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Interactions of Serine Proteases With Cultured Fibroblasts

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This review summarizes the mechanisms by which several serine proteases, particularly urokinase, thrombin, and elastase, interact with cultured fibroblasts. Many of these studies were prompted by findings that interactions of these proteases with cells and the extracellular matrix are important in a number of physiologic and pathologic processes. Two main pathways have been identified for specific interactions of these proteases with fibroblasts. One involves surface binding sites for the free protease that appear to bind only one particular protease. An unusual feature collectively shared by the binding sites for urokinase, thrombin, and elastase is that the bound protease is not detectably internalized by the fibroblasts. The other pathway by which serine proteases interact with fibroblasts involves proteins named protease nexins (PNs). Three PNs have been identified. They are secreted by fibroblasts and inhibit certain serine proteases by forming a covalent complex with the protease catalytic site serine. The complexes then bind back to the fibroblasts via the PN portion of the complex and are internalized and degraded. Recent studies showing that the fibroblast surface and extracellular matrix accelerate the inactivation of thrombin by PN-1 support the hypothesis that the PNs control protease activity at and near the cell surface. The PNs differ from plasma protease inhibitors in their molecular properties, absence in plasma, site of synthesis, and site of clearance of the inhibitor:protease complexes.

Key words: protease nexin, cellular binding sites, extracellular matrix, elastase, thrombin, urokinase, fibroblasts

There is now much evidence that interactions of several serine proteases with cells and the components around them play key roles in a variety of physiologic and pathologic processes. A protease that has been extensively studied in this context is urokinase (see [1] for excellent recent review). The activation of plasminogen to plasmin by urokinase and the resulting extracellular proteolysis has been linked to several important processes involving the movement of cells through tissues. These include mammary gland involution [2], ovulation [3], blastocyst implantation [4], and angiogenesis [5]. Urokinase has also been closely linked to the ability of malignant

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cells to invade and metastasize [1,6]. Finally, urokinase has been related to several nonneoplastic pathologic conditions, some of which involve inflammation and/or tissue degradation, including allergic vasculitis, xeroderma pigmentosum, and pemphigus [1]. Elastase is another serine protease whose interactions with cells and their extracellular matrix have important consequences. Elastase is capable of degrading a number of connective tissue components including elastin [7], collagen [8], proteoglycans [9], and fibronectin [10]. This broad substrate specificity of elastase and its activity at neutral pH endows it with great potential for causing extracellular damage. Elastase has been shown to be directly involved in the pathogenesis of emphysema [11]. Elevated levels of elastase have also been implicated in the pathogenesis of rheumatoid arthritis [12,13] and atherosclerosis [14]. Thrombin, a much more specific serine protease, does not generally degrade extracellular matrix components, although it can cleave a small fragment from fibronectin [15]. On the other hand, thrombin has a potent mitogenic effect on fibroblasts, an effect that could play a role in tissue repair following injury [16,17].

The above proteolytic effects have prompted a number of studies on the mechanisms by which these proteases interact with cells and the extracellular matrix. These studies have revealed cellular receptors for these proteases that have been suggested to play important roles in some of the above processes. They have also led to the identification of cellular components which contribute to the control of proteases in the extracellular environment. The purpose of this review is to briefly summarize the mechanisms by which several serine proteases interact with cultured fibroblasts and what is known about the cellular components involved in these interactions.

TWO PATHWAYS FOR CELLULAR INTERACTIONS OF SERINE PROTEASES

Studies examining the ability of cultured fibroblasts to bind several serine proteases have revealed two general pathways for specific binding. It should be emphasized that these experiments have focused mainly on thrombin, urokinase, elastase, and a couple of other serine proteases. Studies on these interactions are at an early stage, and only preliminary information is available about the binding of certain serine proteases to fibroblasts. These experiments employed traditional receptor binding assays that detect only interactions with a K_d of about 10^{-8} M or higher affinity. Thus, they would not detect the relatively low affinity transient interactions involving proteolysis of cell surface proteins.

Figure 1 diagramatically shows the two general pathways that have been detected for high affinity interaction of serine proteases with cultured fibroblasts. It should be noted that some of the serine proteases examined do not display specific binding to fibroblasts and that certain others bind by one or both of these mechanisms. One pathway involves a surface site which binds the free protease. These sites appear to be relatively specific for only one protease. As will be described in a later section, binding sites have been described for thrombin, urokinase, and elastase. An unusual feature of these sites that contrasts sharply with surface binding sites or receptors for most other kinds of molecules is that the bound protease is not detectably internalized by the fibroblasts.

The second pathway by which serine proteases specifically bind to fibroblasts involves cell-secreted proteins named protease nexins (PNs) [18–21]. As depicted in Figure 1, the PNs are synthesized and released by human fibroblasts into the culture

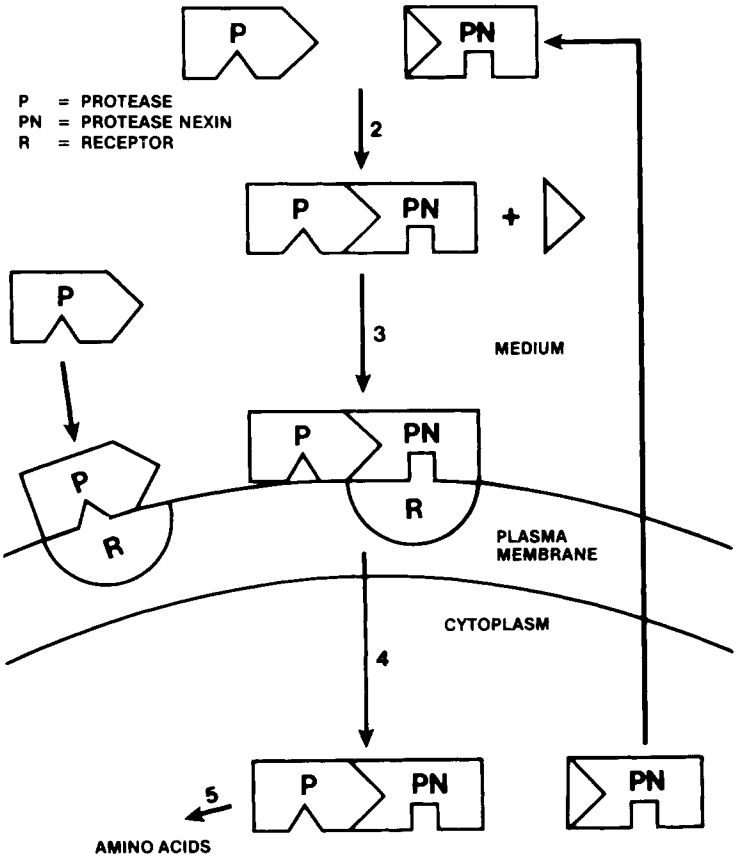

Fig. 1. Two pathways identified for interactions of serine proteases with cultured fibroblasts. See text for explanation.

medium; they bind to certain serine proteases and form a covalent complex involving the catalytic site serine of the protease, thus inactivating the protease. The PN-protease complexes then bind back to the fibroblasts, via the PN portion of the complex, and are rapidly internalized and degraded.

For certain proteases, particularly thrombin and urokinase, it is possible to distinguish between the two pathways by derivatizing the catalytic site serine of the protease with diisopropylfluorophosphate (DIP). The DIP-proteases do not form complexes with the PNs and thus are not bound by that mechanism [18,22]. However, DIP-thrombin [23] and DIP-urokinase [24] bind to cell surface sites for the free protease on fibroblasts.

Cellular Control of Serine Proteases by Protease Nexins

The above brief description of the PNs indicate that they are well suited for controlling certain serine proteases in the immediate environment of cells. Three PNs have been described and recently reviewed [21]. Here, we will briefly summarize some of the general properties of the PNs and relate some recent findings which support the hypothesis that their site of action is at or near the surface of cells in the extravascular compartment.

PN-1

The first PN detected [18,19] is now called PN-1 or PN. It is a protein of Mr 43,000 that rapidly inhibits thrombin, urokinase, and plasmin and less rapidly inhibits tissue plasminogen activator, factor Xa, and the gamma subunit of nerve growth factor [25]. Recent studies have shown that it also forms complexes with proteases inhibited by plasma C1-inhibitor including C1r, C1s, factor X11a, and plasma kallikrein and that it inhibits the latter two proteases as rapidly as C1-inhibitor [26].

Although PN-1 shares some functional similarities with several protease inhibitors found in plasma, including C1-inhibitor, heparin cofactor II, and antithrombin III, its molecular properties are distinct. In addition, there are now several lines of evidence which support a role for PN-1 in the extravascular compartment, in contrast to the well-studied roles of the plasma protease inhibitors in the vascular compartment of the body. First, PN-1 is released by a variety of cultured cells including fibroblasts, heart muscle cells, myotubes, epithelial cells, and fibrosarcoma cells [27]. With fibroblasts, it has been shown that the cells actually synthesize PN-1 [25,28]. In contrast, studies on these cells have not identified a significant release of the corresponding plasma protease inhibitors. Also, PN-1 is not found in significant levels in plasma. Another line of evidence for a site of action of PN-1 in the extravascular compartment comes from the data in Figure 2 which show that cultured human fibroblasts are capable of binding complexes between a protease and PN (^{125}I-thrombin-PN complexes) but do not bind complexes between the same protease and a plasma protease inhibitor (^{125}I-thrombin-C1-inhibitor complexes). Previous studies showed that the bound ^{125}I-thrombin-PN complexes are rapidly internalized and degraded by the fibroblasts [19]. Studies on ^{125}I-thrombin-antithrombin III complexes showed that they are bound and degraded by liver cells [29]. Thus, at this early stage in these studies, the general picture that is emerging is that the PNs are made by certain cells in the extravascular compartment and that these cells can clear protease-

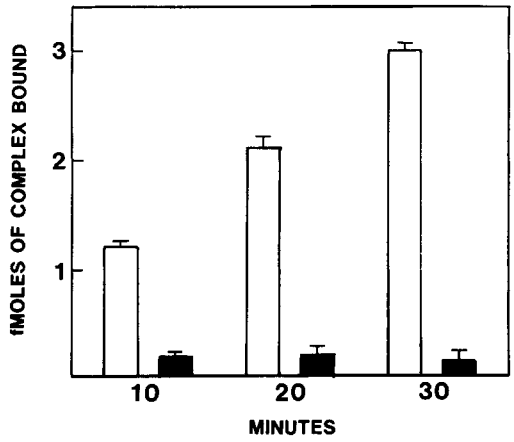

Fig. 2. Binding of ^{125}I-thrombin-PN complexes but not ^{125}I-thrombin-C1-inhibitor complexes to fibroblasts. Complexes were formed between ^{125}I-thrombin and PN or C1-inhibitor. The complexes were purified and the concentration of each was adjusted to 3.8×10^{-14} M. They were incubated at 37°C for the indicated times with 3×10^5 human fibroblasts that had been in serum-free culture medium for 24 hr. Then, the cultures were rinsed and cell-associated radioactivity was measured. Closed bars, ^{125}I-thrombin-C1-inhibitor complexes; open bars, ^{125}I-thrombin-PN complexes.

PN complexes. On the other hand, the liver appears to be the site of synthesis of certain plasma protease inhibitors [30,31] and also the site where complexes with their target proteases are cleared [29].

The data in Figure 3 show another specific interaction between fibroblasts, PN- 1, and thrombin which provides evidence for an additional way that these cells can control certain serine proteases in their environment [32]. For each of the curves shown, a constant amount of ^{125}I-thrombin was present; after the specified time, the extent of complex formation between ^{125}I-thrombin and either PN-1 or antithrombin III was analyzed. These measurements were conducted in the presence and absence of human fibroblasts that had been fixed in 2% paraformaldehyde to prevent their uptake or release of materials. As shown, the fibroblast surface accelerated the reaction with PN-1, but not with plasma antithrombin III. Studies to identify the cellular components responsible for this led to the finding that virtually all of the acceleration can be accounted for by the extracellular matrix [32] and that heparan sulfate and chondroitin sulfate are responsible for the acceleration [33]. Earlier complimentary studies showed that endothelial cells [34,35] but not fibroblasts [35] accelerated the inactivation of thrombin by plasma antithrombin III. Together, these results provide additional evidence for the different sites of action of PN-1 and antithrombin III noted above. Interestingly, the accelerative activity of endothelial cells on the reaction between antithrombin III and thrombin is also due to heparan sulfate [36]. This seeming paradox is likely explained by structural differences in the heparan sulfates, eg, differences in sulfation, on endothelial cells and fibroblasts. Comparisons of the structures of the active heparan sulfate molecules found on endothelial cells and fibroblasts should help elucidate this interesting problem.

An important question regarding PN-1 is what physiologic functions are regulated as a result of its ability to control certain proteases at the cell surface. Although roles in vivo have not been examined, studies in cell culture systems have provided

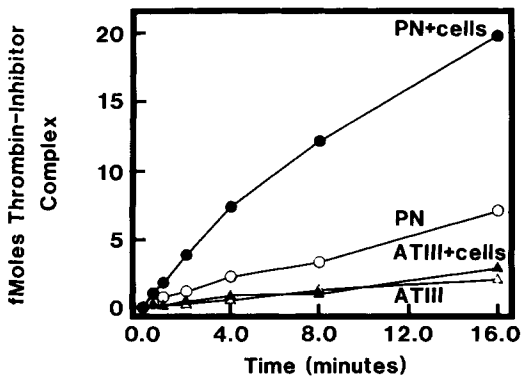

Fig. 3. Fixed human fibroblasts accelerate ^{125}I-thrombin-PN complex formation but not ^{125}I-thrombin-antithrombin III complex formation. Confluent cultures of human fibroblasts were incubated in serum-free medium for 2 days and then fixed with 2% paraformaldehyde. 0.5 ml of medium containing 13.7 nM PN (●) or antithrombin III (▲) was incubated with the cells at 37°C. 13.7 nM PN (○) or antithrombin III (△) was also incubated with culture dishes containing no cells. ^{125}I-thrombin was added to a final concentration of 1.37 nM to initiate the reaction. The reaction was terminated by adding sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis at the indicated times. After electrophoresis, the radioactivity in the bands corresponding to thrombin-PN and thrombin-antithrombin III was measured in a gamma counter. (Reprinted from [32], with permission).

promising clues. For example, added PN-1 shifts the dose-response curve for mitogenic stimulation by thrombin to higher concentrations of thrombin, indicating that responsive cells might actively modulate their response to this mitogen [37]. The PN-1 has also been shown to prevent extracellular matrix destruction by human fibrosarcoma cells [38]. Also, a protease inhibitor from glial cells which resembles PN-1 has been shown to stimulate neurite outgrowth in neuroblastoma cells [39].

PN-2 and PN-3

Much less is known about these PNs. Both were identified by their ability to form complexes with serine proteases that are associated with growth factors: PN-2 with the epidermal growth factor binding protein [40] and PN-3 with the gamma subunit of nerve growth factor [41]. As with PN-1, PN-2 and PN-3 are secreted by human fibroblasts, and their complexes with proteases bind back to these cells and are internalized and degraded.

PN-2 recently was purified to homogeneity [42]. It was isolated as a single chain polypeptide of Mr 106,000 which covalently complexes the epidermal growth factor-binding protein, the gamma subunit of nerve growth factor, and trypsin. The purified protein was metabolically labeled with ^{35}S-methionine, demonstrating that it is a biosynthetic product of the cells. Complexes between PN-2 and the epidermal growth factor binding protein bind to human fibroblasts, fulfilling the criteria for a PN. Although PN-2 forms stable, covalent complexes with the binding protein, enzymatic inhibition studies revealed that the extent of this complex formation is relatively low. Thus, the epidermal growth factor-binding protein was a useful probe for identifying PN-2, but it now appears that PN-2 may be involved with the regulation of other proteases. Studies are now in progress to identify protease targets of PN-2 and to characterize the regulatory roles of PN-2 along the lines described above for PN-1. Studies on PN-3 must await its purification.

CELLULAR BINDING SITES FOR FREE PROTEASES

Thrombin

Early studies examining the mechanism by which thrombin stimulates the proliferation of cultured fibroblasts showed that mitogenic stimulation does not require cellular internalization of thrombin [43]. These studied also identified high affinity binding sites on the surface of these cells for ^{125}I-thrombin [44]. ^{125}I-DIP-Thrombin binds to these sites with same affinity (about 1×10^{-9} M) [23]. From studies using a photoaffinity crosslinking derivative of ^{125}I-DIP-thrombin, the Mr of the sites has been estimated to be 150,000 [45]. These binding sites appear to be clustered on the surface of the cells prior to thrombin binding; interestingly, the bound thrombin is not detectably internalized by the cells [46,47].

Since the major effect of thrombin on these fibroblasts is mitogenic stimulation [17,48], a major question has been the role that these binding sites play in that response. DIP-thrombin binds indistinguishably to these sites and is not mitogenic [23], indicating that ligand binding is not sufficient for mitogenic stimulation and that proteolysis is required for cell activation. Studies on a line of cells that lack the thrombin binding sites have shown that they are the most responsive of any cells yet examined to the mitogenic action of thrombin [48]. Thus, these sites are not necessary for mitogenic stimulation by thrombin. Instead, proteolysis by thrombin at the cell

surface appears to be the primary signaling event for mitogenesis. It should be emphasized, however, that these studies do not rule out a role of the thrombin binding sites in mitogenic stimulation. Indeed, studies have shown that DIP-thrombin potentiates the mitogenic effect of gamma-thrombin, a proteolytically modified form of native thrombin [49]. Thus, the fibroblast surface sites which bind free thrombin may participate in the augmentation of thrombin-stimulated cell division under certain conditions.

Urokinase

Recent studies on the binding of the free two-chain Mr 55,000 form of ^{125}I-urokinase show that it binds with a high affinity to specific sites on the surfaces of monocytes and cells of the monocyte line U937 [50,51] as well as human foreskin fibroblasts [24]. With the Mr 55,000 form as well as the Mr 33,000 form of ^{125}I-urokinase, there was also binding that was mediated by PN, judged by the presence of cell-bound ^{125}I-urokinase-containing complexes of the appropriate sizes. When ^{125}I-DIP-urokinase was employed to eliminate the formation of complexes with PN, it became clear that there are surface sites that specifically bind free Mr 55,000 urokinase but not free Mr 33,000 urokinase [24,50]. Moreover, the amino acid sequence of urokinase that is responsible for binding to these surface sites is located within the first 135 amino-terminal residues of urokinase, a region that is not required for its proteolytic activity and which is lacking in Mr 33,000 urokinase [51].

Like the binding sites described above for free thrombin, the sites on monocytes [50] and on fibroblasts [24] do not detectably internalize bound urokinase. Additional findings that the urokinase bound to fibroblasts dissociates slowly [24] and that the urokinase bound to monocytes is enzymatically active [50] led both groups of investigators to suggest that the bound urokinase could be critically important for cell-mediated activation of plasminogen. Further studies on these interesting binding sites may reveal that they are important for many of the urokinase-mediated processes noted in the introduction.

Elastase

Compared to the cell interactions of thrombin and urokinase, less is known about the mechanism by which elastase interacts with cells. Studies on macrophages showed that these cells have surface sites that bind and internalize elastase; the same sites also bind cathepsin G and lactoferrin [52,53]. Elastase has been identified at the plasma membrane of smooth muscle cells [54], but little is known about the nature of the association.

Recent studies on the interaction of elastase with cultured human fibroblasts identified binding sites for this protease with some unusual properties [55]. Both leukocyte and pancreatic elastase specifically bind to about 180,000 sites on these cells and form covalent complexes with them. The complexes formed when human fibroblasts were incubated with pancreatic ^{125}I-elastase are shown in lane 2 of Figure 4. As can be seen, a band was present at Mr 26,000 which corresponded to an elastase standard. When a large excess of unlabeled elastase was added to measure nonspecific binding this band was reduced but not eliminated (Fig. 4, lane 3). This indicated that free elastase bound to the cells and that some, but not all, of this binding was specific. In addition, a higher molecular weight band at Mr 54,000 was also present. This band was not present when a large excess of elastase was included to

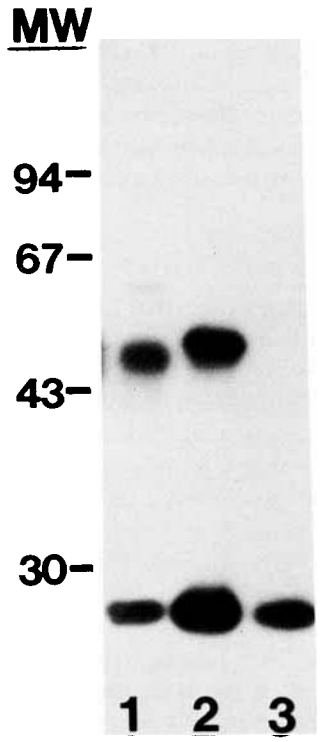

Fig. 4. ^{125}I-Elastase forms complexes with components from calf serum and human fibroblasts. ^{125}I-elastase was incubated with calf serum or human fibroblasts as indicated; the mixtures were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. An autoradiogram was then prepared. Lane 1, ^{125}I-elastase was incubated at 37°C for 10 min with 10% calf serum. Lane 2, ^{125}I-elastase was incubated at 4°C for 30 min with human fibroblasts which had been in serum-free medium for 2 days. Lane 3, like lane 2, except a large excess of unlabeled elastase was included in the incubation to measure nonspecific binding.

measure nonspecific binding (Fig. 4, lane 3). Thus, all of the binding represented by this material appeared to be specific. The cells used in this experiment had been grown in calf serum and then incubated in serum-free medium for 2 days. The Mr 54,000 band was not the product of contaminating calf serum because ^{125}I-elastase formed a Mr 51,000 complex with an elastase-binding component in calf serum later identified as alpha-1-protease inhibitor. Together, these studies show that human fibroblasts possess surface sites that bind and form complexes with elastase. The complexes appear similar to complexes formed between proteases and PN in that the complexes are disrupted by hydroxylamine, and they are not formed when ^{125}I-DIP-elastase is employed in the experiment. However, the cellular component with which elastase forms a complex is not a PN, since it was not detected in the cell culture medium. Also, it was not possible to detect cellular internalization of the cell-bound Mr 54,000 complexes. Thus, the elastase binding sites may represent cell surface components that can bind and inhibit elastase and modulate its action on cells and the extracellular matrix around them [55].

Future Directions

The studies briefly summarized in the introduction have shown that the action of certain serine proteases on cells and the components around them in tissues can produce many physiologic and pathologic effects. The cells, in turn, possess mechanisms to regulate these serine proteases. A principal route for this appears to be via PNs, although surface sites that bind the free protease probably also participate in this control. In future studies, it will be important to further identify the kinds of cells that secrete PNs and to learn how the synthesis and release of these proteins are regulated. For example, do these processes depend on the physiologic state of the cell? Are they altered in some of the diseases mentioned in the introduction?

As summarized above, the surfaces of cells may provide an additional dimension to the regulation of extracellular proteases by accelerating the inactivation of certain serine proteases by the PNs. Currently this acceleration has only been observed for fibroblasts (thrombin and PN-1) and for endothelial cells (thrombin and antithrombin III). Since it is mediated by cell surface/extracellular matrix glycosaminoglycans, it will be important to determine whether it occurs with the other serine proteases that bind glycosaminoglycans, and with PN-2 which is known to bind heparin [42]. In view of the cellular specificity already observed for the acceleration, it will be interesting to further identify the kinds of cells that can accelerate reactions between specific proteases and PNs. This could provide important clues about the sites of action of the PNs and then likely physiologic roles. Another issue that should be explored is the possibility that alterations in cell surface/extracellular matrix glycosaminoglycans known to accompany certain pathologic states [56], including carcinoma [57], might alter the spectrum of active proteases in the extracellular fluid by altering the inactivation of certain proteases by PNs.

Serine proteases also interact with cultured fibroblasts and hydrolyze certain cell surface proteins. These are generally transient low-affinity interactions that are not detected by the procedures used in the studies summarized above. Proteolysis of cell surface proteins could be involved in several of the physiologic and pathologic processes described in the introduction. For example, studies have demonstrated a requirement for proteolysis of one or more cell surface proteins for thrombin-stimulated cell division [23,48], and studies are now in progress to identify protein substrates on fibroblasts for thrombin. This has required the development of procedures to better label surface proteins on these cells and to more effectively resolve them on gels [58].

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