Cell Surface Display of Human Immunodeficiency Virus Type 1 gp120 on Escherichia coli by Using Ice Nucleation Protein

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A new system designed for cell surface display of recombinant proteins on Escherichia coli has been evaluated for expression of eukaryotic viral proteins. Human immunodeficiency virus type 1 (HIV-1) gp120 was fused to the C terminus of ice nucleation protein (INP), an outer membrane protein of Pseudomonas syringae. Western blotting, immunofluorescence microscopy, fluorescence-activated cell-sorting analysis, whole-cell enzyme-linked immunosorbent assay, and ice nucleation activity assay confirmed the successful expression of HIV-1 gp120 on the surface of Escherichia coli. This study shows that the INP system can be used for the expression of eukaryotic viral proteins. There is also a possibility that the INP system can be used as an AIDS diagnostic system, an oral vaccine delivery system, and an expression system for various heterologous higher-molecular-weight proteins.

Surface display of heterologous proteins on live bacterial cells is important for many applications in microbiology, molecular biology, immunology, and biotechnology. A number of bacterial surface display systems have been designed for the expression of various proteins, and these systems have been applied in a variety of ways. Random peptide libraries expressed on the surface of bacteria can be used for rapid screening of ligands and antigens (18). Antibody fragments expressed on the surface of Escherichia coli make possible the rapid screening of "coliclonal" antibody libraries (5). Microorganisms which express heterologous enzymes on their surfaces can be used as a source of immobilized enzymes (6). Bacteria displaying proteins or peptides which have the ability to adsorb pollutants and heavy metals may be applicable for use as whole-cell biosorbents (23). Surface expression of various antigenic determinants may aid the development of new vaccine delivery systems as live vaccines which may be used for immunization without adjuvants, both intravenously and orally (19).

In gram-negative bacterial surface display systems various outer membrane proteins, lipoproteins, and cellular surface structural proteins have been used, including OmpA (7), PhoE (1), LamB (3), TraT (11), immunoglobulin A (IgA)-protease (16), peptidoglycan-associated lipoprotein (8), fimbriulin (12), and flagellin (19). Gram-positive bacterial proteins, such as staphylococcal protein A (10), fibrillar M6 protein (22), and S-layer structure protein (23) have been used as cell wall anchoring motifs. Despite significant study, gram-negative bacterial systems need to be improved in many respects. Most cell surface display systems have limitations for the size of foreign proteins which can be expressed because most anchoring-motif proteins have essential functions in host cells. Display of proteins longer than 60 amino acids may perturb outer membrane structures and cause growth defects (1, 3). Recently, it was reported that an autodisplay system using the C-terminal autotransporter domain from the IgA1 protease-like family of AIDA-I does not severely limit the size of proteins and causes no growth defect (20).

We have studied a new cell surface display system in which the anchoring protein is ice nucleation protein (INP) (13, 14, 15). INP is an outer membrane protein from Pseudomonas syringae which accelerates ice crystal formation in supercooled water (9). It was reported that INP attaches to the bacterial cell surface via a glucosylphosphatidylinositol (GPI) anchor, which has been widely used for attachment of eukaryotic cell surface proteins (26). However, unlike the GPI-anchor system in eukaryotes, the C terminus is free and exposed on the cell surface so foreign proteins fused to the C terminus of INP can be localized to the cell surface. The N terminus of INP is also free and is a candidate for the fusion site for foreign proteins.

In previous studies, we confirmed that INP-based cell surface display has many advantages compared to other gram-negative bacterial surface expression systems. INP has a cylindrical repeating domain which has a catalytic role in the formation of ice crystals. The repeating domain is not essential for membrane anchoring so it can be used as a modular spacer to control the length between a heterologous protein and the cell surface. INP does not lose ice nucleation activity after fusion to a foreign protein, so expression of recombinant proteins on the cell surface can be detected indirectly by ice nucleation activity (INA) assay. When INP is expressed on cell surface aggregates it becomes resistant to protease and remains safely in the stationary phase. Expression of INP fusion protein does not cause a disturbance of membrane structure or host growth defects. Moreover, INP can be overexpressed with higher-molecular-weight proteins on the cell surface. INP can be expressed on various gram-negative bacteria, so a host can be selected according to the application of recombinant bacteria.

We have expressed here the viral envelope glycoprotein gp120 (2, 4), which is derived from human immunodeficiency virus type 1 (HIV-1) fused to the C terminus of INP and INPNC, where INPNC is a recombinant consisting of the N- and C-terminal domains of INP. The approximate molecular mass of gp120, predicted from its primary amino acid se-
sequence. 60 kDa. This may be the largest foreign protein to date that has been expressed on a cell surface. Western blotting analysis, immunofluorescence microscopy, fluorescence-activated cell-sorting (FACS) analysis, whole-cell enzyme-linked immunosorbent assay (ELISA), and INA assay were used to verify the expression of HIV-1 gp120 on the surface of E. coli. Results indicate that the INS system can be used to express large proteins and to develop an AIDS diagnosis kit.

**MATERIALS AND METHODS**

Microbial strains, plasmids, and growth conditions. E. coli JM109 [endA1 recA1 gyrA thi-1 hsdR17 (mcrA-, mcrB-)] and JM109 (atag120 lacI21 lacZ M15] were used for protein expression. pTAI contains inak gene (GenBank accession number AF013159), which encodes INP. pANC3 contains a gene fragment which encodes INPNC consisting of the N- and C-terminal regions of INP. Two expression vectors, pTAIC and pANC3, are able to overexpress pINS in INPNC-hybrid protein under the control of the tac promoter. pLTRENV plasmid DNA (provided by Jinseu Park, Hallym University) was used as a gene source of HIV-1 gp120. E. coli cells were grown in Luria-Bertani (LB) medium (0.5% yeast extract, 1% tryptone, 0.5% NaCl). The culture temperature was maintained at either 37 or 22°C. E. coli cells were grown in Luria-Bertani (LB) medium at 37°C and induced sufficiently for recombination protein. The resulting protein was fusion protein at a dilution of 1:1,000. Prior to microscopic analysis, the cells were washed five times with PBS. Analysis was performed on a confocal microscope (Seoul National University Cancer Research Institute). For further confirmation of surface expression of gp120 with a stable conformation, mouse anti-gp120 monoclonal antibody (MAb) (provided by Jinseu Park, Hallym University) was used as a primary antibody and goat anti-mouse IgG-FITC conjugate was used as a secondary antibody.

FACS analysis. Induced cultures were harvested and resuspended in PBS to an A600 of 0.5. Cells were blocked with PBS containing 3% BSA for 1 h and 30 min at room temperature. After being washed with PBS, cells were mixed with mouse anti-gp120 MAb at a dilution of 1:1,000 and incubated at room temperature. After an extensive washing, the cell-antibody complex was incubated for 1 h and 30 min with goat anti-mouse IgG-FITC conjugate at a dilution of 1:3,000 at room temperature. The cell-antibody complex was then washed with paraformaldehyde. Samples were then analyzed by using a FACSScan (Becton Dickinson).

**Whole-cell ELISA.** Cell culture induced with IPTG at 22°C was harvested by centrifugation, washed three times with PBS, and resuspended in PBS to an A600 of ~0.1. Microtitre plate wells were coated by overnight incubation with 5 × 10^5 intact bacteria at 4°C. Excess cells were discarded, and wells were blocked with 200 µL of PBS containing 3% BSA for 1 h and 30 min at 37°C. All subsequent steps were carried out at room temperature. After the blocking solution was removed, the wells were incubated for 1 h and 30 min with AIDS patient antiserum at a dilution of 1:10,000. After another extensive washing, the cell-antibody complex was incubated for 1 h and 30 min with goat anti-human IgG-alkaline phosphatase conjugate at a final dilution of 1:2,000. Finally, the wells were washed five times with PBS and p-nitrophenyl phosphate (Sigma) was added as a substrate. After addition of 50 µL of 3 N NaOH, the absorbance of each well was measured at 405 nm.

**INA assay.** The measurement and analysis of INA was performed by using the drop-freezing method designed by Vali (25). Cell cultures were harvested and resuspended in distilled water to a concentration of 10^6 cells/mL. The cell suspension was serially diluted at 1:10, and six 5-µL droplets of the diluted samples were dropped onto an aluminum block. The temperature of the block was slowly decreased at a rate of ~0.2°C/min, and the number of freezing droplets was counted. The number of cumulative ice nuclei was calculated by using Vali's formula as follows: N(i) = [ln(1-F)/ln(10)], where N(i) is the cumulative number of ice nuclei, F is the fraction of droplets unfrozen at temperature i, V is the volume of each droplet, and D is the number of dilutions.

**RESULTS**

Construction of INP surface expression vectors. The bacterial envelope of E. coli and other gram-negative bacteria consists of an inner membrane (cytoplasmic membrane), a peptidoglycan cell wall, and an outer membrane. We constructed a recombinant surface displaying system by using INP, an outer membrane protein from P. syringae, as an anchoring motif. The INP gene (inak) is approximately 3.5 kb. INP contains 122 imperfect repeats of the consensus octapeptide (Ala-Gly-Try-Gly-Ser-The-Leu-Tyr). Because the central repeating domain was thought to contribute to the ice nucleation process, the repeating region of INP was deleted to reduce the size of the recombinant anchoring protein. The resulting protein was called INPNC. A diagrammatic representation of the plasmids pTAIgp120 and pTAngp120, encoding INP/HIV-1 gp120 and INPNC/HIV-1 gp120, respectively, is shown in Fig. 1. Surface expression vectors consist of the following parts: (i) the origin of replication from pBR322; (ii) a tac promoter; (iii) a gene source of HIV-1 gp120; and (iv) a reporter gene (lacZ or lacY). The amplified gp120 gene fragment was digested with BamHI and EcoRI and inserted into the same enzyme digested pTAIC and pANC3, generating pTAIgp120 and pTAgp120, respectively.

Expression of INP(gp120) and INPNC(gp120) fusion protein in E. coli JM109. Recombinant E. coli JM109 cells harboring plasmids pTAIgp120 and pTAgp120 were cultured in 5 ml of LB medium containing ampicillin (100 µg/ml) overnight at 37°C. Cultured cells were transferred to new LB medium (5 ml) and grown to an A600 of ~0.4. The amplified gene fragment was digested with BamHI and EcoRI and inserted into the same enzyme digested pTAIC and pANC3, generating pTAIgp120 and pTAgp120, respectively.

Expression of INP(gp120) and INPNC(gp120) fusion protein in E. coli JM109. Recombinant E. coli JM109 cells harboring plasmids pTAIgp120 and pTAgp120 were cultured in 5 ml of LB medium containing ampicillin (100 µg/ml) overnight at 37°C. Cultured cells were transferred to new LB medium (5 ml) and grown to an A600 of ~0.4. The amplified gene fragment was digested with BamHI and EcoRI and inserted into the same enzyme digested pTAIC and pANC3, generating pTAIgp120 and pTAgp120, respectively.

**FIG. 1.** Expression vectors with encoded gene products. Abbreviations: bla, β-lactamase-encoding gene; ori, origin of replication from pBR322; P_tac, tac promoter. (A) Surface expression vector pTAIgp120. (B) Surface expression vector pTAgp120. These expression vectors encode INP(INPNC)/HIV-1 gp120 fusion protein, respectively.
Expression and surface localization of INP (INPNC)/HIV-1 gp120 fusion proteins. Amplified gene fragments encoding gp120 derived from HIV-1 were genetically fused to the C terminus of either INP or INPNC, as described above. In order to investigate whether the subcloned gene constructs could be expressed as fusion proteins, recombinant E. coli cells harboring the two constructs described in Fig. 1 were grown and induced to the same cell density. Western blot analysis then confirmed that the fusion proteins were expressed in E. coli. As shown in Fig. 2, INP/HIV-1 gp120 and INPNC/HIV-1 gp120 fusion proteins were clearly detected. Both fusion proteins were visualized as prominent protein bands with molecular masses of approximately 215 and 90 kDa (Fig. 2, lanes 2 and 3). However, these estimates were not the same as the predicted molecular masses of 179 kDa for INP/HIV-1 gp120 and 87 kDa for INPNC/HIV-1 gp120 as described for other INPs (26).

Immunofluorescence microscopy and FACS analysis for detection of surface localization of the INP (INPNC)/HIV-1 gp120 fusion proteins. To investigate whether the whole INP(INPNC)/HIV-1 gp120 fusion proteins were displayed on the bacterial surface in a stable conformation, immunofluorescence microscopic analysis was carried out with rabbit anti-INPNC serum (Fig. 3) and mouse anti-gp120 MAb (Fig. 4). Recombinant E. coli cells were probed with rabbit anti-INPNC serum as a primary antibody and fluorescently stained with FITC-labeled goat anti-rabbit IgG antibody. As shown in Fig. 3, cells harboring plasmid pTAICD8 (which was a positive control), pTAIgp120, and pTAgp120 (Fig. 3B, C, and D) were efficiently stained by FITC-conjugated antibody. The white spots in Fig. 3 indicated the presence of fluorescein, showing that INP molecules were displayed on the surface of bacteria in a stable conformation. Wild-type E. coli cells were not stained with FITC-conjugated secondary antibody (Fig. 3A). To verify the expression of eukaryotic viral protein gp120 with a biolog-
ically active conformation, recombinant E. coli cells were stained with mouse anti-gp120 MAb. Confocal fluorescence micrographs (Fig. 4) showed that cells harboring the plasmids pTAIgp120 and pTAgp120 (Fig. 4B and C) were intensely stained with FITC-conjugated antibody, while wild-type E. coli and E. coli cells harboring plasmid pTAICD8 had no fluorescent signal (Fig. 4A and B). The surface display of fusion proteins on recombinant E. coli cells harboring the two constructs presented in Fig. 1, along with wild-type E. coli as a control, were further analyzed by FACS analysis. Data were shown in histogram form with the wild type and controls in Fig. 5. The bacterial cells were probed with primary antibodies reactive with gp120 and thereafter were fluorescently stained with an FITC-conjugated secondary antibody. As with previous results, the peaks were shifted to the right against wild-type E. coli.

Whole-cell ELISA. Whole-cell ELISA was also used to confirm cell surface expression of the fusion proteins in cells harboring plasmids pTAIgp120 and pTAgp120 (Fig. 6). To investigate whether INP surface display systems can be used in an AIDS diagnosis kit and in vaccine delivery systems, AIDS patient serum was used as the primary antibody. Wild-type E. coli JM109 gave weak signals when incubated on microtiter wells. In contrast, much higher absorbance values (about four to six times higher) were evident in wells coated with cells containing plasmids pTAIgp120 and pTAgp120, indicating that fusion protein molecules were expressed on the cell surface. This indicated that these systems have potential to be used for AIDS diagnosis and as a live vaccine delivery system.

INA assay. One of the many advantages of the INP-based expression system is that surface expression of foreign proteins can be detected by a simple INA assay because the central repeating domain of INP has a catalytic role in the formation of ice crystals. Deletion mutation experiments of other groups showed that the N and C termini were also important for catalysis of the formation of ice crystals. As shown in Fig. 7, cells harboring plasmids pTAIgp120 and pTAgp120 had higher levels of INA than wild-type E. coli JM109, indicating that the fusion proteins were expressed on the surface of E. coli.

**DISCUSSION**

HIV-1 gp120 was expressed on the cell surface of E. coli fused to either INP or INPNC in order to investigate (i)
whether large proteins, such as gp120, can be expressed by INP-based cell surface display, and (ii) whether recombinant bacteria expressing gp120 exhibit antigenicity with a potential for development of an AIDS diagnostic test. To identify the cell surface localization of fusion proteins, \textit{E. coli} JM109 cells harboring plasmids pTAIGp120 and pTAAgp120 were analyzed by Western blotting, immunofluorescent microscopy, FACS analysis, whole-cell ELISA, and INA assay. The results strongly indicated not only the stable expression of fusion proteins but also the retention of a biologically active conformation of HIV-1 gp120.

The results indicate that large proteins can be expressed on the cell surface and that this cell surface display system can be used in various applications. Cell surface display systems which have been previously studied caused growth inhibition and instability of the outer membrane when large foreign proteins were fused to the cell surface anchoring motif. However, \textit{E. coli} JM109 expressing INP (INPNC)/HIV-1 gp120 fusion proteins was stable in the stationary phase without growth inhibition or other defects (data not shown). We confirmed that the INP system can be used as a surface anchoring motif for proteins as large as 60 kDa.

The INP system also has potential to be used as part of an AIDS diagnosis kit or as an oral vaccine delivery system. HIV-1 gp120 produced by cleavage of precursor gp120 is involved in virus infection, syncytium formation, cell-to-cell transmission, and CD4 tropism (4). The gp120 protein is known for its high immunogenicity and antigenicity (2), so it has been a major candidate for development of an AIDS diagnostic tool and vaccine.

Immunofluorescence microscopy, FACS analysis, and whole-cell ELISA experiments have shown that the anti-gp120 MAb and the anti-gp120 IgG in AIDS patient antiserum binds to gp120 expressed on the \textit{E. coli} cell surface. These results indicate that recombinant \textit{E. coli} expressing gp120 on the cell surface can be used to develop an AIDS diagnostic system or a live AIDS vaccine.

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