Organic Anion Transporting Polypeptide 2B1 and Breast Cancer Resistance Protein Interact in the Transepithelial Transport of Steroid Sulfates in Human Placenta

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ABSTRACT:
The human placenta has both protective and nurturing functions for the fetal organism. Uptake and elimination of xenobiotics and endogenous substances are facilitated by various transport proteins from the solute carrier (SLC) and ABC families, respectively. A functional interaction of uptake and elimination, which is a prerequisite for vectorial transport across cellular barriers, has not been described for placenta. In this study, we examined expression of organic anion transporter (OAT) 4 (SLC22A11), organic anion transporting polypeptide (OATP) 2B1 (SLCO2B1, OATP-B), and breast cancer resistance protein (BCRP) (ABCG2) in human placenta (n = 71) because all three proteins are involved in transmembranal transfer of estrone 3 sulfate (E3S; metabolic product) and dehydroepiandrosterone sulfate (DHEAS; precursor molecule). On the mRNA level, we found a significant correlation of OATP2B1 and BCRP ($R^2 = 0.534; p < 0.01$) but not between OAT4 and BCRP ($R^2 = -0.104; p > 0.05$). Localization studies confirmed basal expression of OATP2B1 and apical expression of BCRP. To study functional interactions between OATP2B1 and BCRP, we developed a Madin-Darby canine kidney cell model expressing both transport proteins simultaneously (OATP2B1 and BCRP in the basal and apical membrane, respectively). Using this cell model in a transwell system resulted in a significantly increased basal to apical transport of both E3S and DHEAS, when both transporters were expressed with no change of transfer in the apical to basal direction. Taken together, these data show the potential for a functional interaction of OATP2B1 and BCRP in transepithelial transport of steroid sulfates in human placenta.

The hormonal system is essential for the maintenance of a wide variety of physiological functions. In addition to peptidic or amino acid-based structures, many hormones are steroids. Various tissues like the adrenal gland, the ovaries, the testes, or the placenta are involved in the synthesis of steroid hormones. However, not all express the entire set of proteins required for this process and therefore are dependent on precursor molecules. An interesting tissue in this context is the human placenta, which is involved in the production of gestagens and estrogens during pregnancy. This function is a pivotal prerequisite for maintaining pregnancy and for physiological functions such as fetal growth. Whereas the syncytiotrophoblast as an important functional cell in placenta expresses all the enzymes necessary for the production of progesterone, the placental estrogen synthesis depends highly on the supply of C-19 steroids because the 17β-hydroxylase/17–20-lyase enzyme is missing (Voutilainen and Miller, 1986; Kallen, 2004). An important source of these precursor molecules is dehydroepiandrosterone (DHEA) and its sulfate (DHEAS), which are produced in the fetal adrenal gland and are available in large amounts in the fetal circulation (up to 10 μM) (Mesiano and Jaffe, 1997). In the placenta, DHEAS undergoes deconjugation by steroid sulfatasises, which have been found in high levels in the syncytiotrophoblast (Salido et al., 1990). The resulting DHEA is used for the placental estrogen synthesis (Kallen, 2004). Another important steroid sulfate is estrone 3 sulfate (E3S), which is synthesized by the fetal liver (Lanoux et al., 1985). The E3S represents a metabolite of estrogen but can also function as a biological inactive pool for this hormone (Miki et al., 2002).

To fulfill their physiological roles, these substances have to enter the syncytiotrophoblast, and later the products of hormone synthesis have to exit the cell. The uptake of steroid sulfates into the syncytiotrophoblast has been characterized to be mediated by the organic anion transporting polypeptide (OATP) 2B1 and the organic anion transporting protein (OAT) 4, which have been described to transport DHEAS.

ABBREVIATIONS: DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; E3S, estrone 3 sulfate; OAT, organic anion transporter; BCRP, breast cancer resistance protein; MDCKII, Madin-Darby canine kidney cells; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; TBST, Tris-buffered saline/Tween 20.
and E3S. Both transporters are expressed in the fetal facing membrane of the syncytiotrophoblast, suggesting an important function in the uptake of fetal-derived sulfates (St-Pierre et al., 2002; Ugele et al., 2003).

Besides these uptake mechanisms, there are also several efflux transporters of the ABC family (e.g., P-glycoprotein, several multidrug resistance associated proteins, and breast cancer resistance protein (BCRP)), which are mainly expressed in the maternal facing membrane of the syncytiotrophoblast (Cordon-Cardo et al., 1990; Nakamura et al., 1997; Maliepaard et al., 2001; Zu Schwabedissen et al., 2006). At present, however, these efflux transporters are regarded as plasental mechanisms to protect the growing fetus against drugs or xenobiotics from the maternal circulation and to remove endogenous compounds from the fetal blood circulation (Young et al., 2003).

Aside from their protective function, these proteins may be involved in the regulation of the hormone synthesis by controlling the intracellular and fetal concentration of precursor molecules, like DHEAS and E3S, thereby interacting with various uptake transporters.

Although uptake and efflux transporter in human placenta are well characterized in terms of their individual function, information regarding their interaction on a functional level is scanty. Therefore, we performed the placental expression of BCRP and the two uptake transporters OATP2B1 and OAT4, which are characterized to transport steroid sulfates (Cha et al., 2000; Tamai et al., 2001; Suzuki et al., 2003a; Nozawa et al., 2004). Moreover, OATP2B1 and OAT4 are shown to be expressed in the basal membrane of the syncytiotrophoblast, whereas BCRP is localized in the apical maternal facing membrane (Maliepaard et al., 2001; St-Pierre et al., 2002; Ugele et al., 2003). We determined the mRNA expression of BCRP, OATP2B1, and OAT4 in a large sample of human placentas to examine a possible coregulation of BCRP and a corresponding uptake transporter. Moreover, we studied localization of OATP2B1 and BCRP in the placental syncytiotrophoblast using confocal laser scanning microscopy. Finally, to elucidate their functional interaction, we developed a model system based on OATP2B1, BCRP, and OATP1B2/BCRP-overexpressing Madin-Darby canine kidney (MDCKII) cells. Using this system, we analyzed the transepithelial transport of E3S, DHEAS, and 17β-estradiol glucuronide.

Materials and Methods

Cell Culture and Substances. After declaration of consent, human placenta tissue samples were obtained from women undergoing cesarean sections or normal birth.

MDCKII cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 2 mM l-glutamine, and 0.1% gentamicin and penicillin/streptomycin (0.5 U/ml and 0.5 μg/ml) and incubated on ice for 1 h under continuous stirring.

RNA Isolation and Analysis. RNA was isolated from 71 placentas (45 term, 26 premature placentas) using RNeasy Mini Extraction Kit (Qiagen, Carlsbad, CA) was used. For quantification of BCRP, OATP2B1, and 18S rRNA, cloned PCR products were used to prepare a dilution series and to calculate a standard curve.

Cloning and Transfection. The full-length OATP2B1 and BCRP cDNA were amplified using reverse-transcribed RNA from placenta (OATP2B1: forward primer, 5'-AGCTCAGCTGACCCAGCTATCGG-3'; reverse primer, 5'-AAAGGACTCGAGGATCTGCGTGGTGC-3'; BCRP: forward primer, 5'-CTGAGATCTCGCTGTGGTTAA-3'; reverse primer, 5'-TGATTGGYAGGTYACAGAAA-3') and cloned into the mammalian expression vectors pcDNA3.1 and pcDNA3.1/hygro (both from Invitrogen). After verifying the sequence by cycle sequencing and matching against the reference sequences (accession number AB026256.1 for OATP2B1 and AF098951.2 for BCRP), MDCKII cells were single-transfected with OATP2B1 and BCRP using the FuGENE 6 Transfection Reagent (Roche, Penzberg, Germany). Cells were selected for antibiotic resistance using 0.6 mg/ml hygromycin B (Invitrogen). Resistant cell clones were characterized for OATP2B1 and BCRP expression using Western blot analysis and immunofluorescence staining. Next, positive BCRP-transfected cells were transfected with pcDNA3.1 vector containing the OATP2B1 cDNA. Here, selection was performed using hygromycin B and neomycin together. Resistant cells were characterized for OATP2B1 and BCRP as described above.

Preparation of Apical and Basal Membrane Fractions. Apical and basal membrane fractions were from two term and two preterm placentas were isolated and characterized as described previously (Grube et al., 2005; Meyer zu Schwabedissen et al., 2005). In brief, 20 g of placental cotyledons were washed with ice-cold phosphate-buffered saline (PBS) and reduced to small pieces. Then, tissue was homogenized in incubation buffer (250 mM sucrose, 10 mM Tris HCl, pH 7.4) supplemented with protease inhibitors (100 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.3 μg/ml pepstatin, and 0.1 μM pepstatin) by 20 strokes (1000 rpm) using the Potter S homogenizer (B. Braun Biotech, Melsungen, Germany) and incubated on ice for 1 h under continuous stirring. After centrifugation at 9000 g, the supernatant was centrifuged at 100,000 g, and the resulting membrane pellets were resuspended in incubation buffer and homogenized with a loose-fitting Dounce B homogenizer. For separation of basal and apical membranes, MgCl2 was added to a final concentration of 10 mM and centrifuged at 2000 g after 10-min incubation on ice. The separated membranes, apical fraction (supernatant) and the basal fraction (pellet), were resuspended in incubation buffer and homogenized with a tight-fitting Dounce B homogenizer. After centrifugation at 100,000 g, the membranes were homogenized using the tight-fitting Dounce B homogenizer and centrifuged at 100,000 g. Then, the pellets of both membrane fractions were resuspended in 1 ml of homogenization buffer, frozen, and stored in liquid nitrogen. An aliquot of the resuspended membrane fractions was used to determine protein concentration by the bichinchonic acid method.

Immunoblot Analysis. Crude membrane fractions were loaded onto a 10% SDS-polyacrylamide gel after incubation in sample buffer at 95°C for 10 min. Immunoblotting was performed using an ice-cooled tank blotting system (Bio-Rad, Hercules, CA) at 370 mA for 1.5 h. After a protein control staining with PonceauS, the blots were blocked overnight with 5% milk in Tris-buffered saline/0.05% Tween 20 (TBST) at 4°C. The OATP2B1 and BCRP
primary antibodies were diluted in TBST containing 5% bovine serum albumin, and the incubation of the blots took place for 2 h at room temperature. The plots were washed with TBST (3 × 5 min and 2 × 10 min) and blocked for another hour with 5% bovine serum albumin in TBST at room temperature. Secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (Bio-Rad) was used at a 1:2000 dilution (2 h, room temperature). Finally, the immobilized antibodies were stained using an enhanced chemiluminescence system (Amersham Biosciences, Freiburg, Germany) and exposed to X-ray films.

**Immunofluorescence Microscopy.** Protein localization was investigated by confocal laser scanning immunofluorescence microscopy in the human placenta and in overexpressing cell lines. For the placental staining, 5-µm cryoslices were used. After drying at 37°C on a heating plate for 10 min, sections were fixed in 100% ethanol at −20°C for 10 min and then washed in PBS (pH 7.3). Blocking was performed in 5% fetal calf serum in PBS for 45 min, followed by incubation with the primary antibodies at 4°C overnight. After three washes with PBS, the sections were stained. OATP2B1 staining with a polyclonal antibody (rabbit, dilution 1:200) was performed as described before (Grube et al., 2005). For BCRP staining, the monoclonal mouse antibody BXP-21 (dilution 1:25) (Alexis Biochemicals, Gruneberg, Germany) was used. The detection was carried out with fluorescence labeled secondary antibodies Alexa Flour 488 IgG (anti-mouse, dilution 1:100) and Alexa Flour 568 IgG (anti-rabbit, dilution 1:200) (Molecular Probes, Eugene, OR). Nuclei counterstaining with TOTO-3-iodide dye (dilution 1:1000) (Molecular Probes) was performed within the Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA).

For staining of the transfected cells, cells were cultured on cover slides and grown to confluence. Before fixation, cells were incubated for 24 h with 2.5 mM butyrate to induce protein expression. After washing with PBS, cells were fixed for 10 min with ethanol (100%); next, cells were washed with PBS again before permeabilization with Triton-X100 (0.1%) for 5 min. After washing with PBS, cells were blocked with 5% fetal calf serum and processed as described above.

**Transport Studies.** Transport studies were performed in a 24-well transwell system. Nontransfected, OATP2B1-, BCRP-, and OATP2B1/BCRP-transfected MDCKII cells were seeded in transwell plates and cultured to confluence. One day before the experiment, the medium in the apical and basal compartments was changed, and cells were incubated with 2.5 mM butyrate for 24 h. The transepithelial transport of radiolabeled E3S, DHEAS, 17β-estradiol glucuronide, and inulin was performed by adding 300 and 200 µl of incubation buffer (140 mM NaCl, 5 mM KCl, 1 mM KH2PO4, 1.5 mM CaCl2, 5 mM glucose, and 12.5 mM Hepes, pH 7.3) to the basal and apical compartments, respectively. Radiolabeled substances were added to the apical compartment to determine apical to basal transport and to the basal compartment to study the basal to apical transport (in each case 1 µCi/ml incubation buffer). The inulin transport was performed to determine the leakage of the cell layer; therefore, inulin was added to the basal compartment (0.4 µCi/ml incubation buffer). After 0.5, 1, 2, and 4 h of incubation, 10 µl were taken from the opposite compartment and mixed with 2 ml of scintillation mixture (Rotiszint, Roth, Karlsruhe, Germany) and measured in a scintillation β-counter (type 1409, LKB-Wallac, Turku, Finland).

**Statistical Methods and Software.** Values are represented as mean ± S.D. Graphs and calculation were prepared using Microsoft Excel (Microsoft, Redmond, WA), GraphPad Prism 3.0 (GraphPad Software, San Diego, CA), and SPSS (SPSS, Chicago, IL) software. Student’s t test and Mann-Whitney U test were performed to determine statistical significance. For correlation analysis of OATP2B1, OAT4, and BCRP, the Pearson test was used. Differences were considered significant at p < 0.05. Three-dimensional analysis of the transfected cells was done using the Volocity software (Improvision, Lexington, MA).
Results

OATP2B1 and BCRP mRNA Expression. Expression of OATP2B1, OAT4, and BCRP mRNA and 18S rRNA was determined in all the placenta samples using real-time PCR technique. Expression of all three transport proteins was normalized to 18S rRNA levels for further calculations. In all 71 samples, mRNA of OATP2B1 and BCRP could be detected with similar levels; however, there was a wide interindividual range [OATP2B1/18S: median = 4.5 × 10^{-6} (25% and 75% percentile: 2.0–8.6 × 10^{-6}); OAT4/18S: median = 11.5 × 10^{-6} (25% and 75% percentile: 11.0–12.1 × 10^{-6}); BCRP/18S: median = 4.1 × 10^{-6} (25% and 75% percentile: 2.1–7.9 × 10^{-6})]. The expression of OATP2B1 and BCRP exhibited a log-normal distribution, whereas mRNA levels of OAT4 were normally distributed (Fig. 1A). Moreover, BCRP and OATP2B1 mRNA expression levels were positively correlated (correlation: 0.534, p < 0.01), an observation missing between BCRP and OAT4 (correlation: −0.104, p > 0.05) (Fig. 1B).

Localization of OATP2B1 and BCRP. Localization of both OATP2B1 and BCRP was studied by Western blot analysis in enriched apical or basal placental membrane fractions and immunofluorescence on placenta slices. The results of the Western blot analysis showed a strong signal in the enriched apical membrane fractions and weak band in the basal one for BCRP (72 kDa); for OATP2B1 (84 kDa), the Western blot exhibited the opposite result. These signals were comparable with the expected bands in the respective overexpression cells (Fig. 2).

The immunofluorescence staining confirmed the expression of both transporters in the placental syncytiotrophoblast, revealing an apical localization of BCRP and a basal one for OATP2B1. The nuclei counterstaining (blue fluorescence) indicates the localization of the multinuclear syncytiotrophoblast (Fig. 3, A–F).

The staining of BCRP-, OATP2B1-, or BCRP/OATP2B1-transfected MDCKII cells also shows a localization of BCRP in the apical and OATP2B1 in the basolateral membrane of the MDCKII cells (Fig. 3, G–I).

Transepithelial Transport Studies. The characterization of the transfected cells with regard to the results of the Western blot analysis and immunofluorescence staining was already mentioned. Using these four cell lines in a transwell system, transport studies with E3S, DHEAS, and 17-estradiol-glucuronide were performed. For all the experiments, inulin transport measurements were carried out to make sure that there was no leakage. Cells were considered to be tight if the inulin transport was less than 1% per hour (data not shown). All three substances—E3S, DHEAS, and 17β-estradiol-glucuronide—were tested for apical to basal and basal to apical transport. For all three compounds, neither a remarkable apical to basal nor a basal to apical transport could be observed in nontransfected MDCKII during 4 h of incubation. Whereas a transport of 17β-estradiol-glucuronide was also not observed in the other three cell lines, all the transfected cells showed an altered transport of E3S and DHEAS. With regard to E3S,
there was no substantial difference between the apical to basal or vice versa transport in the OATP2B1-transfected cells; however, the transport velocity in both directions was much higher compared with nontransfected and BCRP-transfected ones. In contrast, the OATP2B1/BCRP double-transfected cells exhibited a strongly enhanced basal to apical transport of E3S, which was 2.1-fold higher compared with OATP2B1-transfected and more than 11-fold compared with the nontransfected and BCRP-transfected cells after 4 h of incubation. Moreover, the double-transfected cells showed nearly no apical to basal transport compared with the control cells (ratio of basal to apical transport after 4 h: MDCK, 1.60; OATP2B1-transfected, 1.56; BCRP-transfected, 0.47; and OATP2B1/BCRP-transfected, 0.04). The observations for the DHEAS transport were similar to the results for E3S (Fig. 4).

### Discussion

In the present study we investigated expression and function of the uptake transporters OATP2B1 and OAT4, as well as the efflux transporter BCRP, in human placenta and examined functional interaction of OATP2B1 and BCRP in an in vitro system.

The mRNA expression of all the proteins could be detected in each placenta sample; however, the mRNA levels of OATP2B1 and BCRP show wide interindividual variability following a log-normal distribution for both proteins. In contrast, the expression of OAT4 shows a normal distribution. Unlike OAT4, the mRNA expression levels of OATP2B1 and BCRP show a significant correlation, thereby indicating a coregulation or interaction of both genes on the transcriptional level. Whereas regulatory effects for BCRP expression have been described (e.g., a regulation through placental hormones like progesterone and estradiol or the epithelial growth factor) (Wang et al., 2006; Zu Schwabedissen et al., 2006), data on regulation of OATP2B1 expression have not been reported.

In addition to these transcriptional studies, we examined OATP2B1 and BCRP on protein level. Here, a strong expression for BCRP was present in the apical membrane of the placental syncytiotrophoblast, whereas OATP2B1 could be detected in the basal membrane. These results were confirmed both by Western blot analysis and immunofluorescence microscopy and are in line with findings of previous studies (Maliepaard et al., 2001; St-Pierre et al., 2002). The localization of OATP2B1 to the fetal and BCRP to the maternal membrane of the syncytiotrophoblast and the positive correlation of both transporters on mRNA expression level indicated a functional coupling. Such cooperation would be of functional importance because compounds like sulfate conjugates from the fetal circulation could enter the syncytiotrophoblast via OATP2B1 and are subsequently eliminated into the maternal blood by BCRP. To test this hypothesis, we developed a BCRP/OATP2B1-overexpressing MDCKII cell line and respective controls, showing a syncytiotrophoblast-like localization of OATP2B1 and BCRP. Therefore, this in vitro model may simulate the placental syncytiotrophoblast with regard to the expression and localization of BCRP and OATP2B1. In addition, a system like this can be used to study transporter-mediated transepithelial transport processes (Cui et al., 2001). Using this model we could show a strongly increased basal to apical transport of the sulfated steroids E3S and DHEAS in BCRP/OATP2B1 double-transfected cells, whereas non- or BCRP-transfected cells showed almost no transport. The expression of OATP2B1 alone resulted in an increase in basal to apical and apical to basal transport. In contrast, for glucuronide conjugates like 17β-estradiol-glucuronide, which is transported by BCRP but not by OATP2B1 (Tamai et al., 2001; Chen et al., 2003; Suzuki et al., 2003a), neither an enhanced basal to apical nor an apical to basal transport could be observed. Taken together, these findings show a functional interaction between the uptake transporter OATP2B1 and the ABC transporter BCRP in transepithelial transport of E3S and DHEAS. Moreover, our results indicate that the expression of an efflux transporter like BCRP is necessary to get a directional transport across this epithelium because OATP2B1 alone seems to work in both directions; this observation is in line with previous reports of functional coupling of transport proteins (Cui et al., 2001; Kopplow et al., 2005).

With regard to the placenta, these results are of potential physiological relevance because of the important role of this organ in steroid hormone synthesis during pregnancy (for review, see Kallen, 2004). In this context, the placenta is strongly dependent on the uptake of precursor molecules like DHEAS for the estrogen synthesis as the placenta is lacking the 17β-hydroxylase/17–20-lyase (CYP17) (Voutilainen and Miller, 1986). Therefore, a possible source of DHEAS is the fetal adrenal gland (Seron-Ferre et al., 1978). There are some data supporting the idea that the uptake of this molecule to the placental syncytiotrophoblast as hormone synthesizing cell is mediated by the organic anion uptake transporters OATP2B1 and OAT4. Both proteins are localized in the basal, fetal facing membrane of the syncytiotrophoblast (St-Pierre et al., 2002; Ugele et al., 2003). Moreover, DHEAS, which is converted to estradiol by the aromatase pathway, represents the main source of precursor molecules for estrogen synthesis. E3S is another endogenous precursor molecule for estradiol synthesis (Santé et al., 1986). E3S represents the major circulating plasma estrogen and can be formed by sulfation of estrone (Falany, 1997). The formation of the sulfate is mediated by the estrogen

![Fig. 4. Transepithelial transport studies using nontransfected, BCRP-, OATP2B1-, or double-transfected MDCKII cells in a transwell system. Cells were incubated with incubation buffer containing 1 μCi/ml of the respective tritium-labeled substance in the apical or basal compartment, and apical to basal (dotted line) and basal to apical (continuous line) transport was determined. After indicated times, 10 μl of the incubation buffer was taken from the opposite compartment, and radioactivity was determined. The data represent the calculated concentration of the respective substance (E3S, DHEAS, and 17β-estradiol-glucuronide) (mean ± S.D., n = 3).](image-url)
sulfotransferase, whereas the desulfation toward a biological active estrogen is catalyzed by the steroid sulftaase. Again, both enzymes are expressed in the syncyiotrophoblast of the placenta (Miki et al., 2002; Suzuki et al., 2003b). In this context, the expression of an uptake transporter like OATP2B1 in the basal membrane of the syncyiotrophoblast seems to be responsible for the uptake of fetal-derived sulfated precursor molecules (E3S and DHEAS) for the steroid synthesis. The apical expression of BCRP, however, may be involved in the elimination of sulfates like DHEAS or E3S from the fetal circulation into the maternal blood. In addition, BCRP may regulate the placental estrogen synthesis by modifying intracellular E3S concentration, which may function as a pool for inactive estrogen. In this context, it is interesting that BCRP accepts E3S but not estrogen as substrate (Imai et al., 2003). The BCRP expression may also regulate the availability of maternal DHEAS as a precursor for the placental estrogen synthesis, which in return points to the importance of the fetal DHEAS for the steroid hormone synthesis. Finally, the apical BCRP expression may protect the fetal organism from maternal E3S and xenobiotics (Allen and Schinkel, 2002).

Relevance of this functional cooperation is not restricted to the placenta because OATP2B1 and BCRP mRNA are expressed in significant levels in many tissues like liver, intestine, mammary gland, ovary, and testes (Tamai et al., 2000; Maliepaard et al., 2001; Pizzagalli et al., 2003).

In conclusion, our data show an interaction between OATP2B1 and BCRP on an expression and functional level. Moreover, we have evidence that vectorial transport of substrates of OATP2B1, which is mainly expressed in the basal membrane of the syncyiotrophoblast, requires coexpression of an efflux transporter like BCRP in the corresponding apical membrane.

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References

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