NOTES

Human Foamy Virus Polypeptides: Identification of env and bel Gene Products

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Human foamy virus (HFV) proteins were identified in human cells cultured in vitro by immunoprecipitation and immunoblotting with specific antisera. Among several viral polypeptides, four glycoproteins of approximately 160, 130, 70, and 48 kDa were identified in HFV-infected cells. gp130 was shown to represent the intracellular env precursor, and gp70 and gp48 were shown to represent the external and transmembrane env proteins, respectively. The nature of gp160, which shares sequences with the env, bel1, and bel2 proteins, is not yet resolved. In addition, a p62 identified with bel1- and bel2-specific antisera likely corresponds to the bel gene product.

Spumaretroviruses, known also as foamy viruses, induce in tissue culture multinucleated giant cells with a spongy appearance. Foamy viruses have been isolated from various mammalian species, such as Syrian hamsters, cats, bovines, nonhuman primates, and humans (12, 22). No proven pathogenic potential has been clearly demonstrated for any of the virus strains. However, the presence of foamy virus markers in patients with different diseases has been reported (1, 4, 37, 40, 41). In this context, we have recently reported data strongly suggesting the existence of an association between Graves’ disease and the presence of human foamy virus (HFV)-related sequences (17). The genome organization and the replication cycle of the human prototype (HFV) are now well documented (7-9, 15, 21-23, 29, 38). The HFV genome contains open reading frames in its 3′ region which have been shown to code for proteins possessing regulatory functions (13, 30), as is the case for human T-lymphotropic retroviruses and human immunodeficiency viruses. Previously, our group characterized some of the structural proteins of simian foamy virus type 1 (SFV-1) (2, 3). We have also shown by cross-immunoprecipitation experiments with specific antisera that HFV is antigenically closely related to SFV-6 and weakly related to SFV-1 (38). Here we further characterize the HFV polypeptides and present detailed analysis of env and bel gene products in an infected human cell line.

The expression of HFV antigens was examined in the human glioblastoma cell line U373-MG (American Type Culture Collection, Rockville, Md.) in which HFV gives rise to a highly producing replication cycle. Cells were maintained with Eagle’s minimum essential medium (MEM) supplemented with nonessential amino acids, sodium pyruvate, penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% fetal calf serum inactivated at 56°C for 30 min. Cells at about 50% confluence were infected at a multiplicity of infection of 1 with the human isolate of Achong et al. (1), a gift from H. zur Hausen (Heidelberg, Germany).

Polyclonal antisera against whole virus were produced by immunization of rabbits with infected cell lysates. Monoclonal antibodies B4, D11, and A9 against env and bel gene products were prepared in our laboratory as described previously (34). Briefly, monoclonal antibody B4 was derived from mice immunized with pelleted HFV particles, whereas monoclonal antibodies A6 and D11 were obtained from mice inoculated with sonicated HFV-infected cell lysates. The monoclonal antibodies were characterized as immunoglobulin G1 molecules with an affinity for cytoplasmic components, as shown by an indirect immunofluorescence assay. Anti-bel1 and anti-bel2 rabbit polyclonal antibodies were kindly provided by Rolf M. Flügel (Heidelberg, Germany) (20).

When 60 to 70% of infected U373-MG monolayer cells presented a cytopathic effect (6 to 8 days postinfection), they were labeled for 16 h with 50 μCi of [35S]methionine per ml in MEM without methionine but with 5% fetal calf serum. Cells were lysed in 30 mM Tris (pH 7.4)-100 mM NaCl-5 mM MgCl2-1% Triton X-100-0.5% sodium deoxycholate-3 mM phenylmethylsulfonyl fluoride-0.05% sodium dodecyl sulfate (SDS) buffer (about 3 × 106 cells per 250 μl) for 30 min at 4°C. After centrifugation (10 min at 12,000 × g), the supernatant was collected and stored at −20°C. Virus particles were recovered from the clarified culture medium by centrifugation at 100,000 × g for 90 min, and the virus pellet was lysed as described above.

Viral proteins were identified by immunoprecipitation with specific anti-whole virus antisera. Several main viral polypeptides of about 160, 130, 72, 68, and 48 kDa were identified in infected cells (Fig. 1A, lane 2; Fig. 1B, lane 1). A smear was observed between 68 and 80 kDa, and some additional polypeptides were frequently detected at 56, 32, and 26 kDa.

Immunoprecipitation of pelleted virus particles revealed primarily a smear between 70 and 80 kDa, a band at 68 kDa, and a doublet at 46 to 48 kDa. Several minor bands appeared also between 16 and 33 kDa (Fig. 1A, lane 3). Western immunoblot analysis (39) led to a somewhat different intracellular HFV protein pattern (Fig. 1B):
FIG. 1. Characterization of HFV polypeptides by immunoprecipitation and immunoblotting. For immunoprecipitation assays, HFV-infected cells were labeled either with \(^{35}\)S]methylmethionine (50 \(\mu\)Ci/ml; specific activity, 1,245 Ci/mmol; Amersham) for 16 h in MEM without methionine but with 5% fetal calf serum or with \(^{3}H\)glucosamine (100 \(\mu\)Ci/ml; 20 Ci/mmol; Amersham) for 7 h in MEM lacking glucose but supplemented with 1% fetal bovine serum. Western blot analysis was performed essentially as described by Towbin et al. (39). Immunodetection of viral polypeptides was performed by using an anti-whole virus antisera. Electrophoresis was carried out at 15°C in gradient 5 to 15% polyacrylamide slab gels with a Tris-glycine buffer system (16). For fluorography, gels were treated with Amplify before drying. (A) Immunoprecipitation of \(^{35}\)S]methionine-labeled proteins. Lanes: 1, control cells; 2, infected cell extracts; 3, virus particles. (B) Comparative analysis of viral proteins detected by immunoprecipitation and immunoblotting. Immunoprecipitated viral proteins were detected as described above (lane 1) or transferred onto nitrocellulose sheets and autoradiographed (lane 3). Control cells are in lane 2. Viral polypeptides separated by electrophoresis were transferred and analyzed for Western blotting by peroxidase-conjugated anti-rabbit immunoglobulin G antibodies (Biosys) (lane 4). (C) Immunoprecipitation of \(^{3}H\)glucosamine-labeled proteins. Lanes: 1, control cells; 2, infected cell extracts. whereas p72, p68, p62, and p32 were easily identified after blotting (lane 4), the proteins of 160, 130, 70 to 80, and 48 kDa, although efficiently transferred (lane 3), were barely detectable (lane 4).

Metabolic labeling with \(^{3}H\)glucosamine allowed the identification of four glycoproteins among the viral polypeptides described above: three major glycoproteins which band at approximately 160, 130, and 70 kDa and a minor band at 48 kDa detected only after longer exposure of the film (Fig. 1C, lane 2). All of these proteins, absent in uninfected cells, appeared to be env-related HFV polypeptides; the high-molecular-mass glycoproteins of 160 and 130 kDa could be intracellular viral precursors. gp70 and gp48, also present in virus particles, very likely represented the mature env gene products (19).

Further characterization of the env proteins of HFV was performed with an env-specific monoclonal antibody, B\(_4\), prepared in our laboratory. This monoclonal antibody specifically immunoprecipitated the four viral glycoproteins described above (Fig. 2A, lane 4 and 6). Compared with polyclonal anti-whole virus antiserum (lanes 2, 3, and 5), it preferentially recognized the mature env glycoproteins (lanes 4 and 6). The detection of both gp70 and gp48 by B\(_4\) was surprising. Western blot assays performed to elucidate this point were unsuccessful because of the loss of reactivity of HFV glycoproteins after blotting (Fig. 1B, lane 4). However, dissociation of the two mature env proteins was obtained when cells were lysed in the presence of the reducing agent \(\beta\)-mercaptoethanol; under these conditions, gp48 was no longer immunoprecipitated by monoclonal antibody B\(_4\) (Fig. 2B, lane 3). Immunodetection of the high-molecular-mass gp160 and gp130 by B\(_4\) remained unchanged, and a band was clearly visible at 70 kDa. This band likely corresponds to the external env protein, since the smear usually observed between 70 and 80 kDa disappeared. The protein detected at 43 kDa, frequently identified in both noninfected and infected cell extracts, was shown to be actin by using an antiactin antibody (data not shown).

The effects of different glycosylation inhibitors on the processing of HFV env proteins were then examined. Tunicamycin treatment, which prevents the early addition of FIG. 2. Characterization of the env-specific monoclonal antibody B\(_4\). (A) U373-MG cells were infected with HFV and metabolically labeled with \(^{35}\)S]methionine (50 \(\mu\)Ci/ml) for 16 h (lanes 1 to 4) and 6 h (lanes 5 and 6). Noninfected cells are in lane 1; infected cells are in lanes 2 to 6. For comparison, immunoprecipitation was performed with two rabbit anti-whole virus antisera (lanes 1 to 3 and 5) and with monoclonal antibody B\(_4\) (lanes 4 and 6). (B) Noninfected (lane 1) and infected (lanes 2 and 3) U373-MG cells were labeled with \(^{35}\)S]methionine (50 \(\mu\)Ci/ml) for 16 h. Cells were lysed in the absence (lanes 1 and 2) or in the presence (lane 3) of 0.1 M \(\beta\)-mercaptoethanol. Monoclonal antibody B\(_4\) was used for immunoprecipitation.

FIG. 3. Effects of glycosylation inhibitors. Noninfected (lanes 1 and 6) and infected (lanes 2 to 5 and 7 to 11) U373-MG cell extracts were prepared after a 16-h labeling period with \(^{35}\)S]methionine (50 \(\mu\)Ci/ml). Viral proteins were immunoprecipitated with a polyclonal anti-whole virus serum (lanes 4 to 11) or with the env-specific antiserum B\(_4\) (lanes 6 to 10). U373-MG cells were grown in the absence of glycosylation inhibitors (lanes 2, 7, and 11) or in the presence of 1 mM castanospermine (lanes 3 and 8), 2 \(\mu\)g of tunicamycin per ml (lanes 4 and 9), or 1 mM deoxymannojirimycin (lanes 5 and 10).
N-linked carbohydrate side chains to proteins (11, 25, 36), led to a decrease in gp130, giving rise to a new polypeptide of about 100 kDa, as revealed by the polyclonal antisera (Fig. 3, lane 4). Furthermore, an almost complete extinction of the smear in the 68- to 80-kDa region and the disappearance of gp48 were observed (lane 4). These observations were consistent with data obtained with the env antisera: only traces of gp70 and gp48 were identified in infected cell extracts (lane 9).

To examine later steps of maturation of env polypeptides, we used two other inhibitors, castanospermine and deoxymannojirimycin, which inhibit glucosidase and mannosidase, respectively, trimming enzymes involved in the glycosylation process (10, 35). These inhibitors had weaker effects than did tunicamycin. However, both affected the processing of env precursors; thus, while a constant or even increased level of gp130 was detected by the polyclonal antisera, the smear between 70 and 80 kDa was greatly reduced or disappeared and the level of gp48 decreased (Fig. 3, lanes 3 and 5). When removal of glucose residues was inhibited by castanospermine, gp130 and gp48 exhibited higher molecular weights (lanes 3 and 8).

In contrast, deoxymannojirimycin did not affect the molecular size of gp130 (Fig. 3, lanes 5 and 10). A protein of a slightly lower molecular size than 130 kDa was also visualized; its meaning is not yet clear. However, in addition to the decrease in gp48 already noted, the major effect of the inhibitor consisted of a drastic drop in the env surface glycoproteins, as assessed by the absence of a smear between 68 and 80 kDa (lanes 5 and 10).

These data strongly supported the conclusion that gp130 represented the env precursor and that gp70 and gp48 represented the mature external and transmembrane proteins, respectively, of HFV. These results contrasted with a report of Netzer et al. (24), who failed to identify glycosylated HFV proteins in the range of 70 kDa and proposed gp130 as the major env protein. Our claim that the env surface protein is a glycoprotein of about 70 kDa is based on the following facts: (i) proteins between 70 and 80 kDa were detected by specific antisera in infected cell extracts and in virions, and (ii) labeling experiments with [3H]glucosamine and the use of glycosylation inhibitors showed that these proteins are glycosylated. On the other hand, we believe that gp130 is the env precursor because (i) it was not detected in purified virions but only in infected cell extracts; (ii) the processing of gp130 into gp70 and gp48 is in agreement with the published nucleic acid sequence of the HFV env gene, including the cleavage site for generating the surface protein and the transmembrane protein (8); (iii) the shift from 130 to 100 kDa after tunicamycin treatment corresponds to that expected from the complete nucleotide sequence of the env gene; and (iv) the absence of an effect on the level of gp130 of castanospermine and deoxymannojirimycin, inhibitors which act at late steps of the glycosylation process, contrasts with the strong inhibitory effect on gp70.

The molecular size of the env surface protein of HFV is in good agreement with previous results obtained in our laboratory for the SFV-1 isolate (3). It is also consistent with our recent data establishing that a close relationship exists between the env genes of HFV and SFV-1 (14). Differences in the maturation processes of the env products could explain their immunological divergence (38).

As concerns the transmembrane env protein, its molecular size of 48 kDa is very similar to that published by Netzer et al. (24). gp48 is easily visualized in infected cells as well as in virus particles. However, only traces of gp48 were detectable in infected cells after specific labeling with [3H]glucosamine. This finding is consistent with the presence of only three glycosylation sites in the COOH-terminal end of the env gene (6). We have shown that disulfide bonds bind gp70 to gp48, since only treatment by reducing agents was able to completely dissociate the two env proteins, thus preventing their coprecipitation during immunoprecipitation assays. These results are consistent with a number of reports providing evidence for the existence of retroviral glycoproteins as complex oligomeric structures resulting from disulfide bond formation between the external and the transmembrane env protein (6, 18) or from noncovalent binding of these proteins (26-28, 31-33).

Finally, the bel1 and bel2 products present in U373-MG-infected cell extracts were characterized by means of specific antisera. As expected, a protein of approximately 44 kDa (Fig. 4A, lane 4) was immunoprecipitated by a polyclonal anti-bel2 antisera. In addition, p62 and, to a lesser extent, gp160 were also visualized. These last two proteins were revealed also by the polyclonal anti-bel1 antisera (lanes 3 and 6). It should be noted that the bel1 gene product of about 35 kDa was detected only shortly after infection. The best visualization of bel1 resulted from Western blot analysis. In the experiment reported in Fig. 4B, four viral proteins could be identified 22 h postinfection in infected cell extracts with a polyclonal anti-whole virus antisera (lane 4): p72, p68, p62, and p35. The latter corresponds to bel1 since it was recognized by the specific anti-bel1 antisera (lane 5). p62 was also recognized by this antisera (lane 5) and by a monoclonal antibody, D11, prepared in our laboratory (lane 6).

p62 was detectable in immunoprecipitation assays both by the specific polyclonal anti-bel1 and anti-bel2 antisera (Fig. 4A, lanes 3, 4, and 6) and by our two monoclonal antibodies, D11 and A9 (lanes 7 and 8).

Interestingly, D11 resembled the anti-bel2 antisera, since both were also able to detect gp160. However, D11 did not react with the bel1 protein (Fig. 4B, lane 6).
FIG. 5. V8 protease digestion of p62. p62 was immunoprecipitated with different antisera from infected cell extracts prepared as usual after a 16-h period of labeling with [35S]methionine (50 μCi/ml). After polyacrylamide gel electrophoresis, the slab gel was rinsed with water and dried without Amplify. The bands corresponding to p62 were cut from the gel and placed on a second SDS-polyacrylamide slab gel. The protein was digested by 1 μg of V8 protease (Worthington, Freehold, N.J.) in the stacking gel essentially as described by Cleveland et al. (5).

Moreover, peptide mapping experiments demonstrated that the p62 identified by the specific anti-bel antisera and the p62 identified by anti-whole virus sera and by D11 and A6 are the same proteins (Fig. 5).

The presence of bel genes at the 3′ end of the HFV genome is well established. Although the bel1 gene product has clearly been determined to be a transactivating protein (13, 30), the functions of the other bel gene products have not yet been elucidated. Detection of the bel1 protein only at short times after infection is in good agreement with its function. We identified the bet2 product at 44 kDa by using the specific polyclonal anti-bet2 antisera. However, with both the anti-bel1 and anti-bel2 sera, we have mainly detected a protein of 62 kDa. This protein was also specifically detected by the two monoclonal antibodies A6 and D11. The p62, abundant in HFV-infected cells, was characterized by peptide mapping and likely corresponds to the multisplited bel gene product (20).

In addition to the HFV env precursor, gp130, and the mature env proteins, gp70 and gp48, we have identified in HFV-infected cell extracts a high-molecular-mass glycoprotein of 160 kDa. We have shown that gp160 is detectable by the env-specific monoclonal antibody B4 and also by the anti-bell and anti-bel2 antisera. Therefore, although the exact nature of gp160 is not yet elucidated, this glycoprotein cannot merely represent the HFV env gene-encoded precursor as was previously proposed (7, 21).

Experiments are in progress to gain insight into the process of HFV protein biosynthesis and to clarify the unknown functions of the bel1 gene products.

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