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A *gag-env* Hybrid Protein of Human T-cell Leukemia Virus Type I and Its Application to Serum Diagnosis

Tetsuro KUGA,[*1, *5] Motoo YAMASAKI,[*1] Susumu SEKINE,[*1] Masanori FUKUI,[*2] Yoshiharu YOKOO,[*1] Seiga ITOH,[*1, *6] Mitsuaki YOSHIDA,[*3] Toshio HATTORI[*4] and Kiyoshi TAKATSUKI[*4]

[*1]*Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Asahi-machi 3-6-6, Machida-shi, Tokyo 194,* [*2]*Kyowa Medex Co., Ltd., Oote-machi 1-6-1, Chiyoda-ku, Tokyo 100,* [*3]*Department of Viral Oncology, Cancer Institute, Kami-Ikebukuro 1-37-1, Toshima-ku, Tokyo 170 and* [*4]*Second Department of Internal Medicine, Kumamoto University Medical School, 1-1-1, Honjo, Kumamoto-shi, Kumamoto 860*

A fused gene of the *gag* and *env* sequences of human T-cell leukemia virus type I (HTLV-I), the causative agent of adult T-cell leukemia, was constructed *in vitro* and expressed in *Escherichia coli*. The *gag-env* hybrid protein accumulated as insoluble granules with a yield of approximately 12% of the total proteins. In an enzyme-linked immunosorbent assay done with the use of the *gag-env* hybrid protein, all 57 seropositive sera gave positive signals, and none of the sera from normal persons did. This system can produce large quantities of the *gag-env* hybrid protein, which can be used for mass screening of human sera for HTLV-I infection.

Key words: HTLV-I — Hybrid antigen — Antibodies — Diagnosis

Adult T-cell leukemia (ATL) is an unique malignancy of T-cell origin.[1] A retrovirus has been isolated from cell lines derived from patients with ATL[2–4] and named human T-cell leukemia virus type I (HTLV-I).[5] The provirus genome of HTLV-I has been cloned molecularly[6] and the etiological association of HTLV-I with ATL was clearly demonstrated.[7] HTLV-I infection is endemic in southwestern Japan.[8] Three routes of transmission of HTLV-I have been suggested: from mother to child, from husband to wife,[9] and by blood transfusion.[10] To help to prevent such transmission, identification of HTLV-I carriers by screening for antibodies could be done.

Two methods have been developed to detect serum antibodies against HTLV-I: an enzyme-linked immunosorbent assay (ELISA)[11] and the particle agglutination (PA) procedure.[12] At present, HTLV-I particles prepared from culture supernatants of cell lines that produce the virus are used as the antigen in both of these methods. However, the preparation is hazardous and productivity is unsatisfactory. In addition, reactions to cell membrane proteins associated with HTLV-I particles can give false-positive reactions. To solve these problems, we and others have studied the expression of one of the HTLV-I antigens by use of recombinant DNA techniques in microorganisms.[13–18]

In this report, we describe the production of a *gag-env* hybrid protein of HTLV-I in *Escherichia coli (E. coli)*. Preliminary results showed that the hybrid protein can be used for the diagnosis of HTLV-I infection with serum samples.

MATERIALS AND METHODS

Plasmids The plasmids pAFG10 and pEFM2 were used to construct a plasmid (pET17) for the expression of a *gag-env* hybrid protein. Both plasmids were derived from the HTLV-I provirus clone λATM1.[6] In pAFG10, the *gag* gene (nucleotides 1231–1839 of the provirus genome; hereafter nucleotides are specified as reported by Seiki *et al.*[6]) was inserted between the tandemly linked tryptophan promoter and lipoprotein transcription terminator of *E. coli*. Details of the structure of pAFG10 were reported previously.[17] In pEFM2, the truncated *env* gene (nucleotides 5253–6122) was inserted between the same set of promoter and terminator sequences as in pAFG10.

[*5] Present address: Technical Research Laboratories, Hofu Plant, Kyowa Hakko Kogyo Co., Ltd., Kyowa-machi 1-1, Hofu-shi, Yamaguchi 747.
[*6] To whom correspondence should be addressed.

DNA Manipulation Restriction endonucleases and T4 DNA ligase were purchased from Takara Shuzo (Kyoto). These enzymes were used according to the supplier's recommendations.

Sera Sera were obtained from patients with ATL or from healthy volunteers. Antibodies to HTLV-I were detected by strip radioimmunoassay based on Western blotting and indirect immunofluorescence assay.

Preparation of *gag-env* Hybrid Protein *E. coli* K12 strain W3110*strA* was transformed with pET17 and the resulting transformant was cultured as described previously.[19] The *gag-env* hybrid protein was partially purified by the method of Marston *et al.*[20] and further purified by CM-Toyopearl cation-exchange chromatography.

Enzyme-linked Immunosorbent Assay Each well of a 96-well microtiter plate was coated with the *gag-env* hybrid protein (1 μg) adequately diluted with 20m*M* carbonate buffer, pH 9.5, at 4° overnight. After the wells were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 80 (T-PBS), 200 μl of 3% bovine serum albumin in PBS was added and the plate was incubated at room temperature to prevent non-specific binding. Test sera (50 μl) diluted 1:20 were added and incubated at room temperature for 1 hr. After the wells were washed with T-PBS three times, the antigen-antibody complexes formed were detected by incubation with horseradish peroxidase-linked anti-human Ig(Fab) and a peroxidase reaction at room temperature for 15 min in citrate-phosphate buffer, pH 5.0, containing 0.006% H_2O_2 and 0.04% *o*-phenylenediamine. ELISA titers were expressed as the absorbance at 450 nm. Control ELISA tests with antigens prepared from cell lines that produced HTLV-I were done with commercially available ELISA kits (Eitest-ATL; Eisai Co., Ltd., Tokyo) and for these tests, the ELISA titers were expressed as the absorbance at 405 nm.

Results

Bacterial Production of *gag-env* Hybrid Protein Expression of parts of the *env* gene in *E. coli* as fused proteins with peptides of bacterial origin has been reported.[13, 14, 18] Here, we expressed the *env* gene in fused form with the *gag* gene, because the *gag* protein is efficiently produced in *E. coli*.[17] In addition, the hybrid protein is expected to react with antibodies against both the *gag* and *env* gene products. The 5′-terminal half of the *gag* gene in pAFG10 and the 3′-terminal one-third of the *env* gene in pEFM2 were fused as shown in Fig. 1 and the resulting plasmid was named pET17. The two genes were ligated between

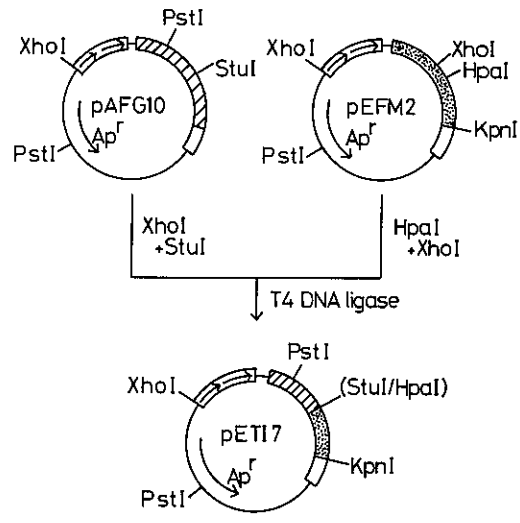

Fig. 1. Construction of the expression plasmid of the *gag-env* hybrid protein. Plasmid pAFG10 was digested with *Xho* I and *Stu* I, and the resulting 0.63-kb fragment containing the tandemly linked tryptophan promoter and the N-terminal half of the *gag* gene (p24) was inserted between the *Xho* I and *Hpa* I sites of pEFM2. The tandemly linked tryptophan promoter is indicated by arrows. The lipoprotein terminator, *gag* gene, and *env* gene are shown by open, hatched, and dotted boxes, respectively.

the *Stu* I site (nucleotide 1607) and the *Hpa* I site (nucleotide 5826) in the *gag* and *env* genes, respectively. The structure of the *gag-env* hybrid protein encoded by pET17 is shown in Fig. 2. It was composed of 228 amino acids; 126 from the *gag* protein and 99 from the envelope protein with Met-Asp at the N-terminus and lysine at the C-terminus from the vector sequences.

E. coli strain W3110*strA* was transformed with pET17. When an extract of a transformant was analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), a stained band at the molecular weight of about 25,000 was detected (Fig. 3). The size of this protein was the same as that expected from the DNA structure. The hybrid protein was accumulated as insoluble granules in the *E. coli* cells. Such insoluble granules are frequently detected when foreign proteins are efficiently produced in *E. coli*.[14, 17, 19] When the cells were cultured in a jar fermenter, the

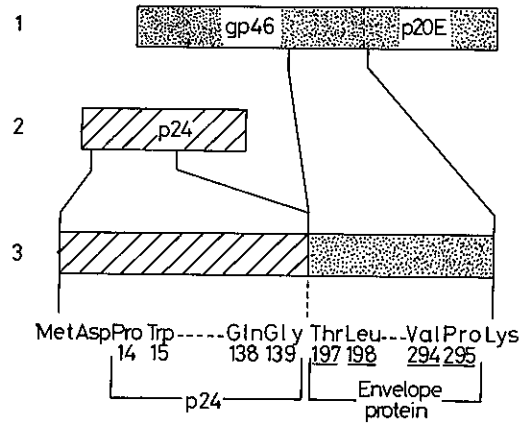

Fig. 2. Structure of the *gag-env* hybrid protein. The portions of the *gag* protein (p24; major core antigen) and the envelope protein included in the *gag-env* hybrid protein are shown. Proteolytically processed products (gp46 and p20E) of the envelope protein are also indicated. Amino acid sequences of the N-terminus, the C-terminus, and the junction site of the hybrid protein are specified. The numbers under the amino acid sequences indicate the position in p24, and the underlined numbers give the position in the envelope protein (numbered without signal sequence). Met-Asp and lysine at the N- and C-termini, respectively, were derived from vector sequences. 1, Envelope protein; 2, *gag* protein; 3, *gag-env* hybrid protein.

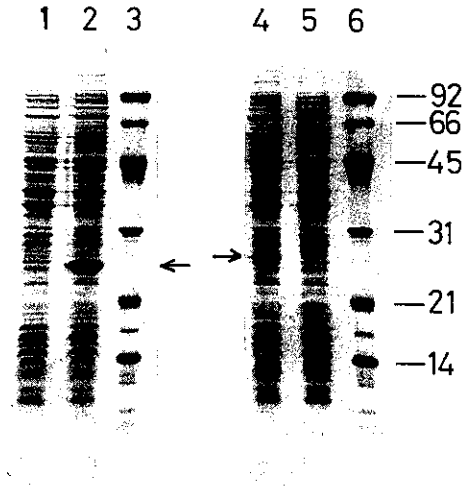

Fig. 3. Production of the *gag-env* hybrid protien in *E. coli*. The *E. coli* K12 strain W3110*strA* harboring pET17 or pAFG10 was cultured and whole-cell extracts were analyzed by SDS-PAGE with Coomassie brilliant blue staining. The *gag-env* hybrid protein and *gag* protein are indicated by arrows. Molecular mass markers are in kilodaltons. Lanes 1, 5, without plasmid; lane 2, pAFG10; lane 4, pET17; lanes 3, 6, molecular mass markers.

yield was 12% of the total protein and pET17 was stably maintained in *E. coli* (100% of the cells contained the plasmid after 48 hr of cultivation). Thus, the *gag-env* hybrid protein was stably produced in large-scale culture.

Further attempts to produce a larger portion of the envelope protein as a fused protein with *gag* protein or to increase productivity were unsuccessful, because the plasmid was deleted from the *E. coli* cells during cultivation (data not shown).

Application of *gag-env* Hybrid Protein to Serum Diagnosis To test whether this hybrid protein could be used to diagnose HTLV-I infection, the hybrid protein was purified to 95% as judged by SDS-PAGE (data not shown) and used in ELISA as described in "Materials and Methods." First, 30 control sera from healthy adult volunteers were surveyed to establish a cut-off level (Fig. 4A). The cut-off level was taken as the mean plus 2.5 times the standard deviation (0.0625 for Eitest-ATL), at which level all 30 sera were judged to be negative. By the same criterion, the cut-off level was 0.0875 for the hybrid protein, and all control sera were again judged to be negative. Next, 57 seropositive sera with antibody titers of $>2^6$ in indirect immunofluorescence (IF) assay were tested. The ELISA titer against the hybrid protein was in a narrower range than in the control ELISA (Fig. 4B). This might reflect the limited variety of epitopes in the hybrid antigen, because it was composed of small portions of the *gag* and *env* antigens. Alternatively, the different amounts of antigens, second antibodies, or both might account for these results. Nevertheless, all sera tested were found to be positive by use of the hybrid protein. One of the 57 IF-positive sera was found to be negative by control ELISA. Similar disagreements of the results of Eitest-ATL and IF tests have been reported by Kamihira *et al.*[21]; the Eitest-ATL gave 3.3% false-negative results. ELISA titers in the two ELISA assays were correlated (r=0.748). The high efficiency of detection of

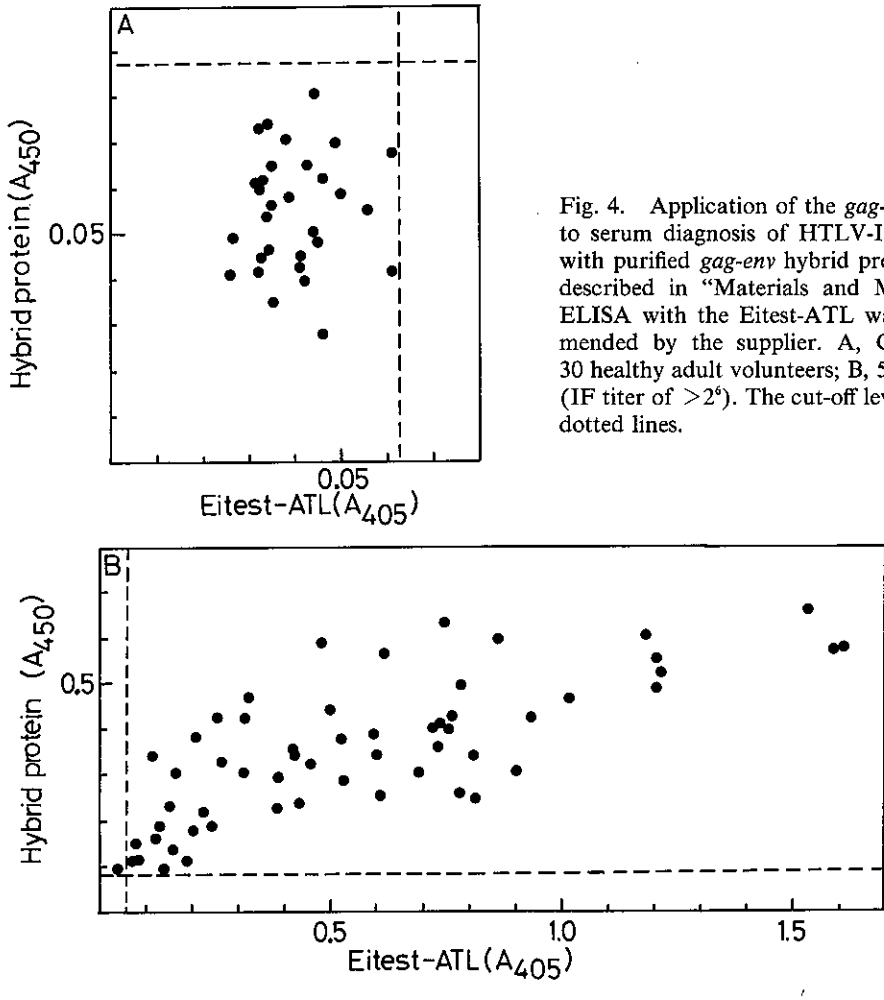

Fig. 4. Application of the *gag-env* hybrid protein to serum diagnosis of HTLV-I infection. ELISA with purified *gag-env* hybrid protein were done as described in "Materials and Methods." Control ELISA with the Eitest-ATL was done as recommended by the supplier. A, Control sera from 30 healthy adult volunteers; B, 57 seropositive sera (IF titer of $>2^6$). The cut-off level is shown by the dotted lines.

antibodies with the hybrid protein indicated that the hybrid protein could be used for serum diagnosis of HTLV-I infection with the same accuracy as the antigen preparation used now.

Discussion

Our first strategy for the production of HTLV-I-encoded antigens was to produce both the core antigen (*gag* protein) and the surface antigen (envelope protein) in unfused form. We succeeded in the efficient production of the *gag* protein (p24: major core antigen) in *E. coli*,[17] but production of the envelope protein was difficult, possibly because of the toxicity of this protein to the cells. In general, two methods are used to produce toxic proteins: production as a fused protein or regulated production by use of a repressible promoter. When fused with β-galactosidase, envelope protein has been expressed in *E. coli*[13] but in our experiments, the plasmid was unstable (fewer than 1% of cells contained the plasmid after 16 hr of cultivation) and mass production was difficult (data not shown). Samuel *et al.*[14] reported the expression of almost the same region of envelope protein as in the *gag-env* hybrid protein by use of the highly repressible P_L promoter and the temperature-sensitive repressor cI857 of bacteriophage λ. However, their method involved rapid heat inactivation of cI857 in an 80°

water bath. Such heat treatment is not practical in mass production. When we tried to express envelope protein by use of P_L promoter and cI857, the envelope protein was not produced when the temperature of bacterial culture was shifted from 30° to 42°. On the contrary, pET17 was stably maintained and the *gag-env* hybrid protein was produced constitutively, because the tandemly linked tryptophan promoter is not repressed by the *trp* repressor.[19] Thus, this *gag-env* hybrid protein seems to be the most favorable way to produce the envelope protein of HTLV-I.

In ELISA tests with the *gag-env* hybrid protein, all 57 seropositive sera reacted. This high efficiency of detection of seropositive sera was the expected result of the fusion of the *gag* protein and envelope protein, because the false-negative rates of the recombinant envelope protein and the *gag* protein are 10%[21] and 20% (unpublished data), respectively. A native epitope recognized by the human immune system has been mapped by Copeland *et al.*[22] in the C-terminal region of the processed envelope protein gp46, and the hybrid protein reported here included this region. Thus, the *gag-env* hybrid protein might contain sufficient epitope(s) for serum diagnosis.

Extensive screening of more samples with *gag-env* hybrid protein is now under way. Preliminary results showed that: 1) the rate of nonspecific reaction was less than 1%; 2) when compared with Serodia-ATLA (Fujirebio Inc., Tokyo) which is a widely used PA kit, 90% of sera gave the same result. Detailed results will be published elsewhere.

This hybrid protein is the only antigen so far reported that could be used for serum diagnosis with the same efficiency as that of antigens prepared from cell lines that produce HTLV-I, and it can also be produced in large-scale culture.

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