Studies on human porin: XIII. The Type-1 VDAC 'Porin 31HL' biotinylated at the plasmalemma of Trypan blue excluding human B lymphocytes

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Abstract In 1989, we demonstrated for the first time the expression of the VDAC 'Porin 31HL' in the plasmalemma of human B lymphocytes, then giving first evidence of a multi-topological expression of VDAC. Meanwhile, data from this and other laboratories support our proposal of a multi-compartment distribution of the channel in mammalian tissues. Here, by biotinylation of plasmalemma-integrated proteins of proven living B lymphocytes, followed by two-dimensional electrophoresis, immuno- and streptavidin affinity blotting, we show that part of the channel molecules can be labelled at the outer membrane of the cells. Thus, by a relevant approach our results invalidate objections concerning putative cross-reactivity of anti-human Type-1 porin antibodies with non-VDAC proteins at the outer cell membrane.

Key words: 'Porin 31HL'; VDAC; Chloride-channels; Biotin; Two-dimensional electrophoresis

1. Introduction

In 1989, by cytotoxicity tests and indirect immuno-fluorescence microscopy, we documented the localization of the voltage-dependent anion-selective channel (VDAC) [1,2] 'Porin 31HL' in the plasmalemma of transformed human B lymphocytes and the accessibility of its N-terminus at the outer cell surface. We then applied antisera against mature 'Porin 31HL' and synthetic peptides of the N-terminal part of the molecule [3]. As a basis, we had purified 'Porin 31HL' from crude B lymphocyte membranes. Sequence analysis proved the protein preparation to be homogeneous and furthermore resulted in the first complete primary structure of VDAC in the animal kingdom [3,4].

Meanwhile, support came from the preparation and characterization of eight mouse monoclonal anti-'Porin 31HL' antibodies: all eight monoclonals are directed against the N-terminal part of the channel molecule [5], they furthermore discriminate between synthetic peptides of the N-terminal region of Type-1 and Type-2 human porin [6–11]. The application of these monoclonal antibodies on normal human T and B lymphocytes and several other mammalian cells by this and other laboratories on the level of light and electron microscopy validated the multi-topological expression of Type-1 mammalian porin [5,12–17]. Recent data on the co-purification of VDAC with the $GABA_A$ receptor [18] are in agreement with our observation on the expression of VDAC in the plasmalemma. Microsequencing of proteins from caveolae of mammalian lung epithelium showed the expression Type-1 in this outer cell membrane compartment [19]. These data were recently reviewed [20,21].

Here, we show by labelling the proteins at the surface of proven living B lymphocytes with Sulfo-NHS-Biotin that Type-1 human porin is found in the cytoplasmic membranes of the cells. Plasmalemma-integrated human porin was detected after two-dimensional electrophoresis by immuno- and streptavidin affinity-blotting techniques. The data were presented at the 39th Annual Meeting of the Biophysical Society of the USA in San Francisco, CA, February 12–16, 1995.

2. Materials and methods

2.1. Cells

Acute lymphoblastic leukemia cells (KM3), grown in RPMI 1640 medium supplemented with 10% fetal calf serum were washed 2 times in phosphate-buffered saline (PBS: 0.137 M NaCl, 0.002 M KCl, 0.008 M Na_2HPO_4, 0.008 M KH_2HPO_4, pH 7.4) and resuspended at 10^8/ml in PBS. Light microscopy with Trypan blue exclusion was used to control viability of the cells before biotinylation.

2.2. Biotinylation

To each ml of cell suspension 200 μl of 20 mM Sulfo-NHS-Biotin (Pierce) in PBS, pH 7.4, was added, and pH was adjusted immediately to 7.4 with Na_2HPO_4 (0.008 M Na_2HPO_4, 0.137 M NaCl, 0.002 M KCl, pH 9.0). The reaction was allowed to proceed for 30 min at 4°C with slow rotation, afterwards cells were washed 3 times with PBS [22–25]. To control labelling results and the viability of the cells, a 100 μl aliquot was incubated for 30 min at room temperature with 25 μg streptavidin-FITC-conjugated (Pierce) as a specific ligand to visualize biotin. After washing 3 times in PBS, the cells were examined by fluorescence microscopy at 540 nm.

2.3. Solubilization

Each ml of labelled cell suspension, containing 10^8 cells, was solubilized with NP40 containing buffer (1% NP40, 1 mM EDTA, 50 mM NaCl, pH 8.0) and 10 μl PMSF protease inhibitor per ml. Suspension was gently mixed for 30 min at 4°C, and lysed cells were centrifuged at $200 \times g$ for 10 min to remove cell debris. The supernatant was then ultracentrifuged at $100{,}000 \times g$ for 30 min at 4°C and the finally resulting supernatant was retained for further analysis.

2.4. Electrophoresis

Proteins were separated by two-dimensional electrophoresis [26,27]

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Abbreviations: VDAC, voltage-dependent anion-selective channel; 'Porin 31 HL', 31 kDa human lymphocyte porin; NHS, *N*-hydroxysulfosuccinimide-ester of biotin; NEPHGE, non-equilibrium pH gradient gel electrophoresis; SDS-PAGE, sodium-dodecylsulfate polyacrylamide gel electrophoresis; FITC, fluoresceinisothiocyanate; PMSF, phenylmethylsulfonylfluoride; EDTA, ethylenediaminetetraacetic acid; PVDF, polyvinylidendifluoride; BSA, bovine serum albumine; HRP, horseradish peroxidase.

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according to isoelectric point by NEPHGE in the first dimension, and according to molecular weight by SDS-PAGE in the second dimension.

Protein precipitate from 100 μl biotinylated solubilized cells (10^7 cells) was dissolved in 40 μl lysis buffer and loaded to NEPHGE gel tube (8 M urea, 2% NP40, ampholines 3.5–10) after short incubation at 80°C. First dimension was run for 6 h at 400 V. NEPHGE gel was equilibrated 2 × 30 min in SDS-PAGE sample buffer for the second dimension and loaded to 12% Laemmli gel for SDS-PAGE, which was run for 4 h at 40 mA.

2.5. Western transfer

SDS-PAGE gel from the second dimension was blotted onto two PVDF membranes (Millipore) simultaneously. They were marked by pricking with a needle through both membranes in a definite pattern to make sure, that they later could be aligned upon another exactly in the same position they had during blotting procedure. Western transfer was performed in a semidry system between horizontal graphite electrodes (Biometra) with a discontinuous buffer system for 40 min at 1 mA/cm² gel. After the blotting procedure reversible Ponceau-S staining was used to control protein transfer and the membranes were blocked at room temperature with Tris-buffered saline (TBS: 0.025 M Tris, 0.15 M NaCl, pH 7.4) containing 3% BSA and 0.01% sodium azide.

2.6. Protein visualization

After washing 3 times in TBS each membrane underwent a different procedure for specific protein visualization. At the first membrane the high affinity of biotin to its specific non-covalent ligand streptavidin (K_d 10^{15} M^{-1}) was used to detect the biotinylated proteins of the preparation. The membrane was incubated with streptavidin-HRP-conjugated (Pierce) 1:1000 in TBS for 1 h at room temperature, washed 3 times with TBS and developed with chloronaphthol and H_2O_2 as substrates for the peroxidase enzyme reaction. At the second membrane the specific binding of monoclonal mouse immunoglobulins against human Type-1 'Porin 31HL' [5,6] (four of them available by Calbiochem-Novabiochem Gmbh) was used to detect the Type-1 porin molecules of the preparation. The antibodies recognize the acetylated N-terminus (11 amino acids) and discriminate Type-1 from Type-2 porin [5,6,7]. The membrane was incubated for one hour with 1:500 antibody/TBS solution containing 1% BSA, 0.01% sodium azide. After washing in TBS, secondary rabbit anti-mouse antibodies, diluted 1:350 in TBS, 0.01% sodium azide, were coupled to primary antibodies for one hour. HRP reaction was visualized with chloronaphthol/H_2O_2 in TBS.

2.7. Detection of biotinylated Type-1 porin

After visualization of protein patterns the membranes were screened for protein spots which have exactly the same position on each membrane, i.e. they react with streptavidin as well as with mouse anti-human porin antibodies. To find the positions, where these spots overlap, the membranes were scanned into a computer, printed onto transparent foils and aligned upon one another. The pattern, which was pricked through the membranes before Western transfer was used for exact alignment.

3. Results

The fluorescence microscopy examination of the streptavidin-FITC incubated, biotinylated cells showed complete biotin labeling (Fig. 1). The samples showed no background fluorescence, thus there was good contrast to identify specific biotin binding. Biotin labelled sites were seen in a continuously

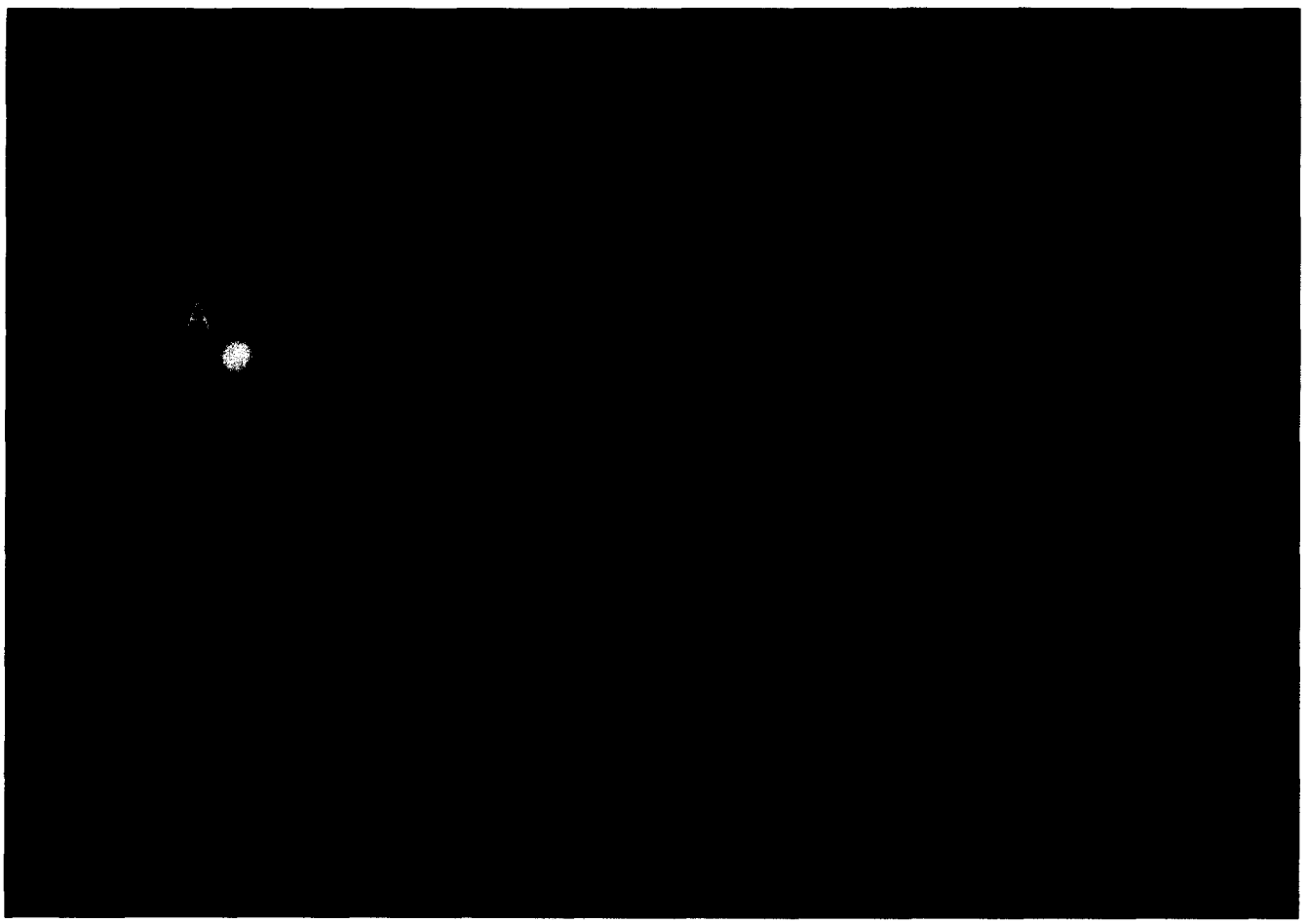

Fig. 1. Fluorescence light micrograph to control biotin labelling. Biotinylated cells were incubated with streptavidin-FITC to visualize the biotin labelling pattern. Notice the continuous labelling pattern and the high viability of cells. (A) Dead cell.

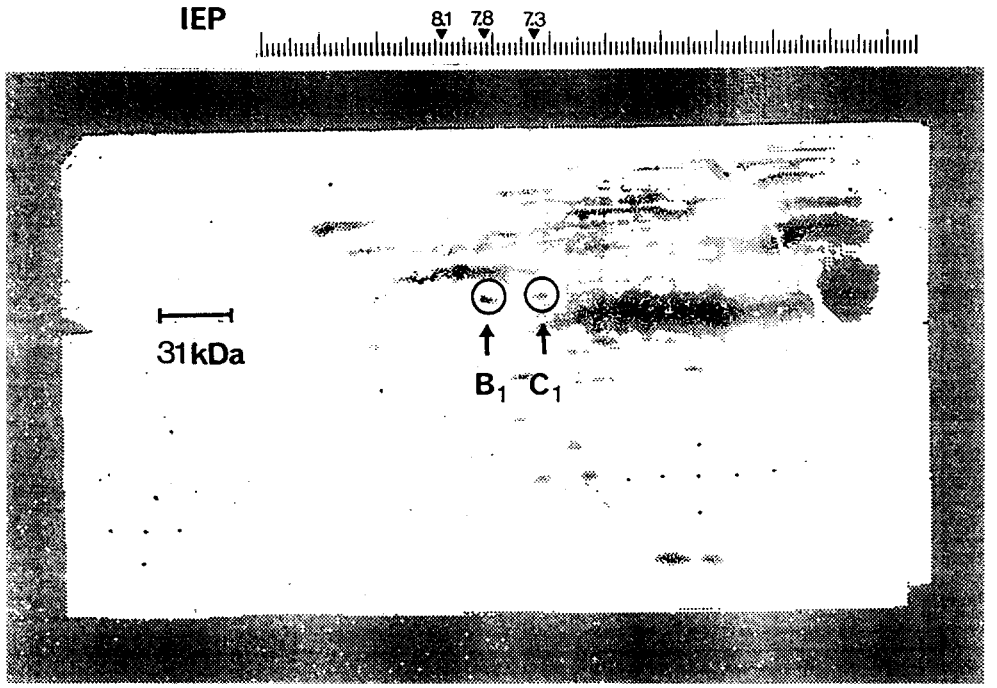

Fig. 2. Printed scan of a streptavidin affinity blot. 'Porin 31HL' was biotinylated at the plasmalemma of human B lymphocytes as visualized by streptavidin-HRP after Western transfer. Notice the two spots at 31 kDa and IEP 7.8 (B_1) and 7.3 (C_1).

fluorescing, thin layer at the outer plasma membrane. The biotin-ester cannot permeate the membrane of living cells. Only few cells, which were not viable enough to exclude the biotin-ester, showed intensive fluorescence of the whole cell body, which indicates biotin labeling of intracellular proteins. We did three experiments, the number of cells showing biotin penetration in any case was less than 4/100.

Evaluating each run, electrophoresis in the first dimension showed good separation of reference pI marker proteins. The NEPHGE gels built up stable pH gradients within the range of 4.5–9.0. After second dimension and Western transfer the streptavidin-HRP treated membranes showed good protein separation according to isoelectric point (IEP) as well as molecular weight (Fig. 2). The mouse anti-Type-1 human porin anti-

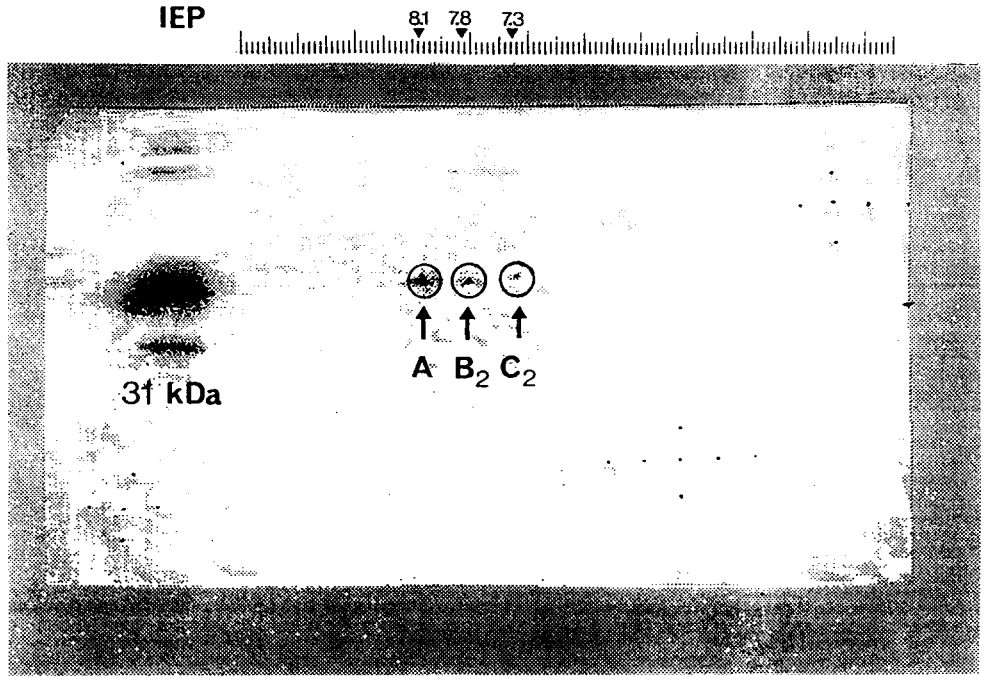

Fig. 3. Printed scan of a mouse anti-Type-1 human porin immunoblot. The second membrane, onto which two-dimensionally seperated proteins of biotin-labelled cells were simultaneously blotted, was treated with monoclonal mouse anti-human porin antibodies. It shows one more spot at 31 kDa and IEP 8.1 (A), which cannot be seen at the streptavidin blot (Fig. 2).

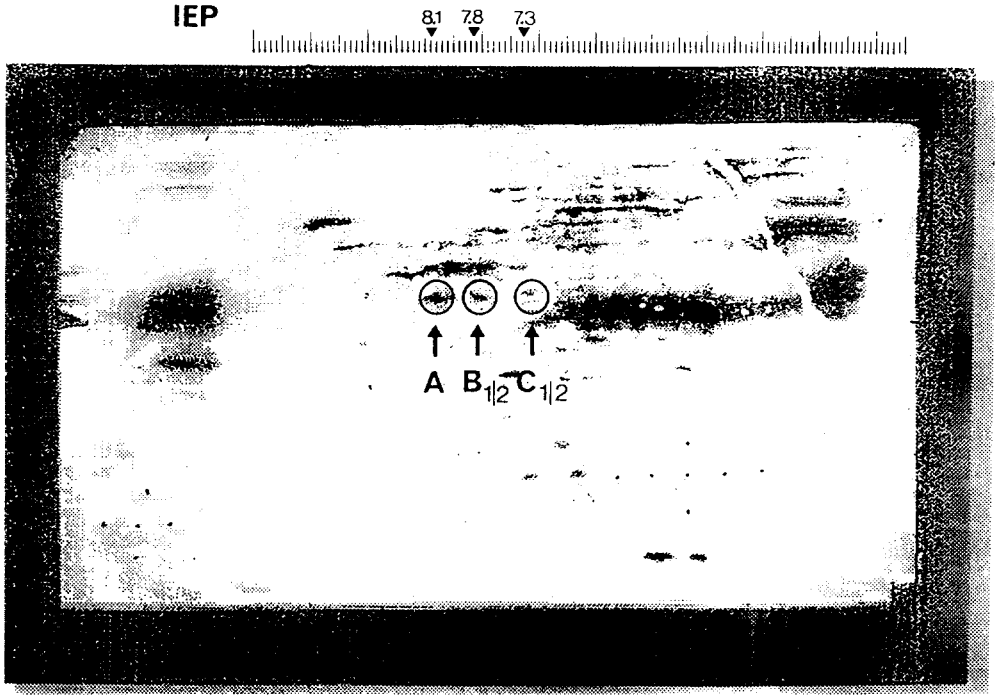

Fig. 4. Synopsis of Figs. 2 and 3. The membranes were transparently printed and aligned upon another. The spots B_1 and C_1 from the streptavidin blot (Fig. 2) show overlapping ($B_{1/2}$, $C_{1/2}$) with B_2 and C_2 from the anti-human porin blot (Fig. 3).

body treated second membranes showed the characteristic two-dimensional pattern for 'Porin 31HL' (Fig. 3). The anti-Type-1 VDAC antibody labelled protein spots were at the molecular weight of 31 kDa and extended over a pH range of 7.3–8.1 (Fig. 3; A, B_2, C_2), as expected for mammalian porin [4,28], indicating that the antigen–antibody reaction is not influenced by biotinylation. The antibody labelled human porin spots, showing no biotinylation at the streptavidin treated membrane, i.e. intracellular porin, were at the more basic IEP (Fig. 3; A). The two biotinylated spots were seen at the more acid isoelectric points as expected by forming of biotin-esters with NH_2-residues (Fig. 2; B_1, C_1; Fig. 3; B_2, C_3).

The computer-scanned and transparently printed membranes showed two spots which were exactly at the same position on each membrane in the aligned foils (Fig. 4; $B_{1/2}$, $C_{1/2}$), representing spots that react with streptavidin-HRP as well as with mouse anti-human-porin antibodies. One of the three anti-Type-1 human porin antibody labelled spots, lying at basic IEP (Fig. 3; A) was not seen at the streptavidin blot (Fig. 2), thus it was not biotinylated.

4. Discussion

By two-dimensional electrophoresis we show that part of the Type-1 VDAC 'Porin 31HL' of highly viable human B lymphocytes can be biotinylated at the outer surface of these cells. This proves that the channel is expressed in their plasmalemma. The procedure of biotinylation ensures that proteins, identified at Western blot membranes by streptavidin, are outer cell membrane integrated. Thus, spots reacting with streptavidin as well as with monoclonal anti-Type-1 human porin antibodies [5,6] represent plasmalemma-integrated Type-1 VDAC. Contamination by biotinylated proteins from inside of subvital cells, which were less than 4% in our preparations, is rather unlikely to cause the reaction at the streptavidin membrane. Densitometric scanning of the anti-Type-1 human porin treated membrane showed, that the two spots which can also be seen at the streptavidin membrane are actually more than 20% of the total VDAC detected (data not shown). However, it has to be mentioned that the spots at the membrane labelled by anti-human porin antibodies cannot be compared directly to those at the streptavidin membrane to give quantitative relations: first, the proteins after two-dimensional electrophoresis were blotted simultaneously onto two membranes; second, detection systems with different affinities and sensitivities were afterwards applied to each of the blotting membranes.

In addition we analyzed glycoprotein vesicles of B lymphocytes purified by lectin affinity chromatography which support our biotinylation data. Here, in relation to whole cell and crude membrane fractions this preparation shows significant enrichment of plasma membrane VDAC. Simultaneously, there is an enrichment of plasmalemma-integrated HLA-DR molecules in the vesicle preparation (Reymann, in preparation). Correspondingly, microsequencing of proteins of caveolin-rich plasma membrane vesicles from lung epithelium revealed Type-1 porin in those plasma membrane domains [19]. These results are in agreement with immunocytochemical data on the multicompartment expression of mammalian VDAC given by this and other laboratories [3,5,12–17]. Furthermore, co-purification studies of VDAC with the outer cell membrane integrated $GABA_A$ receptor [18] support our proposal of the multi-topological expression of VDAC, recently reviewed [20,21].

As a consequence, the paradigm that VDAC is expressed only in outer mitochondrial membranes [1,2] has to be modified. Furthermore, the ideas concerning function and modula-

tion of the channel molecule [8–11] have to be re-estimated. The channel characteristics, highly purified VDAC shows after incorporation into black membranes, have to be seen in context with its plasmalemmal expression. The channel properties of incorporated VDAC are in good agreement with electrophysiological phenomena at the outer cell membrane, which have up to now no known molecular basis [20,21].

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