vif folding needs to be aided by chaperones to prevent it from being packed into insoluble inclusion bodies. Furthermore, the high affinity of GST-vif for the GST column also provides structural folding information. The native state of GST-vif must interact favorably with glutathione sepharose that has two negatively charged groups and one positively charged group on its surface. Future steps include proceeding with expression and purification of GST-vif with the goal of purifying vif. This will consist of PreScission Protease cleavage and further purification steps. Further purification will be obtained by running the sample through an S-100 size exclusion column and/or HPLC. PreScission Protease cleavage will involve off-column cleavage held at varying temperatures and solution conditions to maximize cleavage results. After further purification, crystallization using hanging drop vapor diffusion, as well as the batch under oil method efforts will initiate along with binding assays involving hA3G, and Elongin B and C using the GST-tag or other affinity devices.
Acknowledgements

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Abbreviations

Vif, HIV-1 viral infectivity factor; hA3G, human Apobec-3G, also known as CEM15; SCF, Skp1p-cullin-F-box protein complex, a ubiquitin-ligase; Cd5, Cullin 5; GST, Glutathione S-Transferase; Kan, Kanamycin; Cam, Chloramphenicol; LB, Luria Broth; IPTG, isopropyl-beta-D-thiogalactopyranoside; Tris, Tris Hydroxymethylaminomethane buffer; BME, beta-mercaptoethanol; PMSF, Phenylmethylsulphonylfluoride; PBS, Phosphate-buffered saline; SDS PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; WHO, World Health Organization; rpm, revolutions per minute; g, gravity; NMLW, Nominal Molecular Wight Limit; Hsp, Heat shock protein; S-100, Sephacryl 100.

References


