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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 *bet* 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 B_4, D_{11}, and A_6 against *env* and *bel* gene products were prepared in our laboratory as described previously (34). Briefly, monoclonal antibody B_4 was derived from mice immunized with pelleted HFV particles, whereas monoclonal antibodies A_6 and D_{11} 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 [^{35}S]methionine per ml in MEM without methionine but with 5% fetal calf serum. Cells were lysed in 50 mM Tris (pH 7.4)–100 mM NaCl–5 mM $MgCl_2$–1% Triton X-100–0.5% sodium deoxycholate–3 mM phenylmethylsulfonyl fluoride–0.05% sodium dodecyl sulfate (SDS) buffer (about 3×10^6 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):

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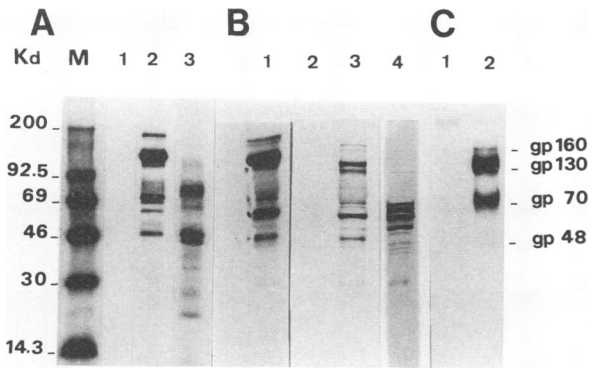

FIG. 1. Characterization of HFV polypeptides by immunoprecipitation and immunoblotting. For immunoprecipitation assays, HFV-infected cells were labeled either with [^{35}S]methionine (50 μCi/ml; specific activity, 1,245 Ci/mmol; Amersham) for 16 h in MEM without methionine but with 5% fetal calf serum or with [^{3}H]glucosamine (100 μCi/ml; 20 Ci/mmol; Amersham) for 7 h in MEM lacking glucose but supplemented with 1% fetal bovine serum. Western blot analysis was performed according essentially as described by Towbin et al. (39). Immunodetection of viral polypeptides was performed by using an anti-whole virus antiserum. 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.

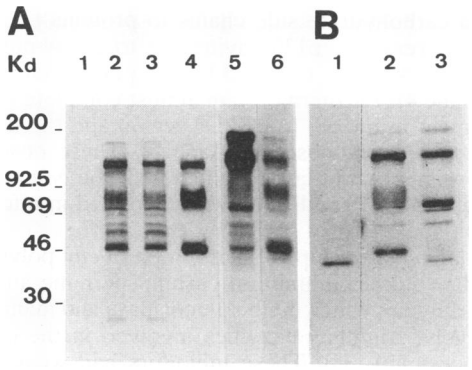

FIG. 2. Characterization of the *env*-specific monoclonal antibody B_4. (A) U373-MG cells were infected with HFV and metabolically labeled with [^{35}S]methionine (50 μCi/ml) for 16 h (lanes 1 to 4) and 6 h (lanes 5 and 6). Noninfected cells are in lane 1; infected cells are in lanes 2 to 6. For comparison, immunoprecipitation was performed with two rabbit anti-whole virus sera (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 μCi/ml) for 16 h. Cells were lysed in the absence (lanes 1 and 2) or in the presence (lane 3) of 0.1 M β-mercaptoethanol. Monoclonal antibody B_4 was used for immunoprecipitation.

whereas p72, p68, p62, p56, 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 antisera (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 β-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

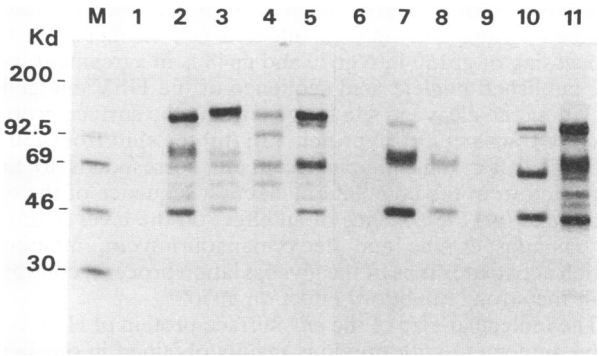

FIG. 3. Effects of glycosylation inhibitors. Noninfected (lanes 1 and 6) and infected (lanes 2 to 5 and 7 to 11) U373-MG cell extracts were prepared after a 16-h labeling period with [^{35}S]methionine (50 μCi/ml). Viral proteins were immunoprecipitated with a polyclonal anti-whole virus serum (lanes 1 to 5 and 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 μg 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 antiserum (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* antiserum: 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 antiserum, 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 [^{3}H]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 [^{3}H]glucosamine. This finding is consistent with the presence of only three glycosylation sites in the COOH-terminal end of the *env* gene (8). 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 *env* 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).

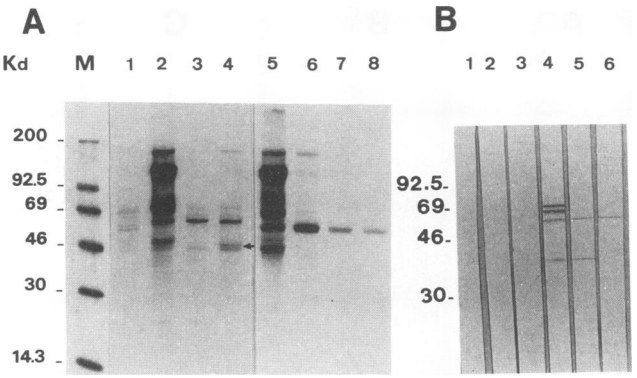

FIG. 4. Identification of *bel* gene products with polyclonal and monoclonal antisera. (A) Noninfected (lane 1) and infected (lanes 2 to 8) U373-MG cells were labeled for 16 h with [^{35}S]methionine (50 μCi/ml). Viral proteins were immunoprecipitated with polyclonal rabbit antisera raised against whole virus (lanes 1, 2, and 5), against *bel1* (lanes 3 and 6), and against *bel2* (lane 4) and also with two monoclonal antibodies, D_{11} (lane 7) and A_6 (lane 8). (B) Noninfected (lanes 1 to 3) and HFV-infected (lanes 4 and 6) U373-MG cell extracts were prepared 22 h postinfection. Viral proteins were detected by Western blotting with a polyclonal rabbit anti-whole virus serum (lanes 1 and 4), the polyclonal rabbit anti-*bel1* serum (lanes 2 and 5), and monoclonal antibody D_{11} (lanes 3 and 6).

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* antiserum. In addition, p62 and, to a lesser extent, gp160 were also visualized. These last two proteins were revealed also by the polyclonal anti-*bel1* antiserum (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 antiserum (lane 4): p72, p68, p62, and p35. The latter corresponds to *bel1* since it was recognized by the specific anti-*bel1* antiserum (lane 5). p62 was also recognized by this antiserum (lane 5) and by a monoclonal antibody, D_{11}, 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, D_{11} and A_6 (lanes 7 and 8).

Interestingly, D_{11} resembled the anti-*bel1* antiserum, since both were also able to detect gp160. However, D_{11} did not react with the *bel1* protein (Fig. 4B, lane 6).

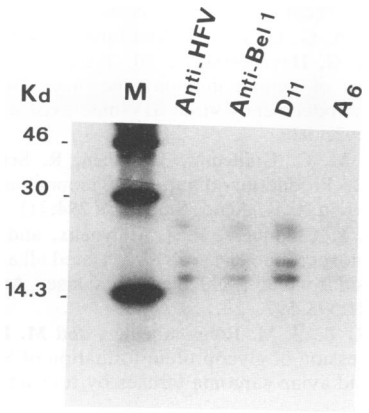

FIG. 5. V8 protease digestion of p62. p62 was immunoprecipitated with different antisera from infected cell extracts prepared as usual after a 16-h period of labeling with [^{35}S]methionine (50 μCi/ml). After polyacrylamide gel electrophoresis, the slab gel was rinsed with water and dried without Amplify. 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-*bel1* antiserum and the p62 identified by anti-whole virus sera and by D_{11} and A_6 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 *bel2* product at 44 kDa by using the specific polyclonal anti-*bel2* antiserum. 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 A_6 and D_{11}. The p62, abundant in HFV-infected cells, was characterized by peptide mapping and likely corresponds to the multispliced *bet* 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 B_4 and also by the anti-*bel1* and anti-*bel2* antisera. Therefore, although the exact nature of gp160 is not yet elucidated, this glycoprotein cannot merely represent an 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 *bel* gene products.

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