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Cleavage of Procaryotically Expressed Human Immunodeficiency Virus Fusion Proteins by Factor X_a and Application in Western Blot (Immunoblot) Assays

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The proteins p15 and p24 of the human immunodeficiency virus (HIV) type 1 gag gene were expressed as fusion proteins in *Escherichia coli* for detecting antibodies against the acquired immunodeficiency virus by Western blot (immunoblot) analysis. These fusion proteins contain amino acids 1 to 375 of the *E. coli* β -galactosidase linked to the viral protein(s) by a recognition sequence for the specific protease factor X_a . They are obtained in large amounts in insoluble inclusion bodies. To avoid ambiguous results caused by cross-reaction of sera with bacterial proteins in Western blots, we purified the recombinant fusion proteins and subsequently removed the bacterial part of the fusions by cleavage with factor X_a . The cleavage mixtures were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes. The viral proteins obtained by this method did not contain any bacterial proteins or protein fragments. Thus, false-positive results in HIV Western blot analysis with bacterially expressed HIV proteins can be excluded with these purified recombinant viral antigens.

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Acquired immunodeficiency syndrome is characterized by a severe disorder of the immune system resulting in opportunistic infections, cancer, and neurological syndromes. The causative agent of acquired immunodeficiency syndrome belongs to the group of human retroviruses called human immunodeficiency viruses (HIV). Soon after the etiological agent was identified, many efforts were directed to developing specific tests for the detection of antibodies in infected individuals. Serological tests for detecting antibodies to HIV are usually based on cell culture-derived viral antigens. Primarily, enzyme-linked immunosorbent assays (ELISAs) are used as screening tests. Additional methods, such as Western blots (immunoblots) and immunofluorescence, are usually performed subsequently to confirm a positive ELISA. A serum sample is defined as HIV antibody positive only if the repeatedly reactive ELISA is confirmed by the expected banding pattern in the Western blot assay (3, 22). A Western blot is considered positive if the antibodies are reactive with multiple viral peptides, i.e., the gag protein p24 and either the transmembrane *env* glycoprotein gp41 or the outer *env* glycoprotein gp120 and its precursor gp160. Sera from a number of HIV-infected individuals may have an indeterminate pattern because of a loss of antibodies to non-*env* proteins in advanced immunodeficiency (20) or a lack of detectable *env*-specific antibodies, as described for the early stages of HIV seroconversion (27). Thus, determination of antibodies to several viral antigens is required for a definite diagnosis. The viral proteins required for Western blot analysis are commonly prepared directly from cell cultures infected with HIV. However, this method is expentic procedures (10, 11, 28). The heterologous expression of HIV proteins or protein fragments in bacteria has turned out to be a particularly effective alternative (4, 9, 12). It is safe and inexpensive, and the yields of viral proteins are high. Additionally, genetic manipulations (e.g., the construction of truncated mutants or strain variants) can be performed very easily with the bacterial expression system. Recent studies have demonstrated the reliability of these recombinant proteins as diagnostic reagents (13, 16, 25). However, none of the bacterially expressed HIV proteins has yet been completely separated from the bacterial background. Therefore, one cannot exclude cross-reactivity of human sera with bacterial material in the Western blot analysis, which might lead to a false-positive result. In this report, we describe the expression of epitopes of the p15 and p24 parts of gag as fusions with a truncated β-galactosidase in *Escherichia coli*. These viral sequences are well conserved among different strains of virus, and the fusion proteins have proved to be immunoreactive (1). To remove the bacterial proteins, we have purified these fusion molecules and subsequently removed the β -galactosidase part of the fusion proteins by proteolytic cleavage with the highly specific blood coagulation factor X_a. The first experiences using these cleaved recombinant HIV proteins with human sera in Western blot assays are described.

MATERIALS AND METHODS

Strains, plasmids, and chemicals. The human T-cell lymphotropic virus type III (HTLV-III) was a gift from R. C. Gallo, Bethesda, Md. (21). The plasmid pBD2 (2) was kindly provided by M. Bröker, Marburg, Federal Republic of Germany. For the expression of fusion proteins, we used *E*. *coli* BMH 71-18 [Δ (*lac-pro*) Δ *thi supE* (F' *lacI*^q Z Δ M15 *pro*⁺)] (14). HIV antibody-positive and antibody-negative sera were obtained from our diagnostic laboratories. The ELISA was performed according to the directions of the manufacturer (Electro Nucleonics International, Stuttgart, Federal Repub-

sive and requires handling of infectious virus. To circumvent these problems, other procedures for obtaining large amounts of HIV proteins or protein fragments have been developed. Synthetic peptides have been shown to be recognized by anti-HIV antibodies and to be useful for diagnos-

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lic of Germany). Horseradish peroxidase-coupled protein A, diaminobenzidine, and Russel's viper venom protease were from Sigma Chemical Co., St. Louis, Mo. Chromozym X and isopropyl- β -D-thiogalactopyranoside were from Boehringer Mannheim Biochemicals, Indianapolis, Ind. All other chemicals are available commercially from Aldrich Chemical Co., Inc., Milwaukee, Wis., Sigma, or Merck Sharp & Dohme, West Point, Pa.

General methods. All recombinant DNA techniques were performed according to standard procedures (17). Oligonucleotides were synthesized on an automatic DNA synthesizer (model 380A; Applied Biosystems) by using the phosphoramidite method (5, 24). Protein concentrations were determined by measuring the optical density at 280 nm (23). Separation of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described elsewhere (6, 15). Western blot analysis. For immunoblotting experiments, proteins were electrophoretically transferred from the acrylamide gels onto nitrocellulose filters (26). After transfer, the nitrocellulose filters were blocked with 1% bovine serum albumin and reacted with antisera diluted 1:200. The blot was stained with horseradish peroxidase-coupled protein A using diaminobenzidine as the substrate. Expression and partial purification of fusion proteins. LB medium (100 ml) containing ampicillin (100 µg/ml) was inoculated with 1 ml of an overnight culture of E. coli BMH 71-18, harboring the recombinant expression plasmid, and grown at 37°C. At an optical density at 600 nm of 1.0, isopropyl-β-thiogalactoside was added to a final concentration of 1 mM, and the culture was shaken for an additional 5 to 7 h. The cells were centrifuged and suspended in 3 ml of lysis buffer (136 mM NaCl, 26 mM KCl, 6.5 mM Na₂HPO₄, 14.6 mM KH₂PO₄, 1 mM CaCl₂, pH 6.8) containing lysozyme (1 mg/ml). The suspension was kept on ice for 30 min, and then it was sonified twice for 30 s each (Sonifier B-12; Branson Sonic Power Co., Danbury, Conn.). After centrifugation, the pellet was suspended in sodium phosphate buffer (3 ml, 50 mM, pH 7). The suspension was centrifuged through a cushion of 40% sucrose (100 ml) in the above buffer. The pelleted fusion proteins were solubilized in 10 ml of 8 M urea-10 mM Tris (pH 8.3), centrifuged to remove insoluble material, and dialyzed against 10 mM Tris, pH 8.3. **Purification of factor X_a.** The blood-clotting protease factor X was purified from bovine blood by selective adsorption to BaSO₄, followed by two chromatographic steps as described by Fujikawa et al. (7). Factor X_1 and factor X_2 were separated by this procedure. Both proteins had final specific activities of 200 U/mg, using N-methoxycarbonyl-D-norleucyl-glycyl-L-arginine-4-nitroanilide (chromozym X) as the substrate (see below).

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proteins as determined by gel electrophoresis, the reactions were carried out either with factor X_{1a} or a mixture of factor X_{1a} and X_{2a} by using an enzyme-to-substrate ratio of 1:500 to 1:12.5 in reaction buffer (150 mM NaCl, 1 mM CaCl₂, 50 mM Tris, pH 8.0) at 4°C.

RESULTS

Construction of expression vector pROS. The high-level expression vector pBD2 (2) that encodes the amino acids 1 to 375 of the E. coli β -galactosidase under the control of its natural inducible *lac* promoter and operator sequence was modified. The new construct pROS is a general expression plasmid for β -galactosidase fusion proteins for subsequent cleavage with factor X_a . We introduced a synthetic 45base-pair (bp) sequence into the BamHI site of the polylinker from pBD2 (Fig. 1) coding for a recognition site of factor X_{a} . Previous experiments have shown that the specificity of factor X_a is not limited to the much-quoted sequence Ile-Glu-Gly-Arg, but it may tolerate several variations (19). We attempted to increase the susceptibility of the engineered cleavage site over all other potential recognition sites that may be present in either the bacterial or the viral part of the fusion protein. For this reason, the factor X_a site was flanked by short stretches of glycine residues to improve the exposure of the desired proteolytic site to the solvent and to give the region additional flexibility to permit facile binding to the protease. Furthermore, a new synthetic 46-bp multiple cloning site was inserted between the Sall and HindIII sites of pBD2, carrying three blunt-end restriction sites, one in each reading frame. Additionally, stop codons in all reading frames were introduced at the 3' end of the synthetic fragment for a defined termination of the fusion protein (Fig. 1). The linker was designed such that pROS still contained a unique *Hin*dIII site. Expression of HIV gag antigens cloned into pROS. For the expression of HIV gag proteins in E. coli, the 1.7-kilobase SstI-BclI fragment of BH10 (residues 681 to 2464, Fig. 2), containing the entire gag gene of HIV type 1, was cloned between the SstI and BamHI sites of the polylinker region of pUC19 (29). The resulting plasmid pHgag is shown in Fig. 2. From this vector, we selected fragments corresponding to different parts of the gag genes for expressing β -galactosidase fusion proteins in pROS (Fig. 2). For further studies, we chose two gag fragments representing major parts of either the p24 or the p15 protein for detection of antibodies against HIV. For this purpose, the 627-bp HindIII-HindIII fragment (nucleotides 1083 to 1710) of pHgag (Fig. 2) was fused in frame to the *lacZ* gene by insertion into the *Hin*dIII site of pROS. The resulting plasmid pSHR2 codes for a large part of p24 and an additional N-terminal 33 amino acids derived from p17. Alternatively, the 750-bp *HindIII-HindIII* fragment (nucleotide 1711 to the HindIII site of pUC19) was introduced into the *HindIII* site of pROS. The plasmid pSHR3 so constructed encodes p15 and an additional Nterminal 56 amino acids derived from p24. E. coli BMH 71-18 cells harboring the plasmids pSHR2 and pSHR3 produce large amounts of the corresponding p24 and p15 fusion proteins upon induction with isopropyl-*β*-*D*-thiogalactopyranoside (Fig. 3). All the proteins investigated were obtained in amounts of about 20% of the total cellular protein. Purification of fusion proteins and separation of their bacterial and viral portions. The expressed p24 and p15 β galactosidase fusion proteins form inclusion bodies and are obtained as insoluble material after cell disruption. Therefore, they can be separated from all soluble E. coli proteins

Activation of factor X. Purified factors X_1 and X_2 were activated with Russel's viper venom protease to factor X_{1a} and X_{2a} essentially as described by Fujikawa et al. (8).

Factor X_a assay. Factor X_a activity during purification was determined with chromozym X as the substrate. A typical assay was carried out as follows. Factor X was activated to factor X_a with Russel's viper venom protease in 1 ml of buffer (150 mM NaCl, 1 mM CaCl₂, 50 mM Tris, pH 8.0) for 15 min at 37°C (8). The solution was allowed to cool to 25°C. After addition of 100 nmol of chromozym X, the reaction was monitored at 415 nm. One unit was defined as the amount of protein hydrolyzing 1 µmol of chromozym X per min at pH 8 and 25°C.

Cleavage of fusion proteins. Since factor X_{1a} and factor X_{2a} showed the same specificity for the cleavage of the fusion

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AspProGlyGlyGlyIleGluGlyArgLysGlyGlyGlyThrSerAsp

FIG. 1. Expression vector pROS for high-level production of cleavable β-galactosidase fusion proteins in E. coli. The construct was obtained from the plasmid pBD2, containing the *lac* promoter and the N-terminal 1,125 bp (375 amino acids) of β-galactosidase followed by the pUC8 polylinker. Two synthetic linkers were inserted subsequently. A 45-bp sequence coding for the factor X_a recognition sequence, flanked by short glycine stretches, was cloned into the BamHI site of pBD2. Furthermore, a 46-bp fragment was inserted between the SalI and HindIII sites of pBD2, containing several singular restriction sites followed by stop codons in all reading frames.

by a simple centrifugation step as described in Materials and Methods. For further proteolytic cleavage with factor X_a , the fusion proteins were solubilized in 8 M urea. The urea was subsequently removed by dialysis, and the fusion proteins remained soluble. The fusion proteins were cleaved with factor X_a at 4°C, at which temperature the protease is very stable and still active and the substrate proteins remain soluble. The reactions resulted in complete cleavage into the β -galactosidase portion (molecular size, 45 kilodaltons) and the viral proteins (29 kilodaltons for the p24 equivalent and 23 kilodaltons for the p15 equivalent), as demonstrated in Fig. 4. Lanes 0 of panels A and B in Fig. 4 show the protein extracts of induced E. coli cells with the fusion proteins encoded by pSHR2 or pSHR3. Lanes 1 to 5 illustrate the cleavage of the fusion proteins by incubation with increasing amounts of factor X_a. At high enzyme-to-substrate ratios, we observed additional degradation products, most likely due to less specific proteolytic cleavage by factor X_a (Fig. 4A, lanes 4 and 5; Fig. 4B, lanes 2, 3, and 4). These results demonstrate that the engineered cleavage site is indeed more reactive than any secondary cleavage sites elsewhere in the fusion protein. Immunoreactivity of cleaved fusion proteins in the Western **blot assay.** The cleaved fusion proteins were tested in immunoblotting experiments. Fusion proteins encoded by pSHR2 and pSHR3 were digested with the protease as described in Materials and Methods; the resulting p24 and p15 fragments

were separated by SDS-PAGE and transferred onto nitrocellulose filters. The strips were probed with an HIVpositive serum. Antibodies reacting with the fusion proteins (Fig. 5A and B, lanes 1) were found, after cleavage of the fusion protein, to be exclusively directed against the viral portions of the fusion proteins (lanes 2 to 5).

While the cleaved p24 equivalent gave a clearly defined band of the expected molecular weight, we found several reactive degradation products of the cleaved p15 equivalent (Fig. 5). This might be caused by degradation of the p15 fusion protein or by some unspecific cleavage with factor X_{a} . It is unlikely, however, that these degradation products still contain β -galactosidase portions, since the experiments with the p24 fusion demonstrate that the same β -galactosidase part is degraded neither in the cell nor by factor X_a.

To demonstrate the necessity of removing the bacterial part of the expressed fusion proteins before their use in diagnostic Western blot analyses, the uncleaved fusion proteins were transferred onto nitrocellulose and incubated with 10 human cytomegalovirus-positive serum samples negative for anti-gag antibodies. One of these samples clearly reacted with the gag fusion protein (Fig. 6, lane 1). However, when the cleaved fusion protein was incubated with this serum sample, the β -galactosidase part of the fusion protein was recognized exclusively and no staining of the viral protein band could be detected (Fig. 6, lanes 2 to 4). Additionally, the serum did not react with gag proteins of purified virions

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FIG. 2. Plasmid pHgag and fragments used for bacterial expression. The entire gag region from clone BH10 (21) was isolated after cleavage with *SstI* (nucleotide 681) and *BclI* (nucleotide 2464) and ligated between the *SstI* and *Bam*HI sites of pUC19 to give the vector pHgag. The coding sequences for gag antigens are indicated by arrows on the inside. The two *Hind*III fragments (627 nucleotides [left] and 750 nucleotides [right]) were used for the construction of the recombinant expression plasmids pSHR2 and pSHR3 by insertion into the singular *Hind*III restriction site of pROS.

in the conventional immunoblot analysis (data not shown). This experiment clearly shows that a Western blot with uncleaved fusion protein would have led to a false-positive result with this serum, whereas the factor X_a -cleaved protein

offers a simple and effective solution to this problem without resorting to virion proteins.

DISCUSSION



FIG. 3. Expression of different *gag* proteins as β -galactosidase fusion proteins. SDS-PAGE (12.5% acrylamide gel) of protein extracts of *E. coli* cells harboring one of four expression plasmids for *gag* antigens stained with Coomassie brilliant blue. Extracts were prepared as described in Materials and Methods. Lanes: 1, pSHR1, containing the *ClaI-HindIII* region of p17; 2, pSHR2, with the *HindIII-HindIII* fragment of p24; 3, pSHR3, encoding the p15 protein; 4, pSHR4, spanning the *XmnI-XmnI* region of p17 and p24; 5, plasmid pROS; M, molecular size standards. Sizes are shown in kilodaltons.

Several attempts to replace HIV proteins purified from the isolated virus by bacterially expressed HIV proteins of protein fragments in serological HIV diagnosis have been reported recently (13, 18). It could be shown that these heterologously produced proteins usually retain their antigenic properties in Western blot analysis of HIV-positive



FIG. 4. Gel analysis of HIV type 1– β -galactosidase fusion proteins cleaved with factor X_a. The enriched fusion protein (500 µg) was incubated overnight at 4°C with increasing amounts of factor X_a (lanes: 1, 1 µg; 2, 5 µg; 3, 10 µg; 4, 20 µg; 5, 40 µg; 0, fusion protein without treatment with factor X_a; M, molecular size standards). (A) Fusion protein extract from pSHR2. (B) Fusion protein extract from pSHR3. Sizes are shown in kilodaltons.



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products can be separated by SDS-PAGE; the purified HIV proteins are blotted directly onto nitrocellulose membranes and are exposed to the test serum. Although the experiments reported here are very encouraging, we cannot be certain if the described method can be applied generally to every desired HIV protein or protein fragment. The investigated p15 fusion protein showed several degradation products (Fig. 3), indicating that probably not every HIV protein is stable, even as a fusion, in E. coli. For selective cleavage of the fusion proteins, we chose the blood coagulation protease factor X_a. Its specificity is very high and its recognition sequence is rather rare. To increase the susceptibility of the desired cleavage site, the recognition sequence was flanked by stretches of glycine for better accessibility of the cleavage site. Additionally, the most preferred amino acid (lysine) was placed C terminally to the cleavage site (arginine) (19). Nevertheless, we observed some cleavage at additional sites of the investigated fusion proteins, when they were digested over a long period or at high enzyme-to-substrate ratios. To further improve the method for routine use, both the viral part and the bacterial part may be engineered to remove the secondary sites. Although only a relatively small number of sera have thus far been tested, the results look very promising for a full-scale study. These tests will have to include other HIV polypeptides, particularly with *env*-specific epitopes. Additionally, the procedure described here should facilitate the production of purified antigens for raising monospecific antibodies and applications in ELISAs. Furthermore, it should be possible to investigate several HIV proteins on one strip in the Western blot with this method, if corresponding factor X_a cleavage reactions are combined before they are applied to the SDS-PAGE gel. Finally, we believe that the method reported here should be of general use for diagnosis of other viral diseases.

FIG. 5. Immunoreactivity of cleaved fusion proteins (fp) with the antiserum of a patient with acquired immunodeficiency syndrome (dilution of serum, 1:200). Proteins of recombinant plasmids were fractionated by electrophoresis on a 12.5% polyacrylamide gel. Proteins were electrophoretically transferred onto nitrocellulose. (A) Fusion protein from pSHR2. (B) Fusion protein from pSHR3. Lanes: 1, uncleaved fusion protein; 2 to 5, fusion proteins incubated with increasing amounts of factor X_a as described in the legend to Fig. 4. β gal, β -galactosidase.

sera and lead to results that are comparable to results with natural HIV proteins. However, in none of these investigations could cross-reactivity of the tested sera with bacterial proteins be excluded, since none of the heterologously expressed antigens had been separated from the bacterial proteins. This problem can be overcome by a bacterial expression system that is characterized by the following advantages. The HIV proteins or protein fragments are produced as β -galactosidase fusion proteins in very large amounts in E. coli. These fusion proteins are obtained as insoluble inclusion bodies and can thus be separated from most bacterial proteins. The fusion proteins were designed to contain a recognition site for the highly specific blood coagulation protease factor X_a . The fusion proteins can be solubilized and are cleaved quantitatively by factor X_a. The cleavage



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FIG. 6. Western blot analysis of cleaved fusion proteins (fp) with serum from an HIV gag antibody-negative sample. Lanes: 1, uncleaved fusion protein from pSHR2; 2 to 4, fusion proteins from pSHR2 incubated with increasing amounts of factor X_a as described in the legend to Fig. 4. β -gal, β -galactosidase.

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