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Expression, purification and biological properties of the carboxyl half part of the HTLV-I surface envelope glycoprotein

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Abstract

The carboxyl half of the surface envelope protein of HTLV-I contains the major immunodominant and neutralizable domains. Using two affinity chromatography steps and a combination of high salt concentration and non-ionic detergent, we purified this part of the envelope protein from *Escherichia coli*. Analysis of some immmunological and biological properties of this protein indicated that it was folded in a way that preserved the correct structure of this domain of the HTLV-I envelope protein. It could be utilized in structural studies to further understand the mechanisms of HTLV-I entry and to better define the component(s) of an effective vaccine.

Keywords: *Escherichia coli*; Human T-cell leukemia virus; Glycoproteins

1. Introduction

Human T-cell leukemia virus type I (HTLV-I) is a human retrovirus associated with two major clinical disorders: a malignant T-cell leukemia termed adult T-cell leukemia or ATL [1–3] and a neurological disease named tropical spastic paraparesis/HTLV-I associated myelopathy or TSP/HAM [4,5]. HTLV-I infection is highly prevalent in patients affected with other syndromes, including polymyositis [6], arthritis [7], infective dermatitis of children [8], uveitis and sicca syndrome [9].

HTLV-I entry into the target cell is mediated by the viral envelope glycoproteins (env). These are two non-covalently linked subunits generated by proteolytic cleavage of an env encoded polyprotein precursor of 61 K: a 46 K surface glycoprotein (SU) responsible for attachment of the virus to a cell receptor, and a 21 K transmembrane glycoprotein (TM) which fuses the viral envelope with the target cell membrane allowing penetration of the viral core into the cytoplasm. These proteins elicit protective immune responses in rats, rabbits and monkeys against HTLV-I infection [10–12]. Several regions involved in viral entry into the target cell have been identified in the HTLV-I envelope glycoproteins by use of neutralizing antibodies or peptides that inhibit fusion [13] and by functional analysis [14,15]. Most of these regions are located in the carboxyl half part of the glycoprotein (gp) 46 SU protein.

Precious information on the three-dimensional structures and functionality of these proteins may be obtained by X-ray analysis of their crystal structure. Recently, the X-ray crystal structure of the receptor-

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binding portion of the gp70 SU of the Friend murine leukemia virus [16], that of truncated versions of the gp120 SU of HIV [17,18] and that of the core structure of the gp41 TM of HIV [19] were solved.

Data on the structure of the HTLV-I envelope glycoproteins are not so far available, even though these proteins or fragments of them have been produced in different heterologous systems [20,21,11,22]. The gp46 SU of HTLV-I produced in chronically infected lymphoid cells has been partially purified by affinity chromatography [23].

As information on the HTLV-I env protein structure will further the understanding of the mechanism of infection by this virus and help in the development of a vaccine, we decided to express in *Escherichia coli* a recombinant protein containing the entire sequence of the HTLV-I SU and the ectodomain of the HTLV-I TM. All attempts to obtain this protein in a soluble form were unsuccessful. Different groups have shown that the carboxyl part of the HTLV-I SU contains major immunodominant domains [amino acid (aa) 175–199 and aa 239–261] as well as regions that elicit neutralizing antibodies (aa 187–199, aa 213–236 and aa 288–317) both in humans and in animals [13]. This part of the protein is also involved in fusion mechanisms of viral and cellular membranes, particularly a domain comprised between aa 170 and 233 [14,24]. For these reasons, we expressed the carboxyl half part of the HTLV-I SU in *Escherichia coli*. Using two affinity chromatography steps and a combination of high salt concentration and non-ionic detergent, we succeeded in obtaining a soluble recombinant protein corresponding to the carboxyl half part of the HTLV-I SU. This protein named pET 2240 env protein was recognized by monoclonal antibodies that bind specifically the native env SU protein expressed at the surface of HTLV-I infected lymphocytes and by antibodies contained in the sera of HTLV-I-positive patients. In addition, the purified pET 2240 env protein inhibited HTLV-I induced cell fusion in vitro. Taken together, these findings indicated that the carboxyl terminal part of the HTLV-I SU expressed in *E. coli* is correctly folded and displays important immunological and functional properties of this domain of the viral envelope protein.

2. Experimental

2.1. Cell lines

The HTLV-I infected cell line 2060 [25] was maintained in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (Biowhittaker, Verviers, Belgium), 100 U/ml penicillin, 100 μg/ml streptomycin and 200 U/ml recombinant Interleukin 2 (Chiron, Suresnes, France). The COS-LTR-LacZ cells [26] were grown in DMEM supplemented with serum and antibiotics as above and 150 μg/ml hygromycin B (Calbiochem, San Diego, CA, USA). The XC-Tat cells [26] were maintained in RPMI 1640 supplemented with serum, penicillin and streptomycin, as above, plus 400 μg/ml geniticin (Sigma–Aldrich, Saint Quentin Fallavier, France).

2.2. Monoclonal antibodies

The monoclonal antibodies MF2, DB4, DF2 and DB5 were raised in Balb/c mice immunized with peptides corresponding to immunodominant regions of the HTLV-I gp 46 [27]. MF2 recognizes an epitope corresponding to the amino acid stretch 190–197; DB4 recognizes the region encompassing amino acids 246 to 252; DF2 specifically recognizes an epitope comprised between amino acids 252 and 261; DB5 recognizes an epitope located in the amino acid stretch 244–252. The monoclonal antibodies 7G5D8 and 4F5F6 were obtained from mice injected with 2060 cells and boosted with partially purified HTLV-I viral glycoproteins from chronically HTLV-I producing cells [28]. The monoclonal antibody 7G5D8 specifically recognizes the region encompassing amino acids 183 to 191, while the monoclonal 4F5F6 recognizes the sequence stretch 175–199 of the surface envelope of HTLV-I, but not smaller sequences as determined by synthetic peptide reactivity.

2.3. Sera

Human sera used in this study were provided by: Dr. J.C. Vernant (La Meynard Hospital, Fort-de-France, Martinique), Dr. J.F. Moreau and Dr. Sar-

thou (Institut Pasteur Cayenne, French Guiana), Dr. S. Sainte-Foie and Dr. C. Hajjar (Centre Hospitalier Intercommunal de Basse-Terre/Sainte-Claude, Guadeloupe), and Dr. M.C. Georges-Courbot (CIRMF, Franceville, Gabon). All sera were heated for 30 min at 56°C before use. The presence of HTLV-I antibodies in these sera was assessed using a commercially available Western blot diagnostic kit (Gene Labs Diagnostics, Singapore).

2.4. Construction of the env expression vector

All restriction enzymes and nucleic acid modifying enzymes used in this study were obtained from Roche Diagnostic, Meylan, France. The envelope gene fragment *Sal*I–*Kpn*I coding for amino acids 165 to 314 was derived from an HTLV-I provirus cloned from 2060 cells [25]. The plasmid DNA was successively digested by the restriction enzymes *Sal*I (5 units/μg DNA) and *Age*I (5 units/μg DNA) and treated by Klenow enzyme (1.5 unit/μg DNA) to obtain blunt ends. The resulting DNA fragment of 446 bp was eluted and ligated to the *Bam*HI and *Xho*I sites of the pET 22B+ vector (*Bam*HI and *Xho*I sites were blunted by Klenow treatment). The pET22B+ vector was obtained from Novagen (Abingdon, UK). The resulting plasmid was named pET 2240 (Fig. 1). In this construct, the HTLV-I env coding sequence was inserted in frame with the leader sequence of the pectate lyase B (*pel*B) gene [29] which allowed expression of the envelope recombinant protein in the periplasmic compartment where disulfide bonds can be formed. Furthermore, this protein had a six histidine tag at the carboxyl end to facilitate purification. The nucleotide sequence of the insert and of the flanking regions in the selected clone pET 2240 was confirmed using the dideoxy sequencing method [30]. For HTLV-I envelope expression, the plasmid pET 2240 was introduced into BL21DE3 bacteria [F- omp T, hsd SB(rB-mB-), gal.dcm (DE3)]. This bacterial strain possesses a sequence coding for the bacteriophage T7 RNA polymerase under control of the Lac promoter (promoter of the lactose operon). The expression of the recombinant envelope protein was induced with IPTG (isopropyl thio-β-D-galactoside), which allowed derepression of the phage T7 RNA polymerase gene and of the envelope sequence inserted into the pET 2240 vector.

2.5. Induction of protein expression and periplasmic fraction isolation

All culture media and ingredients for the growth of bacteria were obtained from Difco Labs. (Detroit, MI, USA). An overnight culture of BL21DE3 con-

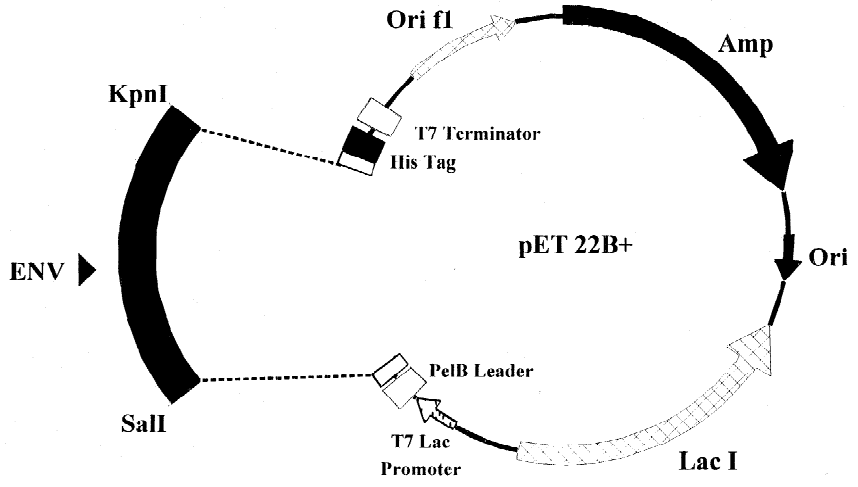

Fig. 1. Construction of the env expression vector pET 2240. The envelope fragment *Sal*I–*Kpn*I was ligated to the *Bam*HI and *Xho*I sites of the pET 22B+ vector. The resulting plasmid containing this envelope fragment was named pET 2240.

taining the pET 2240 plasmid in Luria–Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) was diluted (1:100) in LB medium supplemented with 0.4 *M* sucrose, and incubation continued at 37°C with vigorous shaking until an optical density at 600 nm ($O.D._{600}$) of 0.3 was obtained. At this time, synthesis of HTLV-I envelope was induced by IPTG (Euromedex, Souffelweyersheim, France) at a final concentration of 0.4 m*M*. After 16 h incubation at 25°C, the cells were harvested by centrifugation (Sorvall GSA rotor, 5500 rpm, 15 min, 4°C) and resuspended in 30 m*M* Tris, pH 8, 20% sucrose. After incubation with 1 mg/ml lysozyme for 10 min at 4°C, the suspension was centrifuged (Sorvall SS34 rotor, 9500 rpm, 30 min, 4°C). The supernatant corresponding to the periplasmic fraction was supplemented with protease inhibitors (Complete mini EDTA-free, Roche Diagnostics) and used as a source of recombinant protein in the subsequent chromatographic steps.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The periplasmic proteins and the fractions eluted from the different columns were diluted in phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) (blocking buffer) and incubated overnight at 4°C on NiNTA His Sorb Strip (Quiagen, Courtaboeuf, France). After four washes with PBS containing 0.1% Tween 20 (Sigma–Aldrich), 20 μg/ml of monoclonal antibodies or human sera at 1:100 dilution in blocking buffer were added and incubated for 1 h at room temperature. The plates were washed four times with PBS containing 0.1% Tween 20, and incubated with a peroxidase-labeled goat anti-mouse or anti-human IgG(Fab′)2 fragments (Immunotech, Marseille, France) for 1 h at room temperature. After four washes in PBS containing 0.1% Tween 20, the ABTS substrate (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] [6]), Roche Diagnostic, Meylan, France) was added and the O.D. determined at 405 nm.

2.7. Western blot analysis

The proteins contained in the periplasmic extract or in the chromatographic fractions were separated by electrophoresis in 12.5% SDS polyacrylamide gel described by Laemmli [31] and transferred onto a nitro-cellulose membrane (Hybond C super, Amersham–Pharmacia Biotech, Saclay, France). The membrane was incubated overnight at room temperature in 2% non-fat dry milk and 0.5% BSA in PBS, 20 m*M* Tris, pH 7.4, 0.05% Tween 20. The membranes were then incubated in the same buffer containing 20 μg/ml of MF2 monoclonal antibody specific for the HTLV-I gp 46 for 2 h at 37°C and washed in PBS containing 0.1% Tween 20. The membranes were then incubated with peroxidase-labeled goat (Fab′)2 fragments anti-mouse IgG for 1 h at room temperature. After five washes, the bound antibodies were revealed using the Super Signal chemiluminescence kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

2.8. Purification of pET 2240 env protein from the periplasm

The periplasmic fraction was submitted to a centrifugation at 16 000 rpm for 20 min (Sorvall SS 34 rotor, 4°C) and clarified by filtration through a 0.22-μm filter (Sartorius, Göttingen, Germany).

The filtrate was applied to a HiTrap NHS-activated (1 ml) column (Amersham–Pharmacia Biotech) loaded with the MF2 antibody specific for the surface envelope glycoprotein of HTLV-I, and equilibrated with buffer A containing 30 m*M* Tris, pH 8, 0.15 *M* NaCl. The column was washed with 10 ml of buffer A, and 10 ml of 0.15 *M* NaCl. Then, 100 m*M* triethylamine, pH 11.5 (10 ml) at a flow-rate of 12 ml/h was applied, and the eluted fractions (1 ml) neutralized with 50 μl of 1 *M* Tris–HCl. The fractions containing the HTLV-I protein were identified by Western blotting using the MF2 antibody. The fractions containing the protein of interest were pooled and supplemented with 2 *M* GuHCl (guanidine·HCl, Sigma–Aldrich) and 0.5% *N*-octyl glucoside (Roche Diagnostics). The pooled fractions were applied to a spin column (Amersham–Pharmacia Biotech) or NiNTA agarose (Quiagen) and equilibrated in 20 m*M* Tris, pH 8, 2 *M* GuHCl.

The column was washed with 20 m*M* Tris, pH 8, 2 *M* GuHCl and the bound proteins eluted with 5 ml of buffer containing 20 m*M* Tris–HCl, pH 8, 100 m*M* EDTA, 0.5% *N*-octyl glucoside, 0.5 *M* NaCl without GuHCl.

The proteins contained in the eluted fractions were fractionated on SDS–polyacrylamide gel electrophoresis (PAGE) and revealed by silver staining or Western blot analysis.

2.9. Silver staining

The polyacrylamide gel was incubated three times for 1 h in methanol–water–acetic acid (5:5:1) with gentle shaking. The gel was then incubated for 15 min in 50% methanol, 12% trichloroacetic acid (TCA), 2% $CuCl_2$; 15 min in 10% isopropanol, 5% acetic acid; 15 min in a freshly prepared solution of 0.01% $KMnO_4$; 10 min in 10% isopropanol, 5% acetic acid. After two washes with water (10 min each), the gel was incubated in 0.1% $AgNO_3$ for 10 min and washed with water. Finally, the gel was placed in a solution containing 2% Na_2CO_3, 1 ml/l formaldehyde for 4 to 6 min. The coloration was stopped with 5% acetic acid.

2.10. Inhibition of syncytia formation by pET 2240 env protein

The two-step-purified envelope protein was diluted in 20 m*M* Tris, pH 8 to reduce EDTA and *N*-octyl glucoside concentrations, and concentrated to the initial volume on centricon p10 (Millipore, Saint Quentin Yvelines, France). Serial log 2 dilutions (in 0.5 ml starting at 1 μg/ml) were mixed with 70 000 HTLV-I infected 2060 cells per well in 24-well microplates (Falcon, Becton Dickinson, Plymouth, UK).

XC-Tat and Cos LTR HIV lacZ cells were then added in 0.5 ml (50 000 cells per well) and incubation continued for 24 h. The β-galactosidase activity was revealed by a colorimetric method using CPRG (Roche Diagnostics, Meylan, France) as enzyme substrate as previously described [32]. The percentage of inhibition at various protein concentration was calculated from:

$$\% = 100 - \left[\frac{N_x - N_0}{N_+ - N_0} \right] \times 100$$

where N_x corresponds to the absorbance at 620 nm for a given protein dilution, N_+ to the absorbance of the positive control (coculture with HTLV-I infected cells without the env protein) and N_0 to the absorbance of the negative control (coculture without HTLV-I infected cells).

3. Results

3.1. Expression of pET 2240 protein in bacteria

The pET 2240 plasmid described in Experimental or the pET 22B+ used as a control were introduced into BL21DE3 bacteria. The bacterial cells were grown in LB sucrose medium, and HTLV-I env protein synthesis was induced by addition of IPTG overnight at 25°C. Aliquots of culture medium and periplasmic or cytoplasmic fractions were fractionated by SDS–PAGE and analyzed by Western blotting using the MF2 monoclonal antibody specific for the HTLV-I SU envelope (Fig. 2). In the three fractions corresponding to pET 2240 transformed bacteria (lanes 2, 4 and 6), this antibody revealed a protein of about 22 K. Minor amounts of a degradation product were also detected in the periplasmic

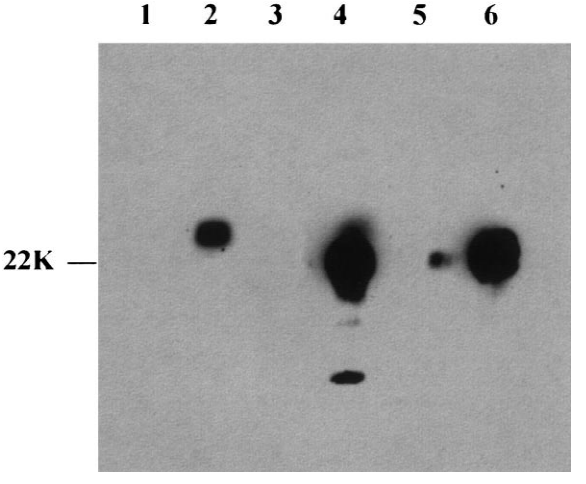

Fig. 2. Detection of the recombinant envelope protein by Western blot analysis. A 15-μl volume of each fraction was analyzed by polyacrylamide gel 12.5% and transferred onto nitrocellulose membranes and revealed with a monoclonal antibody MF2 (20 μg/ml) specific for the HTLV-I envelope. The culture medium, periplasmic and cytoplasmic fractions of bacteria transformed by pET 22B+ were analyzed on lanes 1, 3 and 5, respectively. The culture medium, periplasmic and cytoplasmic fractions of bacteria transformed by pET 2240 were analysed on lanes 2, 4 and 6, respectively.

fraction. No protein was revealed in the same fractions isolated from bacteria transformed by pET 22B+ (lanes 1, 3 and 5). These results indicated that an env protein of the expected size can be induced from the HTLV-I env sequences inserted into the pET 2240. As the secreted protein was found in aggregates or bound to cell debris, we decided to purify the recombinant envelope protein from the periplasmic fraction where disulfide bonds can be formed.

3.2. Purification of the recombinant HTLV-I envelope protein

In order to estimate the amount of recombinant HTLV-I envelope protein in the periplasm of BL21DE3 transformed by pET 2240, the proteins isolated from this cell compartment were fractionated by SDS–PAGE and Coomassie stained (Fig. 3A). Numerous proteins were observed in the periplasm of bacteria transformed by pET 2240 after IPTG induction (total protein concentration = 1.6 mg/ml) (lane 1), while no protein was detected in the periplasm of bacteria transformed by pET 22B+ (lane 2). This suggested that the production of the recombinant envelope protein pET 2240 may induce changes in membrane structure, which allowed passage of cytoplasmic proteins into the periplasmic compartment. The periplasmic fraction after centrifugation and clarification by filtration was supplemented with protease inhibitors, and used in the different chromatographic steps. Firstly, an immunoaffinity column loaded with the MF2 monoclonal antibody was used and the eluted fractions analyzed by SDS–PAGE followed by Western blotting or Coomassie staining (Fig. 3A and B). After Coomassie staining, a major contaminant protein of about 68 K was visible in the eluted fractions in addition to other proteins (Fig. 3A, lanes 3 and 4), while the 22 K recombinant envelope protein was barely visible and only revealed by Western blot analysis (Fig. 3B, lanes 3 and 4). Secondly, the eluted fractions were pooled and loaded onto a nickel column in 20 m*M* Tris, pH 8, 0.15 *M* NaCl and eluted with the same buffer containing 100 m*M* EDTA. Despite an increase in purity, the recombinant env protein was always contaminated by the 68 K protein of *E. coli* (data not shown). We tried various saline concentrations to avoid interactions between these two proteins. We succeeded by adding GuHCl at 2 *M*

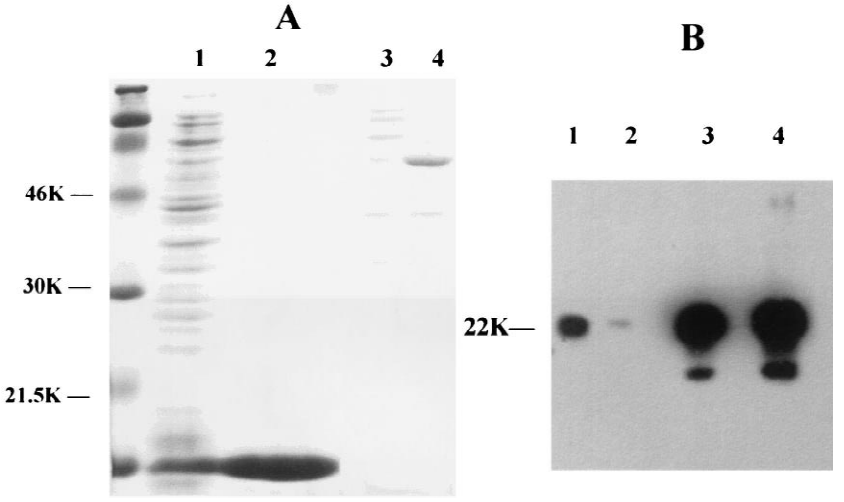

Fig. 3. SDS–PAGE analysis of eluted fractions from immunoaffinity column. Periplasmic proteins of bacteria transformed by PET 2240 were applied to a HiTrap NHS-activated column loaded with MF2 monoclonal antibody. (A) A 50-μl volume of each eluted fraction was analyzed by Coomassie staining (lanes 3 and 4). The total proteins (50 μl) in the periplasm of bacteria transformed by pET 2240 (lane 1) or pET 22B+ (lane 2) are shown. (B) A 10-μl volume of each eluted fraction was analyzed by Western blotting (lanes 3 and 4). The total proteins in the periplasm of bacteria transformed by pET 2240 (10 μl) are shown in lane 1. Lane 2 represents 10 μl of the flow-through of the column.

final concentration before loading onto the nickel column. This step enabled us to obtain a recombinant envelope protein which appeared to be essentially pure as judged by silver staining (Fig. 4, lane 2). At this stage, the envelope recombinant protein tended to form aggregates which sedimented after centrifugation (SS34 rotor, 16 000 rpm, 20 min). We finally succeeded in obtaining a pure protein in a soluble form after addition of 0.5% *N*-octyl glucoside. In some experiments, this protein formed a double band in SDS–PAGE when β mercaptoethanol was used as reducing agent (Fig. 4, lanes 2 and 3). When 0.1 *M* dithiothreitol (DTT) (Roche Diagnostic) was used instead of β-mercaptoethanol, the env protein migrated as one band (Fig. 4, lane 4).

To determine the amount of pET 2240 env protein which had been purified by the procedure described above, we used a Bradford assay and routinely obtained 50 to 100 μg of purified protein from 1 l of culture.

3.3. Probing of the correct structure of the recombinant envelope protein

In order to determine if the recombinant protein structure was correctly formed in bacteria, an ELISA was conducted on the periplasmic proteins isolated from pET 2240/BL21DE3 or pET 22B+/BL21DE3 partially purified by immunoaffinity. In this assay, we employed five different monoclonal antibodies in addition to MF2: DB4, DF2, 4F5F6 and 7G5D8 that recognized peptides of the carboxyl part of the envelope of HTLV-I in ELISA as well as on the surface of virus-producing cells as revealed by immunofluorescence; DB5 that recognized a peptide corresponding to aa 244 to 252 only in a denatured form of the envelope protein. As shown in Fig. 5, MF2, DB4, DF2, 7G5D8 and 4F5F6 at 20 μg/ml recognized the recombinant envelope pET 2240, while DB5, which does not recognize envelope protein expressed on the surface of virus producing cells, failed to bind our recombinant protein.

These results suggested that the two major immunodominant domains of the carboxyl part of the HTLV-I SU were displayed in a similar structure in the recombinant protein produced in *E. coli* and in the envelope glycoprotein SU presented on the surface of virus infected cells.

3.4. Inhibition of syncytia formation by pET 2240 envelope protein

The carboxyl half part of the HTLV-I SU harbors regions involved in fusion mechanisms of viral and

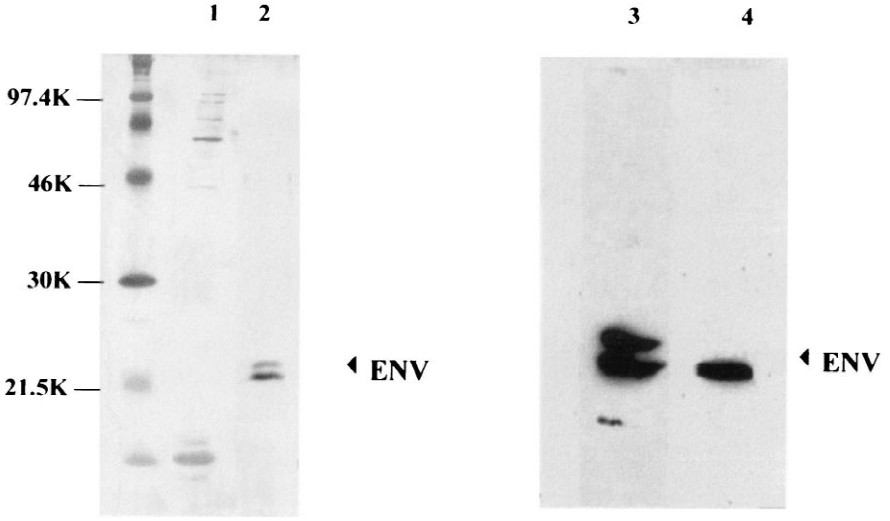

Fig. 4. Analysis of fractions eluted from the nickel column. Lane 1: pooled fractions from immunoaffinity column. Lanes 2, 3 and 4 contained fractions eluted from nickel column revealed by silver staining (lane 2) or Western blotting (lanes 3 and 4). The loading buffer contained β-mercaptoethanol for samples in lanes 1–3, or DTT for the sample in lane 4.

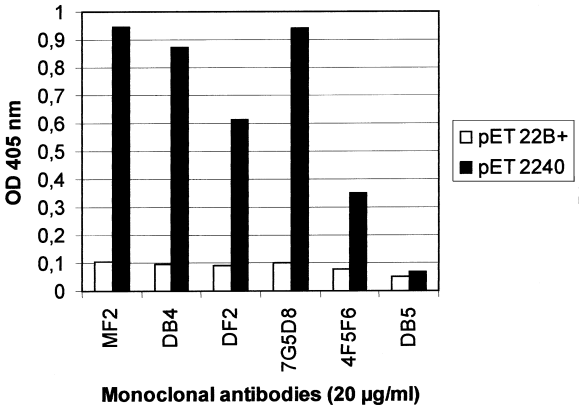

Fig. 5. Recognition of envelope recombinant protein by monoclonal antibodies in ELISA. The periplasmic proteins corresponding to pET 2240/BL21DE3 (black column) or pET 22B+/BL21DE3 (white column) purified by immunoaffinity were incubated overnight at 4°C on NiNTA His Sorb Strip. An ELISA was performed with monoclonal antibodies specific for different epitopes of the HTLV-I SU (20 μg/ml).

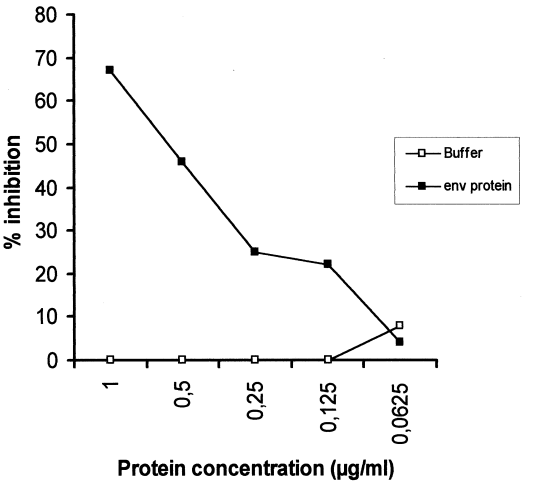

Fig. 6. Inhibition of syncytia formation by pET 2240 env protein. The two-step purified recombinant protein pET 2240 at various serial log 2 dilutions was incubated with 2060 cells and Cos LTR HIV LacZ, XCTat as described in Experimental.

cellular membranes that allow penetration of the viral capsid into the target cell. This fusion mechanism can be partly reproduced in an in vitro assay termed syncytia assay. In this assay, the retroviral envelope molecules present at the surface of HTLV-I infected cells induced fusion with target cells expressing the viral receptor allowing formation of multinucleated giant cells termed syncytia. In this report, a β-galactosidase assay was employed for quantitative evaluation of syncytia formation. For this, HTLV-I producing cells were cocultivated with Cos cells containing the *lac*Z gene under the control of the HIV-LTR promoter and XC cells expressing the transactivator tat protein of HIV. The HTLV-I env proteins induced fusion between the three cells allowing transactivation of HIV-LTR by the tat protein and synthesis of the β-galactosidase. To determine if domains of the HTLV-I SU involved in fusion mechanisms were functional in the pET 2240 envelope protein expressed in *Escherichia coli*, we performed competition experiments between envelope protein expressed on the surface of HTLV-I infected cells and the purified pET 2240 envelope protein in a syncytia assay. Results of a representative experiment presented in Fig. 6 indicated that as little as 1 μg/ml of pET 2240 envelope protein strongly inhibited syncytia formation by HTLV-I 2060 producing cells. This inhibition was dose-dependent and no inhibition was observed in the same type of experiment performed with buffer alone. These observations indicated that the pET 2240 envelope protein produced in *Escherichia coli* was folded in a way that preserved the correct structure of the domains associated with cell fusion of the HTLV-I SU.

3.5. Detection of antibodies specific for the HTLV-I envelope proteins in human sera

The results obtained in the experiments described above indicated that the purified recombinant envelope protein had conserved the immunological and biological properties of the native envelope protein. To confirm these results, we performed ELISA on our recombinant protein with human sera from HTLV-I or HIV-I infected patients as well as sera from uninfected individuals. The results (Fig. 7) showed that HTLV-I positive sera recognized much more periplasmic proteins partially purified from pET 2240/BL21DE3 than those from pET 22B+/BL21DE3. Sera from HIV-I-infected patients or from patients uninfected by retroviruses did not recognize this recombinant protein.

Thus, the pET 2240 protein produced in *E. coli*

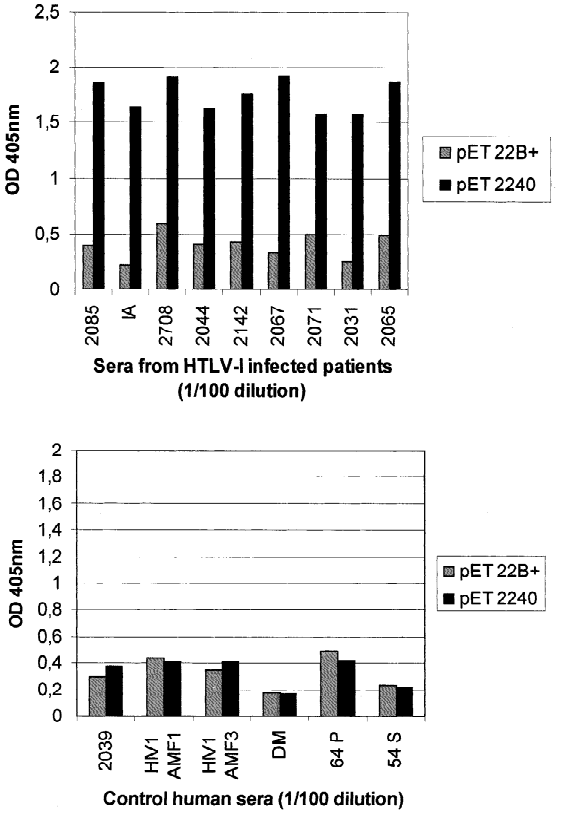

Fig. 7. Recognition of envelope recombinant protein by human sera in ELISA. The periplasmic proteins corresponding to pET 2240 (black column) or pET 22B+ (grey column) purified by immunoaffinity were incubated overnight at 4°C on NiNTA His Sorb Strip with different human HTLV-I-positive or control human sera (1:100 dilution). After washes, the bound antibodies were revealed as described in Experimental.

displayed immunodeterminants recognized by monoclonal antibodies specific for the HTLV-I SU and by antibodies present in infected patients. Besides, it has biological properties similar to those of the native protein expressed on the surface of infected cells. Thus, it might be useful for structural and immunological studies.

4. Discussion

In this report, we described a method for obtaining a pure and soluble form of a 22 K protein corresponding to the carboxyl moiety of the gp 46 surface envelope protein of HTLV-I produced in *Escherichia coli*. We selected an expression system which allowed protein expression in the periplasm, the compartment of bacterial cells where disulfide bridges are formed. In addition, the protein produced in these conditions contained a six-histidine tag at the carboxyl terminal end to facilitate protein purification. The purification procedure comprised three steps. It was complicated mainly by the solubility and the low level production of the recombinant envelope protein.

The first step consisted of two differential centrifugations and a filtration which removed cell debris, membranes and aggregated proteins.

The second step was a NHS-immunoaffinity column using a monoclonal antibody specific for the HTLV-I SU. This step concentrated the envelope protein and removed most cellular proteins. At this stage, the protein was still contaminated by a 68 K bacterial protein and often formed large aggregates. To overcome this problem, GuHCl (2 *M*) and a non-ionic detergent, *N*-octyl glucoside (0.5%) were added to the pooled fractions eluted from the NHS column.

This pool was loaded onto a nickel affinity column, which produced a more than 90% pure 22 K envelope protein. At the concentration currently obtained in the fractions eluted from the nickel column (10–20 μg/ml), the 22 K envelope protein remained soluble in the absence of GuHCl, but 0.5% *N*-octyl glucoside and 0.5 *M* NaCl were both required. The high yield of pET 2240 protein recovered after the different purification steps allowed us to obtain 50 to 100 μg purified protein from 1 l of culture medium. These conditions enabled immunological and biological tests, which showed that the purified envelope protein had comparable immunological and functional properties to those of the native protein expressed on the surface of virus particles or virus-infected cells. Indeed, the pET 2240 protein was recognized in ELISA by monoclonal antibodies that recognized the HTLV-I envelope proteins in immunofluorescence (IF) analysis on living cells, but a monoclonal antibody that recognized denatured envelope protein by Western blot analysis but was negative by IF analysis failed to bind the pET 2240 env protein in ELISA. Finally, the purified protein interfered in an in vitro fusion

assay involving native HTLV-I envelope proteins expressed on the surface of HTLV-I producing cells. This indicated that the domain comprised between amino acids 170–233 which is associated with fusion properties of the native HTLV-I SU was functional in the pET 2240 env protein.

Although it contained only the carboxyl half of the HTLV-I SU, the pET 2240 purified protein may prove of value. Indeed, it has been shown by several groups that this domain contains the major immunodominant and neutralizable epitopes of this protein and is also involved in fusion events following receptor binding.

It could be utilized in structural studies to further understand the mechanisms of HTLV-I entry into the cell and to define molecules that inhibit this crucial step in the retroviral replication cycle.

This molecule could also be employed, alone or in combination with other components, in a vaccine against HTLV-I. An effective vaccine against this virus is feasible as the viruses exhibit few sequences variations world-wide [33] and different versions of the HTLV-I envelope proteins have been shown to prevent infection in animals [10–12]. Evaluation of the capacity of the pET 2240 protein to induce neutralizing antibodies against HTLV-I is underway and preliminary results have indicated that this protein induces such antibodies in rabbits. Finally, the production and purification procedure described in this report enabled us to recover relatively large amounts of purified env protein. This protein could be employed for screening and quantification of specific antibodies in sera of infected people or of antibodies engineered by recombinant technology.

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