RAET1E2, a Soluble Isoform of the UL16-binding Protein RAET1E Produced by Tumor Cells, Inhibits NKG2D-mediated NK Cytotoxicity*

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UL16-binding proteins (ULBPs, also termed as retinoic acid early transcripts, encoded by RAET1 genes), a family of ligands for NKG2D in humans, are frequently expressed by tumor cells and mediate cytotoxicities of natural killer (NK) cells and CD8+ αβ T cells to tumor cells. ULBP1, ULBP2, ULBP3, and RAET1L link to membrane through glycosylphosphatidylinositol, whereas RAET1E and RAET1G contain transmembrane and cytoplasmic domains. Proteolytic cleavage of ULBP2 produces truncated and soluble forms that may counteract NKG2D-mediated tumor immune surveillance. In this study, we report that RAET1E can produce a soluble, 35-kDa protein (termed as RAET1E2) lacking the transmembrane region by selective splicing in tumor cells. The expressions of both RAET1E2 transcripts and protein can be found in different tumor cells and tissues. Preincubation of NK-92 cells, a human NK cell line, with culture supernatants from tumor cell lines expressing RAET1E2 or RAET1E2 gene-transfected COS-7 cells resulted in decreased expression of NKG2D on NK-92 cells. Furthermore, incubation of NK-92 cells with recombinant RAET1E2 protein also decreased the surface expression of NKG2D and resulted in marked reduction in cytotoxicities to MGC-803, HepG2, or K562 tumor cells. Taken together, our data provide strong evidence for an immune escape mechanism of tumors via alternative splicing of ULBP RNA to generate a free soluble ULBP protein, RAET1E2, that may impair NKG2D-mediated NK cell cytotoxicity to tumors.

NKG2D, a C-type lectin-like activating receptor, is expressed on several immune cells including NK cells, CD8+ αβ T cells, and γδ T cells. NKG2D can recognize a group of ligands that represent distant relatives of MHC class I molecules, such as MHC class I-related protein A/B (MICA/B) and ULBPs in human and RAE-1-α-e, H60, and MULT-1 in mice. Although it remains unclear whether NKG2D can directly activate αβ and/or γδ T cells, NKG2D indeed enhances the antigen-dependent activation of T cells and provides both activating and co-stimulatory signals in NK cells (2–13), suggesting that it plays important roles in both innate and adaptive immune responses.

ULBPs belong to a recently identified ligand family for NKG2D in humans. These ligands are frequently expressed by tumor cells and stimulate anti-tumor immune responses mediated by CD8+ αβ T cells and NK cells (14–21). ULBP gene family consists of 10 members, six of which encode potentially functional glycoproteins that are located near the tip of its long arm at q24.2–q25.3, close to the human equivalent of the mouse H2-linked t-complex. This subchromosomal region is similar to a segment of mouse chromosome 10 harboring the orthologous MHC class I-related retinoic acid early transcript loci, Raet1a–d (22). Among them, ULBP1, ULBP2, ULBP3, and RAET1L are linked to membrane through a glycosylphosphatidylinositol anchor, whereas RAET1E and RAET1G contain transmembrane and cytoplasmic domains.

By engaging the NKG2D activating receptor, ULBPs can directly activate NK cells to proliferate and secrete cytokines. Co-stimulation of NK cells with ULBPs and interleukin-12 greatly enhances the production of multiple cytokines and chemokines (17). In addition, in vitro, some target cells insensitive to NK cells can be efficiently lysed when transfected with ULBPs (14, 19, 21). In vivo, the expression of ULBPs correlates with improved survival in cancer patients, and ectopic expression of ULBPs on murine EL4 or RMA tumor cells elicits potent anti-tumor responses in syngeneic C57BL/6 and severe combined immunodeficiency disease mice, which can be strongly enhanced by interleukin-15 (20). Accordingly, Andreas Diefenbach et al. (23) demonstrated the efficacy of using NKG2D ligands as components of vaccine, which may offer new approaches for malignancies. Moreover, it was shown that DNA-based vaccines encoding syngeneic or allogeneic NKG2D ligands together with tumor antigens could markedly activate both innate and adaptive anti-tumor immunity (24, 25).

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2 The abbreviations used are: NK, natural killer; ULBPs, UL16-binding proteins; RAET1, retinoic acid early transcript 1; MICA/B, MHC class I-related protein A/B; MHC, major histocompatibility complex; CHO, Chinese hamster ovary; RT, reverse transcription; mAb, monoclonal antibody; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.
However, accumulating evidence also suggest that NKG2D may be a target for immune escape of tumor cells. It has been reported that tumors could evade NKG2D-mediated anti-tumor response by shedding its ligands from cell surface and then down-regulating the expression of NKG2D (26–33). In addition, it has been recently hypothesized that alternatively spliced forms of RNA transcripts for soluble forms of NKG2D ligands may be produced simultaneously with transcripts for the membrane-bound forms (34). Indeed, by RT-PCR, Bacon et al. (14) found that RAET1G could produce the soluble form RAET1G2 by alternative splicing in the fourth exon (see Fig. 1A), although they failed to confirm the existence of RAET1G2 protein in the supernatants of cultured tumor cells or the peripheral blood of tumor patients. Thus, tumor cells may produce soluble forms of ULBPs to escape NKG2D-mediated cytotoxicity via two different ways. First, the ligands can be proteolytically shed from the surface of tumor cells, such as ULBP2 (31). Such ULBP decay ectodomains are “cut” by proteolysis. ULBP1, ULBP3, and RAET1L may use this mechanism because these molecules are all glycosylphosphatidylinositol-anchoring molecules without transmembrane domain. Second, soluble ligands can be generated by gene-coded novel isoform. Bacon et al. (14) demonstrated that RAET1G2 was generated by alternative splicing of RAET1G RNA. We wonder whether ULBP4 (RAET1E) can generate a soluble ectodomain since RAET1E and RAET1G share common structural features, which are absent in ULBP1–3 and RAET1L molecules.

In this study, we first screened different tumor cells for their RAET1E transcript expression by RT-PCR. Consequently, we cloned and sequenced a novel isoform of RAET1E gene termed RAET1E2. RAET1E2 mRNA and protein expression can be detected in tumor cells positive for RAET1E mRNA. Preincubation of NK-92 cells with culture supernatants from tumor cell lines expressing RAET1E2 (MGC-803, HO-8910, and HepG2 cells) or from RAET1E2 gene-transfected COS-7 cells resulted in decreased NKG2D expression on NK-92 cells. Furthermore, recombinant RAET1E2 protein also decreased NKG2D surface expression on NK-92 cells and inhibited NKG2D-mediated NK cytotoxicities to MGC-803, HepG2, and K562 tumor cells. Taken together, our data provide evidence for an immune escape mechanism of tumors via selective splicing of an ULBP RNA to generate a free soluble ULBP protein, RAET1E2, which in turn impairs NKG2D-mediated immune surveillance.

**EXPERIMENTAL PROCEDURES**

**Primers and Molecular Cloning**—The following PCR primers were used for cloning the genes of RAET1E and a short form of RAET1E2 (see Fig. 1): RAET1E-forward, 5′-ATGCGGAGAATATCTCTGATCCT-3′; RAET1E-reverse, 5′-CTAAGACGCTTCAAGGGC-3′; U4a1 + a2Iden, 5′-TCTCCCACCCAGCTCAGTTAC-3′; U4a1 + a2Race1, 5′-GCCAGAGGCAGCTA-3′; U4a1 + a2Race2, 5′-GTAACCTAGCCTGAGTGGG-3′; AUAP, 5′-GCCCAAGCTCTGAGATT-3′. The cDNAs were synthesized with AP and Moloney murine leukemia virus reverse transcriptase. The expected sizes of PCR products were 792 bp for RAET1E, 630 bp for RAET1E2, and 1128 bp for β-actin.

**Cell Lines**—Twenty-nine tumor and immortalized cell lines were used in this study (see the legend for Fig. 2). The CNE2 human nasopharyngeal carcinoma cell line, Hep-2 human larynx carcinoma cell line, HepG2 human liver carcinoma cell line, MGC-803 human gastric adenocarcinoma cell line, HO-8910 human ovarian carcinoma cell line, and CHO Chinese hamster ovary cells were all cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine at 37 °C in a 5% CO2 atmosphere. HeLa human cervix carcinoma cell line was cultured in McCoy 5A supplemented with 10% (v/v) heat-inactivated fetal calf serum. A375 human malignant melanoma and COS-7 African green monkey kidney cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum. WISH human amnion cell line was cultured in modified Eagle’s medium-non-essential amino acids supplemented with 10% (v/v) heat-inactivated fetal calf serum. NK-92 cell line was kindly provided by Prof. Zhigang Tian from the School of Life, University of Science and Technology of China, and cultured in modified Eagle’s medium-α medium supplemented with 12.5% fetal bovine serum, 12.5% horse serum, and 200 units/ml interleukin-2.

**Production of Recombinant Proteins**—Recombinant RAET1E2 was produced as a six-histidine C-terminal-tagged protein. In brief, purified RAET1E2 PCR fragments were digested with Ndel and Xhol enzymes and linked into prokaryotic expression plasmid pET22b(+). The sequences of the oligonucleotide primers used to amplify the desired sequences by PCR are available upon request. Thirty ml of bacterial inoculum Rosetta-gami™B(DE3) (Novagen) containing RAET1E2 expression plasmids were grown at 37 °C in LB broth overnight. The LB broth in the overnight inoculum was replaced with 1 liter of 2× YT medium and added to a 3-liter shaker flask at 37 °C. The bacterial cell culture was induced at an A590 to 0.6 with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h. The inclusion body was dissolved in 8 M urea and then purified with Amersham Biosciences HisTrap column. Refolding was performed in dialysis tubing. The His-tagged protein molecular weight and purity were confirmed by SDS-PAGE on 15% acrylamide gels and Western blotting.

**Preparation of Monoclonal Antibody (mAb) against RAET1E2**—Prokaryotic expressed human RAET1E2 was used as an antigen to prepare mAb by conventional B lymphocyte hybridoma technique. Positive clones were identified by enzyme-linked immunosorbent assay, immunofluorescence assay, flow cytometry, and Western blotting as described (35).
Western Blotting—For immunoprecipitation analysis of RAET1E2 proteins, cell culture supernatant was incubated overnight at 4 °C with anti-RAET1E2 antibody 6C12 or 8C9 in RPMI 1640 medium. The immunocomplexes were collected after incubation with anti-mouse IgG-agarose beads (Sigma) for 2 h. Cells were lysed by radioimmune precipitation buffer, and samples were separated by 15% SDS-PAGE. Gels were blotted to nitrocellulose membranes (Amersham Biosciences). Membranes were blocked overnight with Tris-buffered saline containing 5% nonfat dried milk and then incubated with anti-RAET1E2 mAb 10F7 or anti-His tag mAb (Qiagen). Immunoreactive bands were visualized with a goat anti-mouse IgG horseradish peroxidase conjugate together with SuperSignal West Pico chemiluminescent substrate (Pierce).

NKG2D Down-regulation Assay—NK-92 cells were cocultured with the culture supernatants from tumor cell lines expressing RAET1E2, including MGC-803, HepG2, and K562 cells, or from RAET1E2 gene-transfected COS-7 cells or supplemented with 1 μg/μl RAET1E2-His tag recombinant proteins for 72 h. The expression profile of NKG2D was assayed by flow cytometry. In brief, cells were incubated with anti-human NKG2D mAb (eBioscience) at 4 °C. The excess probe was washed with phosphate-buffered saline, and the cell-associated fluorescence was analyzed by a FACScan flow cytometer (Beckman Coulter, EPICS XL). Background staining was estimated after incubation with phycoerythrin-labeled isotype antibody.

Cytotoxicity Assays—The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric test was used to evaluate the cytolytic activities of NK-92 cells in vitro. Tumor cells MGC-803, HepG2, and K562 were used as target cells and seeded into 96-well plates with 5 × 10^3 cells/well. NK-92 cells (pretreated with or without HepG2 culture supernatants or 1 μg/μl free form RAET1E2-His tag fusion proteins) as effector cells with variant concentration were incubated with target cells for 4 h. Subsequently, MTT solution (5 mg/ml) was added to each well (15 μl/well) and incubated at 37 °C for 4 additional hours. The reaction was stopped by the addition of 100 μl of Me_2SO to dissolve the tetrazolium crystals. The plate was examined at 570 and 630 nm in a Multiskan microplate reader (Thermo Labsystems), and the percentage of specific lysis was calculated using the following formula

\[
\text{Percent Specific Lysis} = \frac{\text{OD}_{	ext{Target}} - \left(\text{OD}_{	ext{Effector}} + \text{OD}_{	ext{Effector + Target}}\right)}{\text{OD}_{	ext{Effector}}} \times 100\%
\]

where T_{OD} = OD value of target cells, E_{OD} = OD value of effector cells, and (E + T)_{OD} = OD value of effector cells cultured with target cells. The optical density was determined at 570 and 630 nm.

RESULTS

Cloning of a Shortened Form of RAET1E Lacking Transmembrane Region—Sequence alignment between RAET1E cDNA and its genome sequence shows that RAET1E contains four...
FIGURE 2. Expressions of RAET1E and RAET1E2 transcripts in cell lines. A, RAET1E transcripts were detected by RT-PCR in 29 tumor or immortalized cell lines including the 293T human embryonic kidney cell line, MGC-803 human gastric adenocarcinoma cell line, A375 human malignant melanoma cell line, C1R human B lymphoma cell line, Calu-3 human lung adenocarcinoma cell line, CaSkI human cervix carcinoma cell line, CNE2 human nasopharyngeal carcinoma cell line, Daudi human B lymphoma cell line, HCT-116 human colon carcinoma cell line, HEC-1-B human endometrium adenocarcinoma cell line, HeLa human cervix carcinoma cell line, Hep-2 human larynx carcinoma cell line, HepG2 human liver carcinoma cell line, HO-8910 human ovarian carcinoma cell line, HR-8348 human colorectal carcinoma cell line, HT-29 human colon adenocarcinoma cell line, JEG-3/PVH human choriocarcinoma cell line, Jurkat human T cell lymphoma cell line, K562 human chronic myeloid leukemia cell line, MCF-7 human breast adenocarcinoma cell line, MRC-5 human fetal lung cell line, NCI-H520 human lung squamous carcinoma cell line, Pancreas-1 human pancreatic carcinoma cell line, Raji human B lymphoma cell line, Ramos human B lymphoma cell line, RPMI 8226 human peripheral blood myeloma cell line, Skov-3 human ovary adenocarcinoma cell line, U-251 human glioma cell line, and WISH human amnion cell line. RAET1E mRNA was positive in eight cell lines including the 293T human embryonic kidney cell line, MGC-803 human gastric adenocarcinoma cell line, A375 human malignant melanoma cell line, CNE2 human nasopharyngeal carcinoma cell line, Daudi human B lymphoma cell line, HEC-1-B human endometrium adenocarcinoma cell line, HeLa human cervix carcinoma cell line, HepG2 human liver carcinoma cell line, HO-8910 human ovarian carcinoma cell line, and WISH human amnion cell line. Specific primers were used, such that RAET1E transcripts were detected by RT-PCR. B, RAET1E-positive cell lines were rescreened again for RAET1E2 transcript expression (630 bp) by RT-PCR. C, Expressions of RAET1E and RAET1E2 transcripts in primary fresh tumor tissues. Lane 1, uterine cervix cancer; lane 2, ovarian cancer; lane M, DNA marker 2000.

exons (Fig. 1B). We noticed that a termination codon in the intron following exons 1–3 that constitutes the α1 and α2 domains exists. To test whether RAET1E may utilize the intron to produce a shortened form of RAET1E, we designed a pair of primers matching the translation initiation codon and the intron, respectively (Fig. 1B). Unexpectedly, we found that the primers amplified a DNA fragment with the same size as that of α1 and α2 domains. To further analyze this transcript, we performed 3′-rapid amplification of cDNA ends-PCR and obtained the sequence of the whole long cDNA (Fig. 1, C and D). The transcript encodes a shortened form of RAET1E that contains the first 209 amino acids of RAET1E (Fig. 1, C and D) and represents a novel isoform of RAET1E. We termed this novel isoform RAET1E2.
Expression of RAET1E and RAET1E2 in Different Tumor Cells—We first examined the expression of RAET1E and RAET1E2 in 29 different tumor cell lines by RT-PCR. As shown in Fig. 2A, RAET1E transcript was detected in eight malignant tumor cell lines, such as HepG2 human liver carcinoma cell line, MGC-803 human gastric adenocarcinoma cell line, and HO-8910 human ovarian carcinoma cell line. RAET1E2 transcript was detected in all these tumor cell lines that are positive for RAET1E mRNA (Fig. 2B). Also, RAET1E/RAET1E2 transcripts presented in primary fresh tumor cells, such as uterine cervix cancer and ovarian cancer (Fig. 2C). Next, we determined RAET1E and RAET1E2 protein expression in tumor cell lines or primary fresh tumor cells by immunoblotting and detected two bands, 35 and 40 kDa, respectively, in several tumor cell lines positive for RAET1E and/or RAET1E2 mRNA but not in cells negative for their transcripts (Fig. 3A). It should be noted that the difference in molecular weight between RAET1E2 expressed in eukaryotic cells and that in Escherichia coli may be due to the effect of glycosylation. To confirm whether these tumor cell lines can release RAET1E2 protein, the supernatants of HO-8910 ovarian carcinoma cells, MGC-803 human gastric adenocarcinoma cell line, and HepG2 human liver carcinoma cell line were immunoprecipitated with anti-RAET1E2 mAb 6C12 or 8C9 and then detected by another anti-RAET1E2 mAb 10F7 in Western blotting. As shown in Fig. 3B, soluble RAET1E2 protein was detected in the culture supernatants of these tumor cells. Similarly, RAET1E/RAET1E2-transfected CHO and/or COS-7 cells also generated the same bands, demonstrating the specificity of the mAbs for RAET1E (Fig. 3, B and C). In addition, 10F7 mAb was specific only to RAET1E/RAET1E2 as it failed to react with other ULBP-His tag fusion proteins expressed in E. coli cells, whereas all of them could be detected by anti-His tag mAb (Fig. 3D). These data indicate that RAET1E2 can be produced as a secreted protein by tumor cells.

A Novel Isoform of RAET1E in Tumor Cells

FIGURE 4. RAET1E2 decreases the expression of NKG2D on NK-92 cells (left panels) and inhibits their cytotoxicities to tumor cells (right panels). A, NK-92 cells were cultured in the presence of complete medium (brown), MGC-803 (saffron yellow), HO-8910 (green), or HepG2 (sky blue) culture supernatants for 3 days and then analyzed for NKG2D expression as described under “Experimental Procedures.” B, NK-92 cells were cultured in the presence of complete medium (green), culture supernatants of RAET1E2 gene- (orange), or Mock-transfected (Mock) (brown) transfected COS-7 cells for 3 days. C, NK-92 cells were cultured in the presence of complete medium (purple) or that supplemented with 1 μg/μl RAET1E2-His tag fusion proteins (green) for 3 days. Expression of NKG2D on NK-92 cells was assayed by immunofluorescent staining with phycoerythrin-labeled anti-human NKG2D mAb. Data came from one representative of three independent experiments. Cytolytic activities of NK-92 cells (pretreated with or without HepG2 culture supernatants or 1 μg/μl RAET1E2-His tag fusion protein) to HepG2 (B, upper panel), MGC-803 (B, middle panel), and K562 (B, lower panel) tumor cells were evaluated with MTT colorimetric test.
RAET1E2 Decreases the Expression of NKG2D on NK-92 Cells and Inhibits Their Cytolytic Activities—Previous studies demonstrate that high levels of soluble MICA and ULBP2 could be detected in the sera of some tumor patients and that expression of NKG2D was reduced markedly on large numbers of tumor-infiltrating and peripheral blood T cells from individuals with cancer (26–30). Binding of soluble MICA and ULBP2 (sULBP2) with NKG2D could induce endocytosis and degradation of NKG2D, causing severely impaired function of NK cell and tumor-antigen-specific effector T cells (26, 30). To functionally characterize the secreted form RAET1E2, we preincubated NK-92 cells with the culture supernatants of MGC-803 human gastric adenocarcinoma cell line, HO-8910 human ovarian carcinoma cell line, and HepG2 human liver carcinoma cell line for 3 days to determine the effects on NKG2D expression. As shown in Fig. 4A, the culture supernatants of the three tested tumor cell lines induced the reduction of NKG2D expression on NK-92 cells. Importantly, incubation of NK-92 cells with culture supernatants from RAET1E2-transfected COS-7 cells or purified recombinant RAET1E2 protein resulted in dramatic reduction of NKG2D surface expression as well (Fig. 4, B and C). In addition, decreased cytotoxicities of NK-92 cells to HepG2 cells were observed after preincubation of NK-92 cells with culture supernatants of HepG2 cells or anti-NKG2D mAb (Fig. 4D, upper panel). Similarly, NK-92 cell-mediated lysis of MGC-803 tumor cells (Fig. 4D, middle panel) or K562 cells (Fig. 4D, lower panel) was also inhibited by preincubation with recombinant RAET1E2 protein. Together, these data demonstrate that RAET1E2 induces decreased surface expression of NKG2D and inhibits the cytotoxicities of NK cells to tumor cells.

DISCUSSION

The NKG2D ligand system has evolved to function as a sentinel system to alert the immune system in response to infections and tumors since surging evidence indicates that the ligands for NKG2D appear to be stress-inducible molecules. However, malignant transformed cells can exempt themselves from the immune attack mediated by NKG2D via several escape mechanisms. So far, at least four ULBP-related immune escape mechanisms have been documented. First, proteins derived from virus-infected cells such as UL16 could down-regulate the expressions of NKG2D ligands on cell surface and then decrease their susceptibility to NKG2D-dependent cytotoxicity (36–40). Second, shedding of ULBP2 has been described as another immune escape mechanism employed by tumors. This occurs by specific enzymatic cleavages, as seen in the processing of soluble forms of members of the tumor necrosis factor family. Shedding of ULBP2 can generate decoy ectodomain, which may have a blocking role in receptor-lectin interaction. Moreover, it is possible that the binding of ULBP2 ectodomain with NKG2D might lead to internalization, resulting in down-regulation of NKG2D on NK cells. Third, it was hypothesized that alternatively spliced forms of RNA transcripts for the soluble forms may be produced at the same time as transcripts for the membrane-bound forms. Our data provide evidence for an immune escape mechanism of tumors via selective splicing of an ULBP RNA to generate a free soluble ULBP protein. RAET1E2. RAET1E2 has the capacity to down-regulate NKG2D-mediated cytotoxicities to tumor cells. RAET1E2-mediated inhibition of tumor killing effect may be due to both blockade of receptor-ligand interaction and internalization of NKG2D as mentioned above. Finally, some autocrine cytokines such as transforming growth factor-β could selectively down-regulate MICA, ULBP2, and ULBP4 transcripts and then their protein expression on tumor cells (41).

Collectively, proteolytic cleavage at the protein level and alternative splicing at the RNA level may play more important roles in evading NKG2D-mediated immune surveillance as both of them have multiple effects on counteracting activating signals into NK cells. Another key issue is whether the two mechanisms occur independently or synergistically. In other words, the issue is whether alternative splicing can produce soluble ULBP2 or whether proteolytic cleavage will generate ectodomain of RAET1E. In the future experiments, on the one hand, we will use the above cloning strategy to search for the shortened variants of ULBP1–3, RAET1L, and MICA/B; on the other hand, we are going to establish a highly sensitive sandwich enzyme-linked immunosorbent assay platform for RAET1E/E2 to test the proteolytic cleavage possibility. Meanwhile, our finding will facilitate the elucidation of the relations between the quantity of the soluble form of RAET1E in sera and tumor progress or prognosis, especially tested tumors positive for RAET1E/E2 in this investigation, such as hepatic cell carcinoma, gastric cancer, and ovarian cancer. All above efforts will form an important basis for designing a new strategy to diagnose and treat the malignant diseases.

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