Inhibition of Apoptosis after Thymineless Stress Is Conferred by Oncogenic K-Ras in Colon Carcinoma Cells

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ABSTRACT

Ras functions as a molecular switch for several downstream targets and may promote either apoptosis or survival dependent upon the cell system and stimulus. The functional significance of a transfected K-Ras oncogene in influencing apoptosis induced by thymineless stress was examined in a thymidylate synthase (TS)-deficient (TS⁻) colon carcinoma cell line derived from GCjc1 after thymidine deprivation. Oncogenic K-Ras conferred survival in TS⁻ K-Ras clones compared with TS⁺ (untransfected) and TS⁻ pCIneo (vector control). Previously, we had demonstrated that thymineless death involved signaling via Fas/FasL interactions. However, in the presence of oncogenic K-Ras, survival did not involve down-regulation of Fas or FasL expression but did involve members of the Bcl-2 family. Bcl-2 and Bax expression remained relatively constant during thymineless stress in all cell lines. Apoptosis in the presence of wild-type Ras correlated with up-regulated expression of Bak that did not occur in TS⁺ K-Ras clones, whereas survival in these clones correlated with elevated expression of Bcl-xL. Thus, the Bak:Bcl-xL ratio was high in TS⁺ and TS⁻ pCIneo cells undergoing apoptosis, whereas the Bcl-xL:Bak ratio was high in TS⁺ K-Ras clones exhibiting a survival response.

INTRODUCTION

Seventy five to 80% of colon carcinomas demonstrate point mutations in the p53 tumor suppressor gene followed by reduction to homozygosity (1, 2). This leads to deregulated G₁ checkpoint function, thereby conferring a selective growth advantage to these cells. The high frequency of mutations in the p53 gene is preceded in many instances by activation of K-Ras (1). The Ras gene was originally identified as an oncogene of a rat sarcoma virus (the Harvey and Kirsten strains). Later, it was found that many human cancer cells contain mutated alleles of the cellular Ras gene, which causes the transformation of NIH3T3 cells after transfection (3). Approximately 50% of colon cancers demonstrate mutation in K-Ras, predominantly at codon 12 (4, 5), and a substitution of gly→val has been a frequent occurrence in oncogenic Ras protein of human carcinomas. Mutant p53 proteins have shown the ability to cooperate with the Ras oncogene in significantly enhancing cellular transformation (2, 6, 7), and mutation in K-Ras is one of the genetic mutations associated with oncogenesis and progression in colon carcinomas (1). Although it is evident that mutation in the K-Ras gene can confer a selective growth advantage to colon carcinoma cells, it remains unclear as to its functional significance in influencing the ability of these cells to undergo apoptosis in response to stimuli that induce DNA damage.

Deregulation of mechanisms that regulate apoptotic responses are involved in oncogenesis and progression of colon carcinomas. Point mutations in the p53 gene are known to reduce cellular sensitivity to agents that damage DNA (8, 9). Elevated expression of Bcl-2 has also been identified (10), which may extend cell survival due to inhibition of apoptosis (11), and may be associated with decreased anti-Fas sensitivity in addition to decreased Fas expression in tumors of this histotype (12). The role of expression of oncogenic K-Ras in influencing the ability of colon carcinoma cells to undergo apoptosis has been examined in the present study.

Previously, we derived a TS⁺-deficient mutant (TS⁻) from GCjc1 colon carcinoma cells that commits to thymineless death after dThd deprivation. Characteristically, these cells exhibit morphological evidence of apoptosis, and after DNA damage, undergo endonucleosomal cleavage of the DNA (13). This cell clone demonstrates heterozygosity in p53, thereby expressing one wt allele and one with an A→C point mutation at codon 240 (14). Apoptosis is regulated by functional p53 as demonstrated from the transactivation of p50-2 (a wt53-responsive chloramphenicol acetyltransferase reporter construct) and up-regulated expression of endogenous MDM2. DNA damage is linked to the apoptotic machinery of the cells via autocrine signaling through Fas/FasL interactions (15), and delayed apoptosis is associated with up-regulated expression of Bcl-2 (14). Recently, we determined from the sequencing of genomic DNA that TS⁻ cells express a normal K-Ras gene. After transfection of a K-Ras gene mutated at codon 12 (gly→val; Ref. 16), isolated clones demonstrated elevated expression of Ras, an enhanced GTP:GDP binding ratio, and elevated p44/42 MAPK activities, consistent with a functional oncogenic K-Ras protein.

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³ The abbreviations used are: TS, thymidylate synthase; dThd, thymidine; wt, wild type; MAPK, mitogen-activated protein kinase; RT-PCR, reverse transcription-PCR; Ab, antibody; MoAb, monoclonal Ab; IL, interleukin.
tance, however, was that the presence of oncogenic K-Ras conferred resistance to the induction of apoptosis after dThd deprivation. This correlated with elevated expression of the survival factor Bcl-xL, reduced expression of the regulator of deprivation. This correlated with elevated expression of the

MATERIALS AND METHODS

Cell Line. The cloned human colon adenocarcinoma cell line GC/c1 has been described previously (13). A TS-deficient mutant clone selected from GC/c1, TS-, deficient in Ts mRNA and protein, and auxotrophic for dThd has been well characterized (14). Cells were maintained as described previously (15) in the presence of 20 μM dThd.

Growth and Clonogenic Assays. For growth assays, cells were plated in six-well plates (Falcon) at a density of 100,000–200,000 cells/well. After overnight attachment, cells were enumerated at time 0 and 72 h using a Coulter particle counter.

For clonogenic assays, TS-, TS-pClneo or TS-K-Ras clones (nos. 45, 76, 78, and 83) were plated at a density of 3000 cells/well. After overnight attachment, cells were washed in 2 ml of HBSS before dThd deprivation by refeeding with dThd-free medium (15). Alternatively, cells were synchronized in G0 by leucine deprivation (leu- dThd+) for 3 days before dThd withdrawal (leu+ dThd-). At intervals for up to 5 days, cells were rescued with dThd (20 μM) added to individual wells, and clonogenic survival was determined 11 days after dThd restoration, as described previously (15).

Sequencing of K-Ras. Codons 12, 13, and 61 that harbor point mutations in K-Ras were examined by sequencing of PCR products derived from genomic DNA of TS cells according to published procedures (17). The primers used for examination of possible mutations at codons 12 and 13 (K1a and K1b) or codon 61 (K2a and K2b) to yield 162- or 128-bp products, respectively, were as follows, as described (17): K1a, 5'-GGCTGTGAAAATGACTGA-3'; K1b, 5'-GTTCGGACCCAGTAATATGC-3'; K2a, 5'-TTCTTACAGGAAGCAAGTAG-3'; and K2b, 5'-CAAGAAGAGCCGCTTTCCCA-3'.

Transfection of Oncogenic K-Ras. The pSW11-1 clone containing a mutated K-Ras gene subcloned into pBR322 was a gift from Dr. Robert Weinberg, the Whitehead Institute (16). The oncogenic K-Ras CDNA consisted of a 1.1-kb insert cloned from SW480 colon carcinoma cells and contained a g→a substitution at codon 12. Subsequently, the cDNA was subcloned into pClneo (obtained from Dr. Michael Dilling, St. Jude Children’s Research Hospital) and was used for the transfections described.

TS- cells were plated at a density of 400,000 cells/well in six-well plates. The following day, cells were washed with HBSS before overnight incubation with DNA (1 μg; pClneo or pClneo/K-Ras) and lipofectamine (Life Technologies, Inc.; 5 μl) in 1 ml of serum-free OPTI-MEM medium (Life Technologies, Inc.). The medium was subsequently replaced with RPMI containing 10% FCS, and cells were incubated for an additional 24 h before selection in G418. Clones were selected initially in 50 μg/ml G418, surviving clones were isolated, and the G418 concentration was subsequently increased to 300 μg/ml.

Level of Ras Expression. TS- clones transfected with oncogenic K-Ras or pClneo alone and selected in 300 μg/ml G418 were analyzed for the level of Ras expression by immunoprecipitation/Western analysis as described previously (14). Immunoprecipitation was effected overnight using a monoclonal v-H-Ras (Ab-1) agarose conjugate (Oncogene Science). After gel electrophoresis, detection of Ras protein was effected using a primary anti-H-Ras MoAb (Santa Cruz Biotechnology) and a secondary sheep anti-rat IgG-HRP Ab (Amersham), followed by detection using the ECL Western blotting system.

Detection of Guanine Nucleotides Bound to Ras. Determination of the amounts of GDP and GTP bound to Ras, and the elevation in the GTP:GDP ratio in TS- clones expressing oncogenic K-Ras, was conducted as described previously (18). Briefly, cells were starved in phosphate-free medium for 6 h, radiolabeled with [33P]Pi, overnight, and subsequently lysed in the presence of anti-Ras MoAb Y13-259 (Santa Cruz Biotechnology) for 30 min at 4°C. Cells were subsequently scraped, and nucleotides were removed from lysates by incubation for 1 h in a 10% charcoal slurry, followed by repeated centrifugation to remove the contaminating charcoal. Lysates were incubated with anti-rat IgG Agarose (Sigma), and the immunoprecipitates were washed, as described. Immunocomplexes were resuspended in 1 ml KH2PO4 (pH 3.4) and heated to 85°C for 3 min to elute associated guanine nucleotides, which were subsequently resolved by TLC on PEI-cellulose plates in 1 m KH2PO4. GTP and GDP bound to Ras were quantitated using a Molecular Dynamics PhosphorImager.

Activation of the Raf/MAPK Pathway. TS- clones demonstrating elevated Ras expression and enhanced GTP:GDP binding ratios were examined for activation of the Raf/MAPK pathway, which results in enhanced phosphorylation of MAPK (3, 19). Thus, the phosphorylation status of p44 and p42 MAPK was determined using the PhosphoPlus MAPK antibody kit (New England Biolabs), according to the manufacturer’s directions.

Expression of Fas and FasL. Fas expression was determined in cell extracts by a standard ELISA assay, which correlated with results from RT-PCR (15). The capture Ab used was a purified anti-human Fas MoAb (PharMingen), whereas the Ab used for detection was a biotin anti-human Fas MoAb DX2 (PharMingen). FasL expression was determined by RT-PCR as described previously (15). TS-, TS-pClneo, and TS- K-Ras clones were assayed at time 0 and at 72 h after dThd deprivation from G0-synchronized cultures.

Expression of MDM2, Bel-2, Bel-xL, Bax, Bak, and Bel-xS. The ability of TS cells to undergo apoptosis had been shown previously to correlate with functional wt p53 activity, as demonstrated by up-regulated expression of endogenous MDM2. In addition, because enhanced survival may be associated with expression of members of the Bcl-2 family that promote survival (Bcl-2 and Bcl-xL) or regulate cell death responses (Bax, Bak, and Bcl-xS), expression of these genes was also examined by immunoprecipitation/Western analysis at 72 h after release from G0 and dThd withdrawal, as described.
demonstrated elevated expression of the Ras gene in comparison with the PhosphoPlus MAPK antibody kit (New England Biolabs), according to the manufacturer’s directions.

RESULTS

Expression of Ras. When K-Ras derived from TS- cells was sequenced at codons 12, 13, and 61, all sequences were found to be wt. After transfection of a 1.1-kb cDNA of oncogenic K-Ras and selection in a final concentration of G418 of 300 μg/ml, 23 clones were isolated and expanded, and 12 clones were subsequently characterized. Six TS- clones transfected with pClneo alone were also derived. Five of the 12 TS- clones transfected with oncogenic K-Ras (nos. 45, 64, 76, 78, and 83) demonstrated elevated expression of the Ras gene in comparison with the TS-pClneo control clone (Fig. 1). TS- K-Ras clones (nos. 45, 76, 78, and 83) were subsequently evaluated for elevation in the GTP-GDP ratio bound to Ras or alternatively, for enhanced phosphorylation of p44/42 MAPK that would be indicative of the expression of a functional oncogenic K-Ras protein.

Because the elevation in the GTP-GDP binding ratio was anticipated to be ≤100% (18), a human rhabdomyosarcoma cell line transfected previously with and shown to express oncogenic K-Ras (Rh30K-Ras) was included as a positive control (Table 1). The GTP-GDP ratio is shown as a percentage increase in comparison with the same ratio derived in Rh30pClneo. Similarly, the percentage increase in GTP-GDP binding ratio in oncogenic K-Ras-transfected TS- clones was determined in comparison with TS-pClneo (Table 1). A 55% increase in the GTP-GDP binding ratio was observed in the Rh30K-Ras positive control, and in TS- K-Ras clones nos. 45, 78, and 83, the range of elevation in the binding ratio ranged from 38 to 59%.

Table 1 Percentage of increase in the GTP:GDP ratio bound to Ras

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% increase in GTP:GDP</th>
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<tbody>
<tr>
<td>Rh30K-Ras</td>
<td>55</td>
<td>1</td>
</tr>
<tr>
<td>TS K-Ras clones</td>
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</tr>
<tr>
<td>45</td>
<td>59</td>
<td>3</td>
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<tr>
<td>76</td>
<td>Not determined</td>
<td>3</td>
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<tr>
<td>78</td>
<td>43</td>
<td>3</td>
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<td>83</td>
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Thus, TS- clones transfected with oncogenic K-Ras that were used further had demonstrated expression of phosphorylated p44/42 MAPK.

Fig. 2 Activation of p44/42 MAPK in TS K-Ras clones expressing elevated Ras. Phosphorylation of p44 and p42 MAPK was determined using the PhosphoPlus MAPK antibody kit (New England Biolabs), according to the manufacturer’s directions.

Growth and Clonogenic Survival of Oncogenic K-Ras Transfectants. The growth of TS pClneo, and TS- clones nos. 45, 76, 78, and 83 expressing oncogenic K-Ras was compared with the growth of TS- wt cells. TS- demonstrated a doubling time of 32.6 h and TS-pClneo of 28.6 h. TS- clones nos. 45, 78, and 83 showed doubling times similar to that of TS- (32.0–35.2 h), whereas the doubling time of no. 76 was somewhat longer (39.6 h). These four clones were subsequently evaluated for the influence of dThd deprivation on the ability of these cells to undergo apoptosis in comparison with TS- and TS- pClneo.

Cells were plated as described in “Materials and Methods,” and the following day were exposed as asynchronous populations to dThd-deprived or dThd-replete conditions, or alternatively were synchronized in G0 for 3 days by leucine deprivation, followed by release from the block either in the presence or in the absence of dThd. Cells were rescued with dThd (20 μM) at intervals for up to 5 days, followed by determination of the influence on clonogenic survival (Fig. 3). Under both conditions, TS- and TS- pClneo lost 50% clonogenic potential in...
65 h after dThd withdrawal. However, in all four TS− K-Ras clones, considerably enhanced survival was demonstrated in the presence of oncogenic K-Ras. Thus, at 5 days after dThd withdrawal, 5–8% survival was demonstrated in TS− and TS− pClneo, and 53%–69% in all TS K-Ras clones.

Expression of Fas and FasL. Previously, we had demonstrated that the commitment of TS− cells to thymineless death was dependent upon Fas/FasL interactions and correlated with up-regulated expression of FasL when cells were undergoing apoptosis (15). Therefore, the influence of oncogenic K-Ras on the expression of Fas (Table 2) and FasL (Fig. 4) in two TS K-Ras clones demonstrating protection from thymineless death (nos. 45 and 78) was examined at time 0 and 72 h after dThd withdrawal from G0-synchronized cell populations and compared with TS− and TS− pClneo control clones. No difference in Fas expression was detected among the cell lines after dThd deprivation. Additionally, up-regulated expression of FasL was found in all four cell lines under these conditions, irrespective of the commitment to apoptosis.

Expression of Regulators of Cellular Survival or Death.

Expression of the survival factors Bcl-2 and Bcl-xL was examined in TS, TS− pClneo, and TS K-Ras clones (nos. 45, 76, 78, and 83) at time 0 and at 72 h after dThd withdrawal from G0-synchronized cells (Fig. 5). Although there was no elevation in the levels of expression of Bcl-2, Bcl-xL expression was up-regulated in the four TS K-Ras clones. Additionally, levels of expression of the cell death regulators Bax, Bak, and Bcl-xS were also examined. No expression of Bcl-xS was detected (data not shown). Bax expression remained relatively constant after dThd deprivation (Fig. 6). However, Bak was up-regulated at 72 h in TS− and TS− pClneo during apoptosis but was not elevated in any of the TS− clones expressing oncogenic K-Ras (Fig. 6). Thus, when the Bcl-xL:Bak ratio was compared at 72 h after dThd withdrawal, this ratio was elevated in all TS− clones expressing oncogenic K-Ras but not in control TS− and TS− pClneo clones (Fig. 7A). In contrast, comparison of the
Bcl-xl ratios at this time demonstrated a ratio >5 in T5" and T5"pcNeo but <1 in all T5"K-Ras clones (Fig. 7B).

**Functional Activity of p53.** Previously, we had demonstrated that the commitment of T5" cells to apoptosis after thymineless stress correlated with the functional activity of p53, as demonstrated from up-regulated expression of the endogenous p53-regulated gene MDM2 (14). However, after transfection of oncogenic K-Ras, MDM2 was not up-regulated in these clones at 72 h after dThd deprivation from G0-synchronized cultures was as the case for T5" and T5"pcNeo clones undergoing apoptosis at this time (Fig. 8). These data suggest that p53 was no longer functional under conditions of K-Ras activation that promoted survival in response to thymineless stress.

**DISCUSSION**

It is well established that Ras functions as a molecular switch for reentry of cells into the cell cycle between G0 and G1 by transducing extracellular growth stimuli into early G1 mitogenic signals (20, 21). Ras is also required in late G1 for the effective activation of G1 cyclin-dependent kinases, passage through the restriction point, and entry into S phase (22). p21ras functions as a signal transducer in a manner similar to other signal-transducing GTP-binding proteins (23, 24) and initiates a cascade of protein phosphorylation by cytoplasmic serine/threonine kinases including Raf and the MAPKs (25). When Ras is mutated, the GTPase activity is impaired, thereby causing a 10-fold decrease in GTP hydrolysis and an accumulation of the GTP-bound form (3).

The Ras pathway was originally believed to be a linear signaling cascade leading to the sequential activation of Raf, Mek, and p44/42 MAPK (26). However, more recent studies have demonstrated that effector pathways of Ras are highly divergent in mammalian cells (27, 28). In addition to the Raf/MAPK pathway, some small GTP-binding proteins are implicated in Ras-induced cellular transformation, including Rac, Rho, and Ral (29). Analyses of proteins that interact directly with Ras implicate several candidate Ras effectors, including phosphatidylinositol 3'-kinase (30) and RafGDS (31), which are independent of Raf/MAPK. Thus, Ras appears to function as a molecular switch for multiple downstream targets. It is not surprising, therefore, that activation of the Ras gene has promoted both apoptosis (32, 33) and survival (29, 34, 35) in different cell systems in response to various stimuli. Expression of oncogenic Ras in murine fibroblasts has conferred susceptibility to apoptosis after serum starvation (36), exposure to tumor necrosis factor α (37), or after down-regulation of protein kinase C (32). In contrast, expression of an activated Ras gene in IL-3-dependent hematopoietic cells suppressed apoptosis after IL-3 deprivation (29) and prevented apoptosis in intestinal epithelial cells grown in suspension (34). In addition, Ras can suppress c-Myc-induced apoptosis (38). These contrasting roles for Ras may ultimately be dependent upon the multiple signaling pathways that are linked to Ras function.

Both Raf/MAPK (29) and phosphatidylinositol 3’ (38, 39) kinases have been shown to promote survival responses after serum or IL-3 deprivation. In contrast, Ras-dependent apoptosis has correlated with activation of SAPK/JUNK (33) or suppression of cellular protein kinase C activity (32). In the present study to elucidate the role of expression of oncogenic K-Ras on the ability of colon carcinoma cells to undergo apoptosis in response to thymineless stress, expression of activated Ras conferred a survival response in T5"K-Ras clones under these conditions. These results are similar to those reported in hematopoietic cells after IL-3 withdrawal, consistent with activation of the Ras/Raf/MAPK pathway (33).

Previously, we had demonstrated that thymineless death in T5" cells was regulated by signaling via Fas, correlated with up-regulated expression of FasL, and was inhibited by Bcl-2 (15). However, under conditions of thymineless stress, Fas expression remained similar in T5", T5"pcNeo, and in T5"K-Ras clones under conditions where activated K-Ras promoted a survival response. Similarly, FasL expression was up-regulated in response to thymineless stress in all T5" clones expressing oncogenic K-Ras, suggesting that the mechanism of protection from apoptosis induced by thymineless stress was downstream of Fas and its interaction with FasL.

Previously, we had demonstrated that thymineless death in T5" cells was regulated by p53, that T5" cells that were the most resistant to induction of apoptosis after dThd starvation could sustain the accumulation of high levels of Bax, and that a delayed apoptotic response in a subclone of T5", Thy4, correlated with up-regulated expression of Bcl-2 and lack of functional p53 activity (14). Bcl-2 and Bax form homodimers and...
heterodimers, and it is through heterodimerization that survival responses are thought to be mediated (40). The relative Bcl-2: Bax ratios within cells and the balance of Bax homodimers to Bax/Bcl-2 heterodimers are considered to regulate the response to a toxic insult. More recently, expression of other homologous proteins in human cells including Bcl-xL, which can suppress apoptosis (41), and Bak, which is expressed in colon carcinoma cells and has death initiator function (42), have been identified. Ras-dependent pathways leading to both survival and cell death responses have also been associated with downstream regulation of effector molecules that promote either survival (Bcl-2 and Bcl-xL; Ref. 35) or cell death (Bcl-xS; Ref. 33). Thus, expression of the survival factors Bcl-2 and Bcl-xL, and the death-promoting factors Bax and Bak, known to be expressed in TS~ cells, were examined in TS~, TS~pClneo, and four TS~K-Ras clones before and 72 h after dThd deprivation. Of particular interest was that the survival response in TS~K-Ras clones did not correlate with elevated expression of Bcl-2 or decreased expression of Bax. Similarly, the apoptotic response in TS~ and TS~pClneo was independent of these two regulatory factors. However, in the TS~ clones expressing oncogenic K-Ras, the expression of Bcl-xL was up-regulated during the survival response but was not up-regulated in cells expressing wt K-Ras. When levels of Bak expression were determined, this death regulatory factor was elevated in TS~ and TS~pClneo when the cells were undergoing apoptosis but was not elevated in TS~K-Ras clones that were protected from apoptosis. These relationships were even more apparent when the ratios of Bak and Bcl-xL were compared. Data are consistent with those obtained in hematopoietic cells after transfection of an inducible activated Ras gene and IL-3 withdrawal (35). In this study, induction of oncogenic Ras expression resulted in the rapid up-regulation of both Bcl-2 and Bcl-xL, bringing the level of expression equivalent to that seen in growing cells. In addition, Bak expression was not affected, although the level of Bak expressed was not determined. In TS~K-Ras clones, the expression of oncogenic K-Ras appeared to negate the functional
activity of p53, as evidenced by lack of endogenous regulation of the MDM2 gene. It is well known that oncogenic Ras is involved in enhancing cellular transformation and hence may also activate cellular pathways that promote survival responses ultimately leading to deregulation of cellular processes that control apoptosis.

This study has demonstrated that expression of a K-Ras oncogene can significantly affect the ability of colon carcinoma cells to undergo apoptosis in response to thymineless stress. Thymineless death is the mechanism of cell killing associated with 5-fluorouracil in colon cancer and remains the most effective agent for therapy of this disease. The present study has established that mutation in the K-Ras gene in colon carcinoma cells is involved not only in providing a selective growth advantage to these cells but also in regulating the expression of downstream effector molecules that regulate the response of these cells to stimuli that damage DNA. We have established that the presence of oncogenic K-Ras reduces the ability of TS+ cells to undergo apoptosis after dThd deprivation. The presence of activated Ras may therefore influence the regulation of apoptosis in malignant epithelial cells treated with agents that target TS.

REFERENCES

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