JOURNAL OF VIROLOGY, Oct. 1989, p. 4464–4468
0022-538X/89/104464-05$02.00/0

Vol. 63, No. 10

Effects of Mutations in Hyperconserved Regions of the Extracellular Glycoprotein of Human Immunodeficiency Virus Type 1 on Receptor Binding

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Received 10 April 1989/Accepted 21 June 1989

Sequence comparison of the human immunodeficiency virus type 1 and type 2 *env* genes revealed the presence of six linear regions in the extracellular glycoprotein that are highly conserved. To investigate the functional significance of these regions, we made short deletions in each and assayed the ability of the mutated proteins to bind CD4 antigen. Small deletions in four of the highly conserved regions drastically reduced receptor binding. Some deletions interfered with the maturation of the envelope glycoprotein, but maturation did not necessarily correlate with the ability to bind CD4 antigen.

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome, selectively infects human monocytes and T lymphocytes that express the CD4 antigen on their surfaces. The CD4 molecule is the receptor for HIV (2, 7, 15), and its interaction with the viral particle through the viral gp120, while not sufficient, is required to initiate a productive infectious cycle in most cases (14). The HIV *env* gene encodes two proteins that are translated as a single precursor of 160 kilodaltons (kDa) that is cleaved to give rise to the gp120 surface (SU) protein (11) and a gp41 transmembrane protein. Syncytium formation, which results from cell-to-cell fusion after HIV infection in vitro, is also dependent on gp120-CD4 interaction (12, 23).

HIV type 2 (HIV-2) and related simian immunodeficiency viruses encode extracellular glycoproteins that are antigenically distinct from HIV-1 gp120, yet all use CD4 as the viral receptor (21, 22). Among the structural protein products of the HIV genome, SU shows the most heterogeneity, with 80% overall amino acid identity between different isolates of HIV-1 and not more than 40% identity between sequenced isolates of HIV-1 and HIV-2 (5). This variability is confined to certain regions interspersed with domains that are highly conserved among different isolates of HIV-1 and HIV-2. Conservation of clearly delineated sequences among two species of HIV that share common biological properties is likely the result of selective pressure to maintain them.

We aligned the predicted amino acid sequence of the part of the *env* gene encoding SU from the HIV-1 BRU isolate (24) with that of HIV-1 MAL, a divergent isolate from Zaire (1), and that of HIV-2 ROD (5) by aligning the cysteine residues of each sequence. Comparison of these different proteins allowed us to define six regions highly conserved between HIV-1 and HIV-2 (Fig. 1).

To establish a relationship between the primary structure of the HIV-1 envelope glycoprotein and its biological properties, to understand the functional importance of the highly conserved regions, and to determine whether regions of homology between HIV-1 and HIV-2 are important for the virus-receptor interaction, we deleted small stretches of amino acids (underlined in Fig. 1B) in each of six hyperconserved regions without removing any conserved cysteine residues. Deletions were made by oligonucleotide-directed mutagenesis in a fragment containing a modified HIV-1 *env* gene (BRU isolate) in which two cleavage sites between gp120 and gp41 were removed (6). Insertion of this mutated fragment into recombinant vaccinia viruses (called VV-env-cs here; cs stands for cleavage site mutation) led to the synthesis of a noncleaved product of the *env* gene that was more tightly anchored at the cell surface than is the wild-type envelope protein (6) and thus has the potential to be of more value as a vaccine candidate. This better association of the envelope protein at the cell surface also allowed more precise evaluation of expression of the mutated envelope glycoprotein at the cell surface.

Envelope mutants expressed in recombinant vaccinia viruses. Each mutated HIV-1 *env* gene was introduced into vaccinia virus to facilitate expression. BHK cells were infected with the different recombinant vaccinia viruses in the presence of [^{35}S]cysteine, and labeled cell extracts were immunoprecipitated with monoclonal antibody (MAb) 110-4. This antibody is directed against a region of gp120 that does not overlap with any of our deletions (from residues 303 to 323 [13]). Infection of cells with VV-env-cs resulted in the synthesis of an *env*-encoded protein that appeared as a doublet with a band of 170 kDa and a weaker band of approximately 160 kDa (Fig. 2). Pulse-chase experiments indicated that the lower band was the precursor to the upper band which was the result of additional glycosylation (data not shown). Infection of cells with vaccinia virus containing the wild-type *env* gene (with unmutated cleavage sites) resulted in the synthesis of the 160- and 120-kDa proteins but not the 170-kDa protein (data not shown).

Cells infected with vaccinia viruses containing each deleted and nondeleted *env* gene produced approximately similar amounts of envelope protein (Fig. 2). This result indicates that the deletions did not grossly interfere with the synthesis and the stability of the *env*-encoded protein. VV-env5-cs produced proteins that were indistinguishable in size from those synthesized by the parental VV-env-cs. However, the pattern of *env*-specific proteins produced by each of the other mutated *env* genes was different from the parental pattern. The envelope glycoprotein produced by

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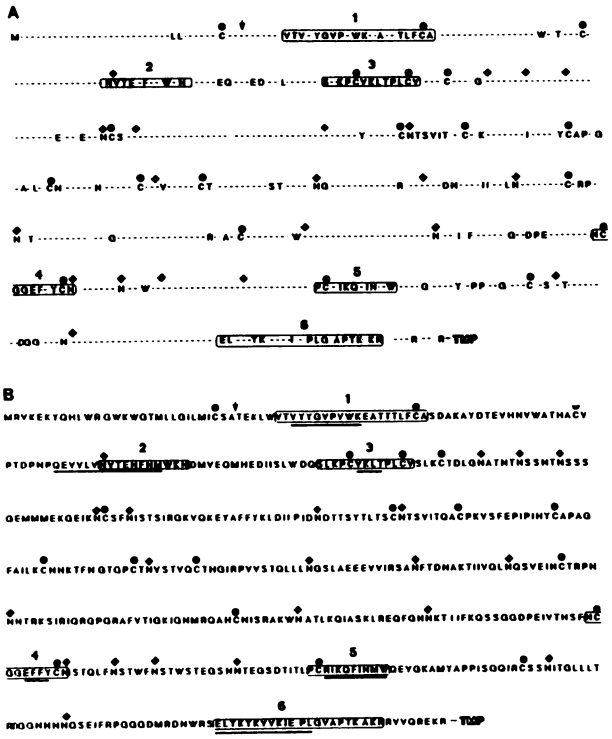

FIG. 1. Amino acid sequence of the extracellular glycoprotein of HIV-1 (isolate BRU). One-letter amino acid abbreviations are used. Cysteine residues (●) and potential sites of glycosylation (◆) are marked. The arrow indicates the position of the cleavage site between signal peptide and extracellular protein. TMP, Transmembrane protein. The six hyperconserved regions between HIV-1 (BRU and MAL isolates) and HIV-2 (ROD isolate) are boxed. (A) Amino acids that are not identical in each of the three sequences are represented by dashes; otherwise, the amino acids are shown. Amino acids that are insertions of one sequence relative to another are not shown. (B) Complete amino acid sequence of HIV-1 (BRU isolate) extracellular glycoprotein, amino acids deleted by site-directed mutagenesis are underlined. Deletions were as follows: 1, residues 38 to 46 (nucleotides 5913 to 5939); 2, residues 82 to 95 (nucleotides 6045 to 6101); 3, residues 120 to 123 (in addition, valine 119 is replaced by alanine) (nucleotides 6161 to 6169); 4, residues 386 to 389 (nucleotides 6957 to 6968); 5, residues 424 to 432 (nucleotides 7074 to 7094); 6, residues 487 to 499 (nucleotides 7262 to 7300). The numbering system used is based on that for the BRU strain (16, 24). Mutants were confirmed by dideoxy sequencing (20). Details of mutagenesis are available on request. Virus produced from each deleted plasmid is named VV-env*x*-cs, where *x* stands for the hyperconserved region of envelope that was deleted.

VV-env1-cs appeared as a doublet, like the parental VV-env-cs glycoprotein, but the stoichiometry of the two species was inverse. VV-env2-cs, VV-env4-cs, and VV-env6-cs produced a glycoprotein that migrated as a single band of 160 kDa. Interestingly, VV-env3-cs, which had a deletion of only three amino acids, produced the smallest protein (130 kDa) although no potential glycosylation site was removed. Immunoprecipitations with a human polyclonal serum and with an MAb raised to a peptide in the transmembrane protein gave results identical to that obtained with MAb 110-4 (data not shown).

The number of amino acids deleted in each clone is too small to explain the observed differences in molecular weight (Fig. 2). Treatment of the envelope proteins with endoglycosidase H or production of the proteins in the presence of

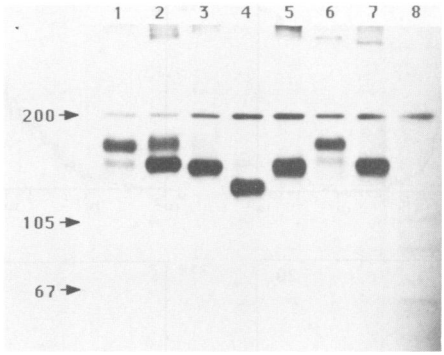

FIG. 2. Analysis of HIV-1 gp160 produced by recombinant vaccinia virus-infected cells. BHK cells infected with recombinant or wild-type vaccinia virus were labeled for 16 h with [^{35}S]cysteine (100 μCi/ml). Labeled cell extracts were immunoprecipitated with MAb 110-4 and analyzed by electrophoresis on a 7.5% polyacrylamide gel, followed by fluorography. Lanes 1, VV-env-cs; 2, VV-env1-cs; 3, VV-env2-cs; 4, VV-env3-cs; 5, VV-env4-cs; 6, VV-env5-cs; 7, VV-env6-cs; 8, wild-type vaccinia virus. Apparent sizes (in kilodaltons) are shown on the left. VV-env-cs is called VVTGeLAV-1139 in reference 6.

glycosylation inhibitors (castanospermine and monensin) showed that in general, the differences in size could be explained by differences in complex sugar content (data not shown).

Detection of gp160 on the surface of CEM cells infected with recombinant vaccinia viruses. Because of differences in maturation of the *env* proteins produced by the various recombinant viruses, we suspected that the deletions may also influence transport to the cell membrane. Expression of gp160 on the surface of CEM cells (a CD4$^+$ human lymphocyte cell line) infected with recombinant vaccinia viruses was evaluated by flow cytometric immunofluorescence analysis (Fig. 3). More than 90% of the cells were infected, as determined by labeling with polyclonal antibodies against vaccinia antigens. Different patterns of cell surface expression were obtained for different recombinant viruses: VV-env5-cs, which had the same size as VV-env-cs, was expressed at the cell surface; VV-env3-cs was also exported to the cell membrane, but the level of fluorescence intensity was lower than that for VV-env-cs or VV-env5-cs. In contrast VV-env2-cs, VV-env4-cs, and VV-env6-cs, which produced proteins of the same size and thus may have been altered in processing in a similar way, did not efficiently reach the cell surface. Finally, an intermediate result was obtained with cells infected with VV-env1-cs; approximately half poorly expressed envelope protein at the cell surface. Thus, the different deletions made in the gp160 *env* gene altered processing of the glycoprotein in a manner correlated with changes in transport to the surface of infected cells. Except in the case of VV-env3-cs, the appearance of the top band (170 kDa) was correlated with expression of the glycoprotein at the cell surface.

Binding to CD4 antigen. To study receptor binding, we used a test based on the ability of an MAb against CD4 (OKT4) to immunoprecipitate preformed complexes of CD4 and HIV envelope proteins (15). Membrane and cytosol of ^{35}S-labeled cells were extracted with 1% Nonidet P-40 (Sigma Chemical Co.) and incubated with 50 ng of soluble CD4 (3), and the formation of gp160-CD4 complex was assessed by immunoprecipitation of the complex with excess MAb OKT4. The specificity of the reaction was confirmed

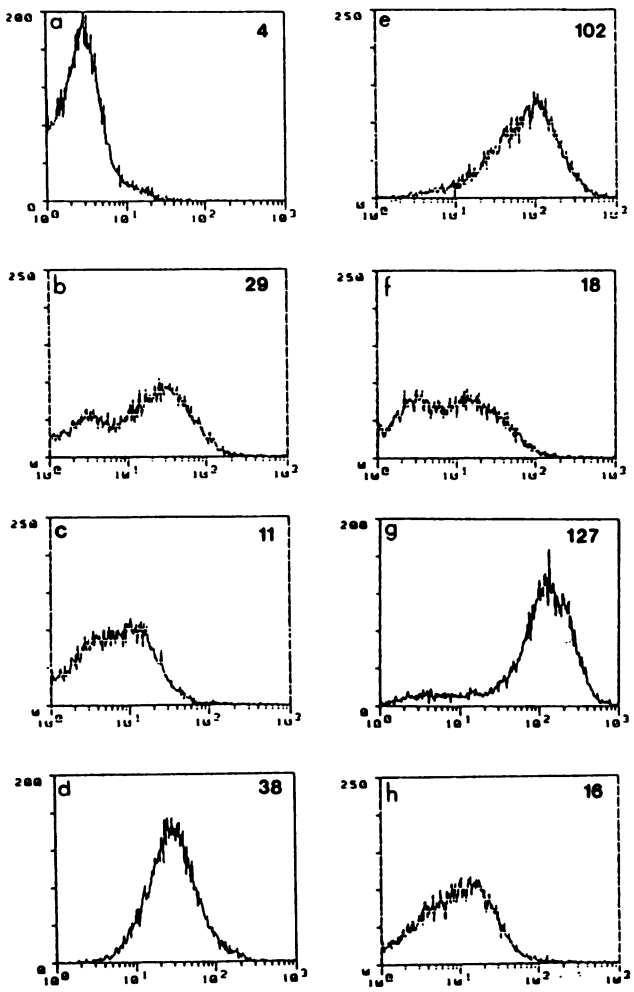

FIG. 3. Expression of gp160 at the surface of CEM cells infected by recombinant vaccinia virus. In each panel, the mean of fluorescence intensity for MAb 110-4 is indicated at the top right. (a) Wild-type vaccinia virus; (b) VV-env1-cs; (c) VV-env2-cs; (d) VV-env3-cs; (e) VV-env-cs; (f) VV-env4-cs; (g) VV-env5-cs; (h) VV-env6-cs. Comparable results were obtained with MAb 41-1 (Genetic Systems), which recognizes an epitope in the extracellular portion of the transmembrane protein.

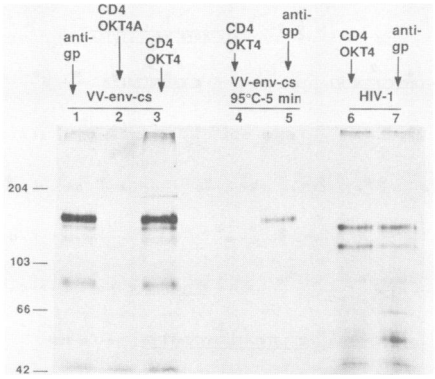

FIG. 4. Coimmunoprecipitation analysis of gp160 binding to soluble CD4. BHK cells infected with recombinant vaccinia virus VV-env-cs (lanes 1 to 5) or CEM cells infected with HIV-1 (lanes 6 and 7) were labeled for 16 h with [^{35}S]cysteine (100 μCi/ml). Labeled cell extracts were immunoprecipitated with antiglycoprotein MAb 110-4 or incubated with soluble CD4 and immunoprecipitated with anti-CD4 MAb OKT4A or OKT4 (as indicated). In lanes 4 and 5, protein extract (the same as in lane 1) had previously been heated to 95°C for 5 min. Immunoprecipitates were analyzed by electrophoresis on a 7.5% polyacrylamide gel. Apparent molecular sizes (in kilodaltons) are shown on the left.

by the fact that MAb OKT4A, which recognizes an epitope on CD4 involved in the interaction with the envelope protein (15), was unable to immunoprecipitate the gp160-CD4 complex (Fig. 4). Furthermore, the precipitation of gp160 by MAb OKT4 was dependent on addition of exogenous CD4 (data not shown). Heating the extract to 95°C for 5 min completely eliminated the coimmunoprecipitation of gp160 with CD4 (Fig. 4). In addition, there was no discernible difference in our test between the ability of the wild-type HIV envelope protein to bind to the receptor and that of the envelope protein with its cleavage sites mutated (Fig. 4). This finding indicates that cleavage of the envelope precursor is not necessary for binding to CD4. The fact that the 170- and 160-kDa species of glycoprotein reacted equally with the CD4 antigen (Fig. 4 and 5) indicates that the hyperglycosylation that takes place when the cleavage site is mutated does not interfere with the ability to bind to CD4.

We then tested each mutant for the ability to bind to the CD4 receptor. VV-env1-cs and VV-env3-cs showed a high efficiency of binding (Fig. 5). This ability was weakly reduced for VV-env1-cs as compared with VV-env-cs. In contrast, binding by VV-env2-cs, VV-env4-cs, VV-env5-cs and VV-env6-cs was drastically reduced. These results suggest that regions, 2, 4, 5, and 6 either constitute a part of the gp120 binding region or contribute to create or maintain the tertiary structure of the binding site.

It should be noted that the two mutants that were still able to bind CD4 antigen had distinct altered patterns of processing. VV-env1-cs was not well expressed at the cell surface, and only the smallest product of the doublet had the capacity to bind CD4 antigen; VV-env3-cs reached the cell surface and was the smallest glycoprotein (130 kDa) produced with the recombinant vaccinia viruses.

The affinity constant for the CD4-gp120 interaction is 10^{-9} M (10). Results presented here show that four hyperconserved regions of gp120 are essential for proper CD4 binding. It was of interest to compare the locations of these regions with those reported by others. Previous work demonstrated that a mutant gp120 that lacked the N-terminal 30 amino acids bound with high affinity to the CD4 receptor, whereas a deletion of the first 164 amino acids resulted in an apparent loss of CD4-binding ability (4). Our data show that a deletion within this region, deletion 2 (from residues 82 to 95 [Δ82-95]), completely abolished the binding, whereas deletion 1 (Δ38-46) had a small effect on binding and deletion 3 (Δ120-123) had no effect at all. On the other hand, it has been shown that a proteolytic fragment of the envelope protein that does not contain the N-terminal end can bind to CD4$^+$ cells (17). This finding suggests that sequences at the N terminus may be necessary to create, but not necessarily to maintain, a structure capable of binding CD4.

Concerning the C terminus of the gp120, we have shown that three deletions (Δ386-389, Δ424-432, and Δ487-489) disrupted CD4 binding to gp120. These regions are close to but different from those described by others. Insertional mutagenesis revealed that residues near positions 363, 419, and 472 (corresponding to 368, 424, and 477 in our sequence) affected binding whereas deletions from residues 399 to 406

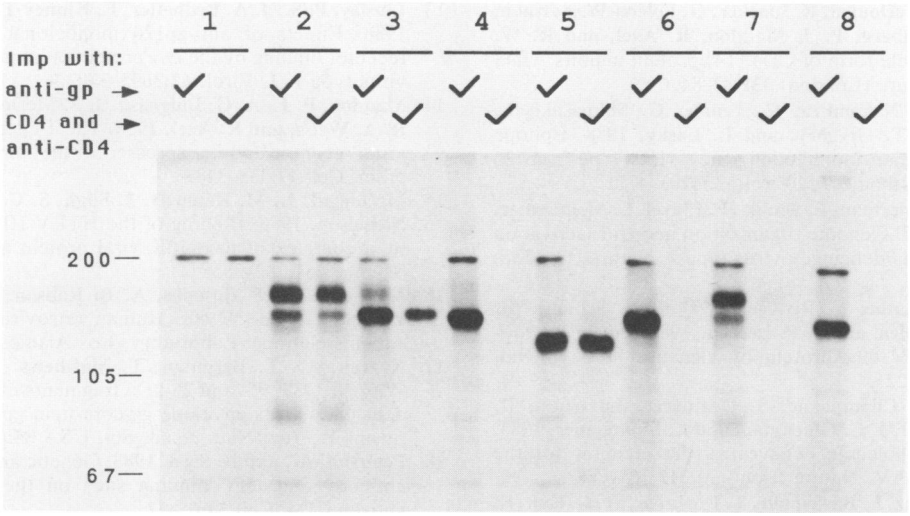

FIG. 5. CD4-binding ability of mutant gp160. BHK cells infected with recombinant or wild-type vaccinia virus were labeled for 16 h with [^{35}S]cysteine (100 μCi/ml). Labeled cell extracts were immunoprecipitated with antiglycoprotein MAb 110-4 or incubated with soluble CD4 and immunoprecipitated with anti-CD4 MAb OKT4. Antibody-antigen complexes were precipitated with protein A-Sepharose, denatured with sodium dodecyl sulfate, 2-mercaptoethanol, and boiling, and analyzed by electrophoresis on a 7.5% polyacrylamide gel. Lane 1, wild-type vaccinia virus; 2, VV-env-cs; 3, VV-env1-cs; 4, VV-env2-cs; 5, VV-env3-cs; 6, VV-env4-cs; 7, VV-env5-cs; 8, VV-env6-cs. Apparent molecular sizes (in kilodaltons) are shown on the left.

had no effect (8). In comparison, our deletion 4 (Δ386-389) shows that residues essential for a binding structure exist in this region of the protein.

Dowbenko et al. (4) have shown that MAbs that block CD4-gp120 interaction recognized epitopes from residues 414 to 429 and residues 427 to 438 (according to our nomenclature). These epitopes map to the same place as our region 5 (residues 424 to 432). It is interesting to note that of all of the deletions of hyperconserved regions shown here, only deletion of region 5 had no discernible effect on glycoprotein maturation (Fig. 2 and 3). Lasky et al. (10) showed that deletion of a region just downstream (431 to 442) can also eliminate binding. We have also recently shown that some point mutations in this same region can eliminate binding (A. Cordonnier, L. Montagnier, and M. Emerman, Nature (London), in press).

Finally, Linsley et al. (13) demonstrated that CD4-specific binding of gp120 produced by a recombinant vaccinia virus was dependent on the presence of the 44 C-terminal amino acids. Our results also suggest that the C-terminal portion is critical for binding, since its removal by deletion 6 (Δ487-499) abolished binding. However, the epitope recognized by MAb 110-1, an antibody that does not block gp120-CD4 interaction (13), maps exactly to region 6. Therefore, this region probably behaves as a structural component of the glycoprotein.

Deletions in the highly conserved region of the envelope protein led to the synthesis of glycoproteins of different sizes. Furthermore, these alterations were accompanied by changes in transport of the glycoprotein to the cell surface. Because some of these altered proteins (VV-env1-cs and VV-env3-cs) still bound to CD4, we argue that the exact carbohydrate structure of the envelope glycoprotein is not important for binding to the receptor. Indeed, wild-type envelope produced in the presence of several others inhibitors of complex carbohydrate processing (castanospermine, deoxynojirimycin, and monensin) had no effect on binding of the glycoprotein to CD4 in our in vitro test (data not shown). It has recently been shown that deglycosylated envelope protein in vitro also retains the ability to bind to CD4 (E. Fenouillet, B. Clerget-Raslain, J. Gluckman, D. Guetard, L. Montagnier, and M. Bahraoui, J. Exp. Med., in press). It should be noted that most of the potential glycosylation sites are not conserved between HIV-1 and HIV-2 (Fig. 1).

Although the deletions are distantly located with respect to each other on the primary structure, it is likely that some are closely located in the three-dimensional structure of gp120 and contribute to a discontinuous binding site. Alignment of HIV envelope glycoprotein gp120 with immunoglobulin heavy-chain constant regions (14) showed that two blocks (residues 40 to 92 and 406 to 445), corresponding to our regions 2 and 5, in which deletions abolish binding, share significant sequence and structural homologies with immunoglobulin C domains. Although the domains of CD4 necessary for binding to gp120 have been relatively easy to define (9, 18, 19), the domains of gp120 necessary for binding to CD4 appear to be more complexly arranged. Additional investigation will be aimed at a more precise definition of those gp120 amino acid residues required for receptor binding.

We thank A. Regnault for technical assistance, R. Olivier for help with fluoresence-activated cell sorting, M.-P. Kieny and Transgène for protocols and reagents for vaccinia viruses and for virus 1139, Genetic Systems for antibody 110-4, and the laboratory of R. Axel for soluble CD4. We also thank K. Peden for helpful discussions and H. Blough, L. Chakrabarti, M. Guyader, and K. Peden for comments on the manuscript.

A.C. was supported by a fellowship from la Fondation pour la Recherche Médicale. M.E. is a fellow of the Leukemia Society of America.

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