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Acidic Residues on the N-Terminus of Proinsulin C-Peptide Are Important for the Folding of Insulin Precursor[1]

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To investigate the role of C-peptide in the folding of insulin precursor, a series of C-peptide mutant proinsulin genes were constructed, overexpressed in *Escherichia coli* and the proteins purified. Correct disulfide linkages of these proteins were confirmed by both tryptic peptide mapping and insulin receptor binding analyses. *In vitro* refolding experiments were performed with the purified proteins and showed that mutations on the glycine-rich middle segment of C-peptide, GGGPGAG, and deletion of the C-terminal pentapeptide, EGSLQ, as well as mutations on the two pairs of dibasic residues at the two ends of C-peptide did not significantly affect the refolding yields. However, both alanine replacement mutation and deletion of three highly conserved acidic residues (EAED) at the N-terminus of the C-peptide resulted in serious aggregation during refolding. The results indicate that the highly conserved acidic N-terminal part of C-peptide is very important for insulin precursor folding, and that C-peptide may have some intramolecular chaperone-like function in the folding of insulin precursor.

Key words: C-peptide, intramolecular chaperone, mutation, proinsulin, protein folding.

C-peptide is an enzymatic cleavage product that arises from proinsulin during maturation. It is stored in secretary granules and eventually released into the bloodstream in equal molar amounts with insulin. An essential role of C-peptide in the biosynthesis of insulin is linking the A and B chains together and making inter-chain disulfide bond formation into an intramolecular process. Since chemically cross-linked A and B chains, even if intact, can fold to give the correct disulfide linkages of insulin, the C-peptide has been regarded as biologically inert for a long time. However, C-peptide was recently demonstrated to have beneficial effects in type I diabetes mellitus (*1*). Although not specified yet, the specific binding receptor for C-peptide has been found on many types of cell membranes (*2*). The C-peptide signal transduction pathway is involved in MAPK (*3*). It is thought that C-peptide is a biologically active peptide hormone with multiple physiological functions (*4*).

Many proteins, including most extra-cellular proteases and some peptide hormones, are synthesized as precursors with additional N-terminal propeptides, which are proteolytically cleaved to form the active mature molecules (*5*). The role of the propeptide in protein folding has become the focus of many investigations since the discovery of the dependence of subtilisin on its propeptide for proper folding in 1987 (*5*). The propeptides essential for protein folding are termed intramolecular chaperones (*6*). More and more propeptide-dependent protein folding systems have been characterized since the work of Shinde and Inouye (*7*), including many proteases, such as subtilisin (*8*), αLP (*9*), and carboxypeptidase Y (*10*), as well as some peptide hormones such as GCAP-II (*11*) and NGF (*12*). An intramolecular chaperone fulfills the criteria for a molecular chaperone (*13*) in that it assists the folding of the protein to which it is linked, but is not a component of the final mature and functionally active structure. Intramolecular chaperones differ greatly from molecular chaperones. Proteins can fold spontaneously under proper conditions even in the absence of their molecular chaperone (*13*). However, in the absence of intramolecular chaperones, most proteins that rely on them can not fold into their functionally bioactive states (*5–12*). Interestingly, intramolecular chaperones can function *in trans*, namely, a covalent attachment is not required for them to facilitate protein folding (*14, 15*). As in the case of subtilisin, intramolecular chaperones are proven to confer structural information for folding (*8*). In this respect, intramolecular chaperones are also called steric chaperones (*16*).

Other than as a connecting peptide, the role of C-peptide in proinsulin in the folding of the insulin precursor has rarely been reported. Varandani and Nafz once showed that the addition of C-peptide had a small, but not significant, effect on the renaturation of scrambled insulin in the presence of protein disulfide isomerase and DTT (*17*). Several years ago we reported that double-C-peptide human proinsulin has a higher refolding yield than wild type proinsulin at higher protein concentrations (*18*). We thus hypothesizd that proinsulin C-peptide may play an intramolecular

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Abbreviations: αLP, alpha lytic protease; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; GCAP-II, guanyl cyclase activating peptide-II; LB, Luria Bertant medium; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; RPC, reverse phase chromatography; SDS, sodium dodecyl sulfonate; WT, wild type.

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Position 1 3          15          29    35
   Human  RREAEDLQVGQVELGGGPGAGSLQPLALEGSLQKR
   Monkey RREAEDPQVGQVELGGGPGAGSLQPLALEGSLQKR
   Bovine RREAEGPQVGALELGGGPGAGG_____LEGPPQKR
   Rat-1  RREAEDPQVPQLELGGGPEAGDLQTLALEVARQKR
   Pig    RREAENPQAGAVELGGGLGG__LQALALEGPPQKR
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Fig. 1. **Linear depiction of the sequences of C-peptides from different species.** The amino acid sequences are numbered as indicated and include two pairs of dibasic amino acids flanking both the N- and C-termini. The grayed residues indicate relatively conserved regions of C-peptide among different species.

chaperone-like role in the folding of insulin precursor. For this purpose, we designed a series of C-peptide mutant proinsulins according to the sequences of C-peptide (Fig. 1), and compared their *in vitro* refolding with that of wild type proinsulin.

MATERIALS AND METHODS

Materials—Temperature-inducible expression vector pBV220 was used to express target proteins in *Escherichia coli* strain DH5α. Plasmid pJG103 (derived from pBV220) contains the gene encoding wild type human proinsulin. Taq DNA polymerase and restriction endonucleases were purchased from Promega. DTT was from Serva. Trypsin with an activity of 10,400 units/mg was purchased from Sigma. ^{125}I-Insulin was kindly provided by the Navy Radioimmunoassay Technique Center (Beijing, China). Other chemicals of analytical grade were local products.

Construction and Cloning of Recombinant Plasmids—The C-peptide mutant proinsulin genes were obtained by site-directed mutagenesis by PCR using pJG103 as a template. In total, nine C-peptide mutant proinsulin genes were constructed, *i.e.* (numbering of amino acids of C-peptide according to Fig. 1), 1-2A (R^1, R^2→A), 3-6A (E^3, E^5, D^6→A), 15-20L (G^{15}, G^{17}, G^{19}, A^{20}→L), 15-20Q (G^{15}, G^{17}, G^{19}, A^{20}→Q), 15-20KQ (G^{15}, A^{20}→K; G^{17}, G^{19}→Q), 15-20K (G^{15}, G^{17}, G^{19}, A^{20}→K), 34-35A (K^{34}, R^{35}→A), Δ3-6 (deletion of sequence EAED from 3 to 6), Δ29-33 (deletion of sequence EGSLQ from 29 to 33). Expression plasmids containing the genes encoding 15-20L, 15-20Q, 15-20KQ, and 15-20K mutant proinsulins were constructed as follows. The proinsulin gene fragment between the *Eco*RI (B chain N-terminus) and *Xho*I (more than half of C-peptide from its N-terminus) sites in pJG103 was amplified with 5′-primer and the corresponding 3′-mutant primer. After digestion with *Eco*RI and *Xho*I, the amplified fragment was cloned into the same two sites in pJG103 to replace the native fragment. The expression vectors for 1-2A, 3-6A, 34-35A, Δ3-6, Δ29-33 were constructed as follows. Mutant proinsulin genes were obtained using nested-PCR, digested with *Eco*RI and *Bam*HI (A chain C-terminus), and then cloned into the *Eco*RI and *Bam*HI sites of pBV220. Competent *E. coli* strain DH5α cells were used to transform these recombinant plasmids. All mutant genes were confirmed by DNA sequencing.

Expression and Purification of C-Peptide Mutant Proinsulins—The C-peptide mutant proinsulins were expressed as inclusion bodies in *E. coli*. Five hundred milliliters of logarithmic growth phase bacteria were inoculated into 5 liters of LB medium with 50 mg/liter of ampincillin and cultured at 30°C for 2 h. After a 6-h induction at 42°C, the cells were harvested by centrifugation. The cell pellet was then suspended in 4 volumes of STET buffer containing 0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 5% Triton X-100, pH 8.0, and ultrasonicated. Inclusion bodies were collected by centrifugation at 10,000 ×*g* at 4°C for 15 min, dissolved in 1.5 volumes of buffer containing 0.2 M NaCl, 0.1 M Tris-HCl, 1 mM EDTA, 8 M urea, 1 mM DTT, pH 8.0, and stirred overnight at 4°C. After centrifugation at 10,000 ×*g* at 4°C for 15 min, DTT was added to the supernatant to a final concentration of 20 mM, and the solution was incubated at 37°C for 2 h. After reduction, the solution was diluted with 5 volumes of ice-cold distilled water. The reduced target protein was precipitated at the isoelectric point and collected by centrifugation at 10,000 ×*g* at 4°C for 10 min. The protein precipitate was suspended in water and solubilized by adding 2 N NaOH. The solubilized protein solution was then diluted with 0.05 M glycine-NaOH buffer, pH 10.8, to an absorption at 280 nm of about 1.0, and kept at 4°C for 24 h. The refolded protein was concentrated by ultrafiltration and purified by Sephadex G-50 gel filtration chromatography (column 150 cm × 1.8 cm). The target fraction was pooled and loaded onto Resource™ RPC (Amersham Pharmacia Biotech, 3 ml) for further purification. The RPC column was equilibrated with distilled water containing 0.1% trifluoroacetic acid (TFA), and eluted with a linear gradient of 0–100% acetonitrile at 1.5 ml/min over 20 min. The purified proteins were then lyophilized and stored at –20°C. The yields of C-peptide mutant proinsulins were in a range of 10–40 mg from 5 liters of cell culture.

In Vitro Refolding Assay—The purified proteins were dissolved in buffer (to a protein concentration of 0.3, 0.6, or 0.9 mg/ml) consisting of 0.2 M NaCl, 0.1 M Tris-HCl, 1 mM EDTA, 8 M urea, pH 8.0, and DTT at a ratio of 80:1 (molar amount of DTT to that of protein SH). The mixture was incubated at 37°C for 2 h to allow complete reduction of the protein. Then, the mixture was dialyzed against 2,000 volumes of buffer containing 0.05 M glycine-NaOH, pH 10.8, for 24 h for protein refolding. The dialyzing buffer was changed twice at 4 h intervals during the early stages of the refolding process. The refolded sample was examined in 15% native PAGE and the refolding yield was measured by computational analysis of the PAGE gel with Glyko Bandscan software (Glyko, USA). Non-reducing (no reducing reagents in the sample loading buffer, other than the protein markers) SDS-PAGE was also used to analyze the refolded protein samples.

Tryptic Peptide Mapping Assay—C-peptide mutant proinsulins were digested with trypsin to test their abilities to form insulin-like molecules. The purified protein was dissolved in 0.05 M Tris-HCl, pH 7.2, to a protein concentration about 2.0 mg/ml. Trypsin was added to the protein solution at a substrate/enzyme ratio of 50:1 (w/w), and the mixture was incubated at 37°C for 30 min. The reaction was stopped by the addition of an equal volume of 2× native PAGE loading buffer. The tryptic peptides were then analyzed immediately by 15% native PAGE.

Insulin Receptor Binding Assay—C-peptide mutant proinsulins were tested for their ability to compete with ^{125}I-insulin in binding to insulin receptor. Insulin receptor was partially isolated as crude membranes from human placenta according to the method previously described by Fujita-Yamaguchi *et al.* (*19*). The proinsulin proteins were

diluted into a series of concentrations in KRB (0.119 M NaCl, 1.2 mM $MgSO_4$, 0.03 M HEPES, 0.05 M KCl, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 0.01 M $NaHCO_3$, pH 7.4) buffer. To 50 μl of proinsulin sample, an equal volume of properly diluted ^{125}I-insulin in KRB buffer and 6-fold diluted insulin receptor in KRB buffer in 2% of bovine serum albumin were added and the samples were mixed. The mixtures were incubated overnight at 4°C and centrifuged at 10,000 ×*g* for 5 min. The radioactivity of the pellets was determined by gamma ray detection. The concentration of wild type proinsulin producing 50% inhibition of ^{125}I-insulin binding to receptor was set as 100%, and the activities of the C-peptide mutant proinsulins were compared.

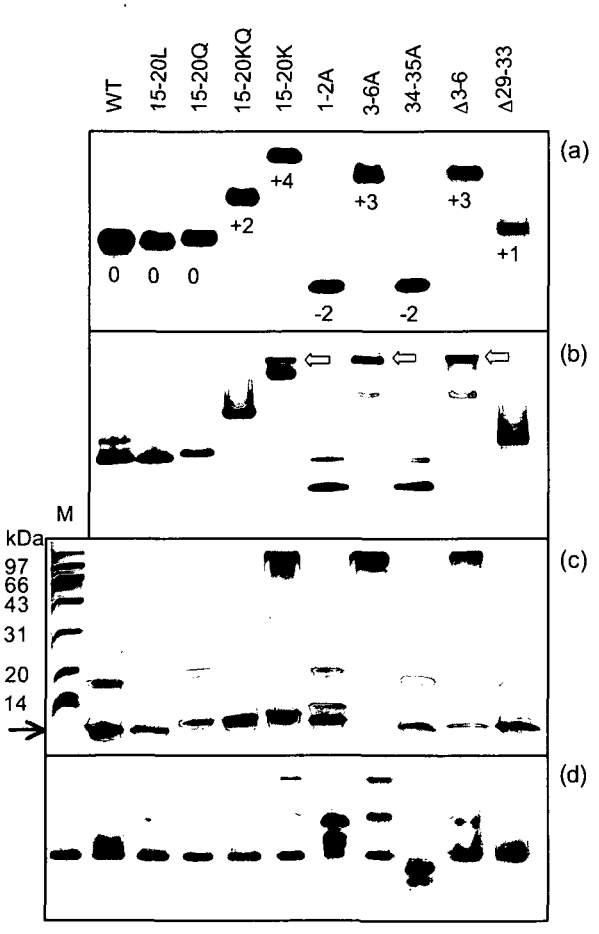

Fig. 2. **PAGE analyses of C-peptide mutant proinsulins.** (a) 15% native PAGE analysis of purified C-peptide mutant proinsulins. The number under each band shows the net charge changes as compared with wild type proinsulin. (b) 15% native PAGE analysis of in vitro refolding of wild type and C-peptide mutant proinsulins. Refolding was carried out at a protein concentration of 0.6 mg/ml. (c) 15% non-reducing SDS-PAGE analysis of the *in vitro* refolded products in (b). Lane M represents the protein molecular weight markers. The proinsulin samples were treated in SDS-PAGE loading buffer in the absence of DTT, while the protein markers were treated in the presence of DTT. Bands denoted by → are in the correctly folded state. (d) native PAGE analysis of the trypsin digests of different C-peptide mutant proinsulins. Lane M represents porcine insulin as a control. The enzymatic digestion reaction was performed in 0.05 M Tris-HCl, pH 7.2, at 37°C for 30 min at an E/S ratio of 1:50 (w/w).

RESULTS

Preparation of C-Peptide Mutant Proinsulins—The wild type proinsulin and its nine mutant proteins were prepared in a similar way. The expressed proteins were in the inclusion body form, and a refolding process was included in our purification procedure. The refolded proteins were further purified by two steps of column chromatography. The purity of the proteins was acceptable for our investigation as shown by native PAGE analysis (Fig. 2a). Protein identity was confirmed by both mass spectroscopy and amino acid composition analyses (data not shown).

Comparison of the In Vitro Refolding of C-Peptide Mutant Proinsulins—As shown by the native PAGE gel in Fig. 2b and the plot in Fig. 3, no significant differences were observed among the refolding products of the wild type, 15-20L, 15-20Q, 15-20KQ, 1-2A, 34-35A, and Δ29-33 proteins. The refolding yields of these mutant proinsulins were in the same error range as that of the wild type for protein concentrations from 0.3 to 0.9 mg/ml. Even at the high protein concentration of 0.9 mg/ml, the refolding yields were about 80%. When refolded at 0.3 mg/ml, the refolding yield of 15–20K was above 80%, But its refolding yields decreased dramatically at both 0.9 and 0.6 mg/ml. In striking contrast to the high refolding yields of the wild type and C-peptide mutant proinsulins, the refolding yields of 3-6A and Δ3-6 were much lower, especially at high protein concentrations. The refolding yield was below 18% at 0.9 and 0.6 mg/ml, and close to 50% when at the low protein concentration of 0.3 mg/ml. On both the native PAGE gel (Fig. 2b) and the non-reducing SDS-PAGE gel (Fig. 2c), some proteins are visible at the top of the separation gel in lane 15–20K, and much more in lanes 3-6A and Δ3-6 (indicated by blank arrows in Fig. 2b). It is obvious that these proteins are aggregates formed during *in vitro* refolding since they moved easily into the 5% stacking gel, but were retarded at the top of the 15% separation gel, indicating a very large molecular mass. This can also be inferred from the results of non-reducing SDS-PAGE analysis (Fig. 2c, lane 15-20K, lane 3-6A, lane Δ3-6). Non-reducing SDS-PAGE analysis showed that the aggregates were large molecules cross-linked inter-molecularly by disulfide bonds.

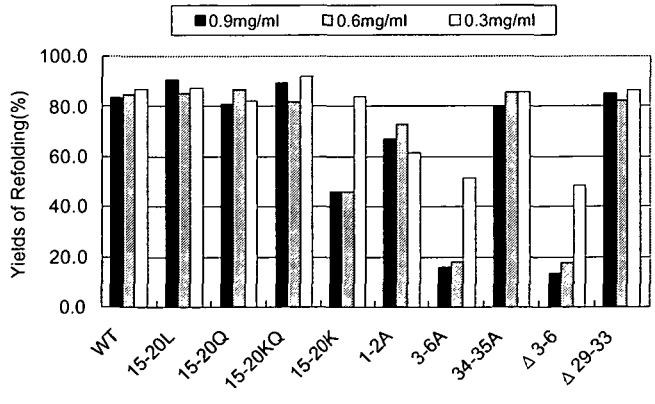

Fig. 3. **Yields of *in vitro* refolding at different protein concertations.** Data were obtained by computational analysis of native PAGE for refolded proinsulin proteins at different concentrations by Glyko Bandscan gel analyzing software (Glyko, USA).

TABLE I. **Insulin receptor binding assay of different C-peptide mutant proinsulins.**

	Relative activity (%)
WT	100.0
15-20L	81.0
15-20Q	84.9
15-20KQ	110.8
15-20K	116.9
1-2A	13.8
3-6A	249.9
34-35A	7.2
Δ3-6	110.0
Δ29-33	97.3

Confirmation of Correct Disulfide Bridges of C-Peptide Mutant Proinsulins—Except for 34-35A, all other C-peptide mutant proinsulins could be converted to des-B30Thr-insulin by trypsin digestion as judged by comparing the relative mobilities of the tryptic products on native PAGE gel with that of native porcine insulin (Fig. 2d). The deletion of B30Thr does not affect the relative mobility on native PAGE gel as compared with insulin. As with wild type proinsulin, des-B30Thr-insulin as the major tryptic product of 15-20L, 15-20Q, 15-20KQ, 15-20K, 3-6A, Δ3-6, and Δ29-33 suggests that those proinsulin mutants might have folded correctly with native disulfide bonds. It was observed to be a little harder to convert 1-2A to des-B30Thr-insulin, probably because the disappearance of the dibasic amino acid residues reduced the affinity of trypsin to the B29Lys-B30Thr cutting site. Still some des-B30Thr-insulin can be seen in Fig. 2d. The inability of 34-35A to be converted to des-B30Thr-insulin is due to the mutation of dibasic amino acid residues linking the insulin A chain.

The receptor binding assay indicated that most of the C-peptide mutant proinsulins were as potent as wild type proinsulin in competing with ^{125}I-insulin for binding to insulin receptor, except 1-2A and 34-35A, whose relative activities were only 13.8 and 7.2%, respectively, compared with wild type proinsulin (Table I). Since insulins with incorrect disulfide bonds give almost no receptor binding activity (*20, 21*), this again suggests that all mutant proinsulins could fold correctly to different degrees. The reason that 1-2A and 34-35A show lower receptor binding activity is probably due to the disappearance of the two positive charges close to the insulin part in both cases. Interestingly, the disappearance of three negative charges in 3-6A mutant proinsulin had the effect of increasing its receptor binding activity to some extent (Table I).

DISCUSSION

The role of the propeptide in protein folding has been widely studied in recent years (*5–12, 14–16*). Although some progresses have been made in elucidating the physiological role of the proinsulin C-peptide (*4*), the chaperone-like function of C-peptide in the folding of the insulin precursor has rarely been reported. Under appropriate conditions, insulin with full native biological activity can be obtained in reasonably good yield from either scrambled insulin or reduced insulin A and B chains (*22, 23*), suggesting that the insulin A and B chains contain sufficient structural information for correct folding. Since some data have shown that the addition of C-peptide has a small effect on the renaturation of scrambled insulin (*17*), and double-C-peptide proinsulin gives a higher refolding yield than wild type proinsulin at high protein concentration (*18*), we propose that the proinsulin C-peptide may play an intramolecular chaperone-like role in the folding of insulin precursor.

The amino acid sequences of C-peptides from different species are more variable than those of the insulin A and B chains (*4*), but they do have several relatively conserved sequences, such as the N-terminal acidic region, the glycine-rich middle segment and the C-terminal highly conserved pentapeptide (Fig. 1). The glycine-rich middle segment of C-peptide has been found to possess nearly the full biological activity of intact C-peptide (*1*). The C-terminal pentapeptide of C-peptide was observed to elicit the full activity of intact C-peptide in stimulating Na^+/K^+-ATPase (*24*). In order to know the possible effects of different regions in C-peptide on the folding of insulin precursor, a series of C-peptide mutant proinsulins were prepared. The results of the *in vitro* refolding experiments indicate that hydrophobic (15-20L) and hydrophilic (15-20Q, 15-20KQ, 15-20K) mutations in the glycine-rich middle part do not significantly affect the *in vitro* refolding of insulin precursor. Replacement of the dibasic amino acids at the two ends of C-peptide with alanine also has little effect on the refolding of insulin precursor, as does the deletion of the C-terminal peptapeptide. However, mutations in the N-terminal highly conserved acidic region of C-peptide do have significant effects on the *in vitro* refolding of insulin precursor as compared with wild type proinsulin. A mutation of E^3, E^5, D^6 to A^3, A^5, A^6 results in the formation of large aggregates during *in vitro* refolding. Similar results are obtained by deleting this EAED sequence. These results indicate that the highly conserved acidic region in the N-terminus of C-peptide is very important for the folding of insulin precursor. One may argue that changes in the isoelectric point may have affected *in vitro* refolding under the experimental conditions in this study, and we believe that isoelectric point changes may have some effects on refolding behavior. In our case, however, this factor is not very important. In fact, 15-20KQ has two more positive charges than wild type proinsulin, but its refolding yield is as high as that of the wild type. 15-20K has one more positive charge than 3-6A and Δ3-6, but its refolding yield is much higher than those of 3-6A and Δ3-6 under the same conditions (Figs. 2, a, b, and c, and 3). All mutant proinsulins except 34-35A could be converted to native insulin-like molecules and gave similar tryptic peptide maps (Fig. 2d), indicating that these mutant proinsulins have a high incidence of correctly paired disulfide bonds. The insulin receptor binding assay (Table I) also indicates that all mutant proinsulins folded correctly as evidenced by their relatively high receptor binding activities.

A propeptide may facilitate protein folding by greatly decreasing the energy barrier that blocks the further transformation of the folding intermediate into the native state (*25, 26*). Although C-peptide has been thought to lack a stable secondary structure either within the proinsulin molecule or in its intact form, based on structure simulation, the N- and C-termini of C-peptide show a tendency to form an α-helical structure (*1*). The N-terminus of C-peptide elicits the characteristics of an α-helix in high concentrations of trifluoroethanol (*27*). It is possible that the N-terminus of C-peptide interacts with the insulin A and B chains to sta-

bilize the folding intermediates to favor correct folding. We conclude that the N-terminal acidic region of C-peptide is important for the folding of insulin precursor and that C-peptide has some intramolecular chaperone-like function.

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