Binding and Invasion of Liver Cells by *Plasmodium falciparum* Sporozoites

ESSENTIAL INVOLVEMENT OF THE AMINO TERMINUS OF CIRCUMSPOROZOITE PROTEIN*

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§ The abbreviations used are: CS, circumsporozoite; TSR, thrombospondin type I repeat domain; GAG, glycosaminoglycan; HSPG, heparan sulfate proteoglycan.

The circumsporozoite (CS) protein on their surface, which is involved in the attachment of sporozoites to liver cells. CS protein is a member of the thrombospondin type I repeat (TSR) domain family and possess a single copy of TSR domain toward its carboxyl terminus. We show by a direct measurement the correlation between the binding activity of various segments of the CS protein and their ability to inhibit the invasion of liver cells by the sporozoites. We made eight truncated versions of *Plasmodium falciparum* CS protein to elucidate the role of various regions in the binding and invasion process. Deletion of the TSR domain actually enhanced binding activity by 2-3-fold without the loss of receptor specificity, indicating that TSR may not be the only domain in defining the specificity of binding. These same deletions blocked invasion of live sporozoites more efficiently than proteins that include the TSR domain. Deletion of as little as six amino acids from amino terminus or both. Characterization of these mutants uncovers the crucial role of amino terminus of CS protein in these processes.

*Plasmodium* sporozoites display circumsporozoite (CS) protein on their surface, which is involved in the attachment of sporozoites to liver cells. CS protein is a member of the thrombospondin type I repeat (TSR) domain family and possess a single copy of TSR domain toward its carboxyl terminus. We show by a direct measurement the correlation between the binding activity of various segments of the CS protein and their ability to inhibit the invasion of liver cells by the sporozoites. We made eight truncated versions of *Plasmodium falciparum* CS protein to elucidate the role of various regions in the binding and invasion process. Deletion of the TSR domain actually enhanced binding activity by 2-3-fold without the loss of receptor specificity, indicating that TSR may not be the only domain in defining the specificity of binding. These same deletions blocked invasion of live sporozoites more efficiently than proteins that include the TSR domain. Deletion of as little as six amino acids from amino terminus or both. Characterization of these mutants uncovers the crucial role of amino terminus of CS protein in these processes.

The circumsporozoite (CS) protein is the predominant surface antigen of *Plasmodium* sporozoites and performs numerous functions for the parasite. It is vital for the development of sporozoites (1), helps parasite to invade salivary glands in mosquitoes (2), and has also been implicated in the binding of sporozoites to liver cells (3, 4). Once inside, CS has been shown to inactivate the protein synthesis machinery of the host cell (5). CS protein is one of the key targets recognized by the host immune system and has emerged as one of the prime candidates for an anti-malarial vaccine (6-8).

CS protein can be roughly segmented into three parts, namely (i) the amino terminus region-containing region I, (ii) a central repeat region, and (iii) a carboxyl terminus segment containing TSR domain (Fig. 1A). Region I has been implicated in the invasion of salivary glands in mosquitoes and in the inhibition of protein synthesis in liver cells (2, 5). The central repeat segment is a low complexity region containing multiple copies of species-specific repeat peptides (NANP in case of *Pl. falciparum*) with no obvious sequence homology to any known protein. The carboxyl terminus of CS demonstrates sequence conservation across the species (9) and its region II-plus has been implicated in liver cell binding and subsequent inhibition of protein synthesis (3-5). Region II plus is an 18-amino acid region and is a part of a larger TSR domain, which was initially discovered in thrombospondin (10).

TSR is an ancient eukaryotic domain (11) now known to be present in more than 300 different proteins (12), including surface antigens of pathogenic microorganisms (13) as well as metalloproteases of mammalian origin (14). Although the role of TSR domain in these diverse proteins has largely not been defined, in a few cases it has been shown to interact with cellular and extracellular matrix (15, 16), specifically heparan sulfate, a ubiquitous cell surface polysaccharide, CD36 and various other GAGs (17).

Sporozoites, after being injected into the circulation, traverse through the cytosol of several cells before finally invading a hepatocyte (18). Given that the TSR domain receptors are ubiquitous on host cells, it is difficult to envision that the TSR domain alone is responsible for specific binding and invasion of hepatocytes. To address this issue, we have constructed variants of the CS protein lacking either the TSR domain or residues from amino terminus or both. Characterization of these mutants uncovers the crucial role of amino terminus of CS protein in these processes.

EXPERIMENTAL PROCEDURES

Materials

*Escherichia coli* strain BL21(DE3) and vector pET11a were obtained from Novagen (Madison, WI). Heparin, fucoidan, chondroitin sulfate A, chondroitin sulfate B, dextran sulfate, chondroitinase ABC, and heparinase I of *Flavobacterium harpium* was obtained from Sigma. Restriction enzymes and cell culture supplies were purchased from Invitrogen. Heparin-Sepharose column was obtained from Amersham Biosciences, Inc. The hepatoma cell line (HepG2) was purchased from ATCC (Manassas, VA). Anti-mouse-alkaline phosphatase conjugate was from Pierce. Monoclonal antibody 2A10 directed against the (NANP) repeat domain was a kind gift from Dr. Robert Wirtz (Centers for Disease Control and Prevention, Atlanta, GA).
Construction of CS Deletion Mutants

Carboxyl Terminus Deletions—Plasmid pCS1 was used as template for the construction of these deletion mutants. Plasmid pCS1 contains DNA sequence encoding for a mature CS protein in pET11a, a T7 promoter-based E. coli expression vector (19). Two CS DNA sequences lacking 90 and 109 amino acids from the carboxyl terminus were amplified by PCR. The oligonucleotides used for amplification were designed such that an NdeI and a BsiWI restriction site was introduced at 5' and 3' ends, respectively. The insert was digested and cloned in pET11a, giving rise to plasmids pCS20 and pCS21 lacking 90 and 90 amino acids from the carboxyl terminus, respectively. Plasmid pCS20 only encodes for the first 97 amino acids from the amino terminus and has been described elsewhere (20).

Amino Terminus Deletions—DNA encoding truncated versions of CS, lacking 5, 14, 23, 28, and 90 amino acids from the amino terminus, were amplified by PCR from plasmid pCS1 and cloned in vector pET11a giving rise to plasmids pCS19, pCS16, pCS17, pCS6, and pCS7, respectively.

Carboxyl and Amino Terminus Deletions—Plasmid pCS20 was used as a template to amplify a fragment of CS lacking 28 amino acids from the amino terminus and 109 amino acids from the carboxyl terminus. The truncated fragment was cloned in pET11a, giving rise to plasmid pCS25.

Expression, Localization, and Purification of Recombinant Proteins

All the constructs were expressed in bacterial strain BL21, containing T7 RNA polymerase gene under the control of lac promoter, as described (19). Briefly, cells were transformed with the desired plasmid and the culture was expanded in super broth (pH 7.2), containing 100 μg/ml ampicillin. At A600 of 1.0, cells were induced for 4 h with 2 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested, and the periplasm was separated by a gentle osmotic shock without disrupting the cell membrane. Except for pCS7, CS proteins from all the constructs were purified on a heparin-Sepharose affinity column as described (19). Briefly, cells were transformed with the desired plasmid and the culture was expanded in super broth (pH 7.2), containing 100 μg/ml ampicillin. At A600 of 1.0, cells were induced for 4 h with 2 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested, and the periplasm was separated by a gentle osmotic shock without disrupting the cell membrane. Except for pCS7, CS proteins from all the constructs were purified on a heparin-Sepharose affinity column as described (19). Periplasmic extract containing CS7 was purified on a Q-Sepharose (anion exchange) column. Subsequently, all the proteins were subjected to gel filtration chromatography.

Cell Binding Assay

Hepatoma cell line HepG2 was used to evaluate the binding activity, and the assay was performed as described (3). Briefly, cells were fixed with 4% paraformaldehyde followed by blocking with Tris-buffered saline containing 1% bovine serum albumin. Proteins were incubated with cells for 1 h, followed by anti-CS monoclonal antibody for 30 min and alkaline phosphatase coupled anti-mouse IgG for 30 min. 1 mM 4-methylumbelliferyl phosphate was used as substrate, and fluorescence was measured in a fluorometer with excitation at 350 nm and emission at 460 nm. The sensitivity of the fluorometer was adjusted such that the fluorescence activity in a given assay was within the maximum detection limit of the instrument.

Binding Activity of CS Mutants in the Presence of GAGs

For evaluating the effect of various GAGs on the binding activity of the carboxyl terminus deletions, 50 nM recombinant proteins were co-incubated with different concentrations of heparin, fucoidan, chondroitin sulfate A, chondroitin sulfate B, or dextran sulfate for 15 min at 37 °C before the addition of this mix onto the cells. For amino-terminal deletions, 50 nM protein was co-incubated with 5 nM heparin. The assay was continued as described above.

Effect of Heparinase I and Chondroitinase ABC on the Binding Activity of CS-Heparin Complex

HepG2 cells were treated with log dilutions of heparinase I or chondroitinase ABC or buffer for 2 h at 37 °C. The cells were washed three times with Tris-buffered saline, followed by addition of 50 nM recombinant protein and incubation at 37 °C for 1 h. Fluorescence was measured as described above.

Competition Binding Analysis

For competition analysis, HepG2 cells after blocking were incubated with different concentrations of CS27 or peptide (MRGSSSNTRVLNELYNDNAC) for 60 min before adding the protein. The experiment was continued as described above.

RESULTS

Cloning, Expression, Localization, and Purification of Deletion Mutants of CS—Eight different deletion constructs of CS lacking residues from amino or carboxyl terminus or both were created (Fig. 1A). Plasmids pCS19, pCS16, pCS17, pCS6, and pCS7 lacked the first 6, 14, 23, 28, and 90 amino acids from the
amino terminus, respectively. Plasmids pCS21 and pCS20 encoded truncated versions of CS where 90 and 109 amino acids from the carboxyl terminus were deleted. Plasmid pCS25 had deletions on both ends and lacked 28 and 109 amino acids from amino and carboxyl termini, respectively. Authenticity of all the clones was verified by sequencing (data not shown). All the constructs were expressed in BL21 (data not shown). Numerous recombinant proteins, when secreted into the periplasm, have been shown to fold correctly by the periplasmic protein folding machinery of the E. coli (24, 25). Periplasm was used as source to purify the proteins. CS protein possess heparin binding properties, which have been attributed to region I and the TSR domain of the protein (26). All as the mutants had at least one of the two domains intact, heparin-Sepharose column was used for purification. All the deletion mutants except CS7 bound onto the heparin-Sepharose column. Protein CS7, although containing both Region I and TSR domain, did not bind to the heparin-Sepharose column and had to be purified onto an anion exchange column. This suggested that the CS-heparin interaction is not solely dependent on region I and TSR domain of the protein. Repeats are not responsible for these interactions as CS27, a repeat-less protein, coding only for the first 97 amino acids from amino terminus also bound to the heparin column with equal efficiency. Protein containing fractions were pooled and purified to apparent homogeneity by size exclusion chromatography (Fig. 1B). The proteins eluted from the column within the range of their stipulated sizes, indicating that they were maintaining a monomeric configuration. Authenticity of the proteins was verified by amino terminus sequencing and mass spectrometry (data not shown).

The Effect of Deletions on the Binding Activity of CS Protein—Binding activity of deletion mutants was evaluated on HepG2 cells. Carboxyl terminus mutants CS20 and CS21, though without the TSR domain, demonstrated an 2–3-fold enhancement in the binding activity in comparison to the native protein CS1 (Fig. 2A). The binding activity was dose-dependent and specific, as it showed saturation at higher concentrations. Although deletion of the TSR domain led to an increase in activity, truncations from the amino terminus rendered CS inactive. Loss of the first six amino acids (CS19) led to a 75% decrease in the binding activity of the (Fig. 2B). Deletion of more than six amino acids (proteins CS16, CS17, CS6, and CS7) resulted in a complete loss of activity (Fig. 2B), although these proteins could still bind to the heparin column (data not shown). Protein CS25, where the first 28 amino acids of the active protein CS20 were removed, also became inactive (Fig. 2B). These mutants remained inactive even when tested at higher concentration (data not shown). The difference in binding activity of various constructs was not because of a difference in recognition of the bound protein by the antibody, as monoclonal antibody used in all these experiments recognize the central repeat region of the protein, which was not mutated, and on a Western blot recognized all the mutant proteins with equal efficiency (data not shown).

Binding Specificity of TSR Domain Deletion Mutants—CS protein binds to a GAG moiety of liver cell surface proteoglycan (4, 27). The first GAG binding site to be discovered in CS protein is the TSR domain in the carboxyl-terminal region. To determine whether TSR domain deletion mutants (CS20 and CS21) were also specifically binding to a GAG-based cell surface receptor, their binding activity was evaluated in the presence of five different GAGs (heparin, fucoidan, dextran sulfate, chondroitin sulfate A, and chondroitin sulfate B). For both the TSR deletion mutants (CS20, CS21) and the native protein, heparin was the most potent inhibitor of binding with an IC50 of 2–3 μM (Fig. 3A, Table I). The involvement of non-specific and low-affinity receptors in binding was ruled out because complete inhibition of binding of both the truncated and full-length protein was obtained at 50 μM heparin (Fig. 3A). Dextran sulfate had a similar inhibitory effect on the truncated proteins (IC50 2.2–3.8 μM), but a 10-fold excess of dextran sulfate was needed to inhibit the activity of the native protein (IC50 = 32 μM), indicating that dextran sulfate could be binding to both the amino terminus and the carboxyl terminus of CS1 (Fig. 3B, Table I). Fucoidan had an IC50 of 250 μM for all the three proteins (Fig. 3C, Table I). In contrast, chondroitin sulfate A, B, and E are ineffective in inhibiting the binding of full-length CS protein to HepG2 cells (28). The inhibition profile of various GAGs on the binding of TSR deletion mutants was similar to the full-length protein, suggesting that all the three proteins are binding to the identical receptor.

Previously we have shown that, at substoichiometric concentrations, heparin was able to substantially enhance binding of CS protein to HepG2 cells (29, 30). A similar profile was seen
with the mutants, where submolar concentrations of heparin improved the binding activity by 3-fold (Fig. 3A). Dextran sulfate also showed a similar profile for enhancement, whereas fucoidan promoted the binding even when present at molar excess (Fig. 3, B and C).

Binding Activity of TSR Deletion Mutant on Heparinase I- or Chondroitinase ABC-treated Cells—As TSR domain deletions were binding through a GAG-based receptor, the GAG moiety of the receptor was characterized. Binding activity of TSR deletion mutants and the full-length protein was evaluated on HepG2 cells that had been treated with either heparinase I or chondroitinase ABC. Heparinase I primarily cleaves heparin-like regions of heparin-like GAGs (31, 32), whereas chondroitinase has been widely used in depolymerization of GAGs and proteoglycan chondroitin sulfates (33). Treatment of cells with heparinase I resulted in 50–80% decrease in the binding activity of both TSR deletion mutants (CS20, CS21) and the full-length protein (CS1). This indicates that both the TSR deletions and the full-length protein are specifically interacting with heparin-based GAGs. The treatment of HepG2 cells with chondroitinase ABC did not affect the binding of either of the three proteins (Fig. 4).

**Activation of Amino Terminus CS Deletion Mutants by Heparin**—Recently we have shown that the binding activity to HepG2 cells in otherwise inactive CS proteins can be restored in presence of substoichiometric concentrations of heparin (30). To evaluate the capacity of heparin to restore the binding on HepG2 cells, 50 nM inactive amino terminus deletions (CS6, CS7, and CS19) were pre-incubated with 5 nM heparin before being added to the cells. Although heparin did show partial activation of CS19, which lacks only six amino acids, it was unable to activate the other inactive amino terminus deletions (Fig. 5). This indicates that the residues involved in the activation process are localized in the amino terminus of the protein.

**Competition of CS Protein Binding by Peptide**—Deletion of the first six amino acids resulted in a loss of binding activity; thus, a peptide representing the first 20 amino acids of CS1 was synthesized and used as a competitor to inhibit the binding activity of CS1 protein. The peptide failed to inhibit the binding activity (data not shown). On the other hand, CS27, representing the amino terminus of CS protein, inhibited the binding of CS1 and CS20 by 65 and 35%, respectively (Fig. 6), suggesting that the residues involved in binding are distributed across the amino terminus region of the protein and might be close together in the tertiary structure of the protein.

**Inhibition of Sporozoite Invasion**—In a double-blinded experiment, full-length (CS1), and truncated proteins (CS21, CS19, and CS25) were used to compete with *P. falciparum* sporozoites during liver cell invasion. Only intracellular sporozoites that successfully invaded the liver cells in the presence of various concentrations of the full-length or the deletion mutants were counted. The results of this assay corroborated the findings of the binding studies, as at comparable concentrations, CS21, protein lacking the TSR domain, effectively stopped *P. falciparum* sporozoites from invading HepG2 cells and showed very significant inhibition (89%) at the lowest concentration (1 μg/ml) tested. Full-length protein CS1 showed only 36% inhibition at the same concentration (Table II). At the same time, CS19, lacking the first six amino acids, showed minimal inhibition (11%), whereas CS25 was unable to inhibit invasion at all the concentrations tested. CS19 was able to inhibit invasion by 88% at the highest concentration tested (20 μg/ml), whereas similar levels of inhibition were obtained by CS1 at 5 μg/ml concentration, suggesting that CS19 is 4-fold less efficient in inhibiting invasion with respect to the full-length protein.

![Fig. 3. Binding activity of carboxyl terminus deletions in the presence of GAGs.](attachment:image.png)
The CS protein has long been of interest because of its putative role in parasite targeting and invasion of the liver. We constructed eight different deletion mutants of mature CS protein to study what role its various regions might play in the binding and invasion of liver cells by the parasite.

Once the proteins were purified, the contrast between CS deletions and the full-length protein in cell binding was evaluated, and the results were subsequently compared with their potential to inhibit invasion of liver cells by live sporozoites. Surprisingly, in the absence of TSR domain, the truncated protein not only bound to HepG2 cells, it actually showed a 2–3-fold enhancement over the full-length protein (Fig. 2A). In contrast, deletion of the first six residues from amino terminus alone (CS19) resulted in a major loss of binding activity and deletion of more than six residues totally inactivated the protein (Fig. 2B). Binding activity of active TSR deletion mutant (CS20) was also lost when the first 28 amino acids were deleted (CS25), clearly demonstrating that a major binding motif of CS is located in the amino terminus region of the protein. Curiously, although deletion of the first few amino acids (CS19, CS16, CS17, CS6) resulted in a loss of binding to HSPG on HepG2 cells, these proteins had bound to the heparin column. Heparan sulfate, although structurally related to heparin, has low levels of sulfation and contains a glucuronic acid residue in place of the flexible iduronic acid residue found in heparin. Both heparin and heparan sulfate, because of their negative charge, interact with positively charged amino acids in a given protein. In protein CS6 (lacks 28 amino terminus residues), 22% of the remaining amino terminus residues (15 of 67) are positively charged. Binding of CS6, CS16, CS17, CS19, and CS25 to heparin column can be attributed to the flexibility and high sulfate content of heparin, which would have allowed it to interact with positively charged residues of CS protein that otherwise may not be a part of the binding motif involved in interaction with HSPG-based receptor on liver cells.

Could the binding of TSR domain deletions be the result of nonspecific interaction of these truncated proteins with liver cells? To investigate this proposition, we competed the binding of TSR domain deletions (CS20 and CS21) with five different GAGs. These GAGs have been regularly used as competitors by several investigators to evaluate the specificity of CS protein binding to liver cells (4, 27, 28). Reduction in binding activity of these TSR domain deletions in the presence of heparin was comparable with the inhibition profile of the full-length protein CS1 (Fig. 3A). This indicates that, like the full-length protein, the TSR deletion mutants bind through a GAG-based receptor on hepatocytes. Additionally, both the deletion mutant and full-length CS protein lost their binding activity when HepG2 cells were pre-treated with heparinase I (Fig. 4). These experiments indicated that, like the full-length protein, the TSR domain truncated proteins were binding through a heparin-based receptor on hepatocytes.

We have recently shown that heparin, at substoichiometric concentrations, enhances the binding activity of CS protein presumably by cross-linking a large number of CS molecules (29, 30). An enhancement in the binding of TSR domain deletions in the presence of submolar concentrations of heparin indicated that residues involved in enhancement did not relate to the TSR domain. In contrast, proteins truncated at proximal end of the amino terminus, as CS19, lacking the first...
six amino acids, showed only partial activation. Proteins with deletions extending beyond those six amino acids could not be activated (Fig. 5), indicating that the residues involved are located toward the amino terminus of the protein and this phenomenon is not because of nonspecific aggregation of the proteins by GAGs.

An enhancement of CS binding at substoichiometric concentrations of GAGs is not an isolated biological event. Previously, heparin has been shown to mediate adhesion of *Leishmania* amastigotes to cellular receptors (34), and, recently, McCormick et al. (35) have also demonstrated that heparin can enhance the binding of *P. falciparum* infected erythrocytes to human microvascular endothelial cells. Similarly, *T. gondii* tachyzoites show an increased infectivity in the presence of low concentration of sulfated glycoconjugates (36). Hence, a carefully modulated response to varying concentrations of GAGs could be a fine-tuned mechanism that these pathogens have adapted for their propagation and survival.

A peptide including the entire amino terminus (CS27) served to compete with the entire protein for binding to cells. To further elucidate the contribution of this amino-terminal region in binding, we tried to inhibit binding of the CS protein with a peptide containing only the first 20 amino acids of mature *P. falciparum* CS protein (Fig. 6). The 20-mer peptide did not inhibit binding, which may indicate that the residues involved in interaction are not contiguous in the primary structure of the amino terminus, although in a tertiary conformation they could be present close to one another and an intact amino terminus might be essential for providing the correct structural conformation.

A similar situation exists in fibroblast growth factor, another heparin-binding protein, where eight amino acids involved in heparin binding are widely distributed (Asn-27, Arg-120, Thr-121, Lys-125, Lys-129, Gln-134, Lys-135, and Ala-136) but are present close to one another in the tertiary structure of the protein (37, 38). A multiple sequence alignment of the amino terminus of CS protein from various species does suggest the presence of at least four conserved positively charged residues (Arg-9, Lys-34, Lys-43, Arg-46), which could be responsible for the binding activity of CS protein.

An intact amino terminus is required for the binding of CS protein to HepG2 cells. Is an intact amino terminus a prerequisite for invasion? To investigate this proposition, full-length and truncated proteins were evaluated for their capacity to inhibit the invasion of HepG2 cells by *P. falciparum* sporozoites. The results, where sporozoites that successfully invaded the hepatocytes were counted, were consistent with the binding profiles. The TSR domain deletion (CS21) completely inhibited invasion of sporozoites at the lowest concentration. The truncated protein, missing only six amino acids at the amino terminus, was required in a 20-fold excess to achieve the same level of inhibition. More extensive deletions of the amino terminus were totally ineffective as competitors, clearly showing that an intact amino terminus is not only required for binding, it is also a prerequisite for the invasion process. Recently it has been shown that, prior to invasion, attachment of sporozoites to liver cells requires binding of CS protein to cell surface HSPG (39). The inhibition of invasion by TSR deletion mutants could have occurred by effectively competing out sporozoites from attaching on to the liver cells, although blocking subsequent steps that result in invasion could not be ruled out.

We have recently shown that the TSR domain could be effectively neutralized, by converting a conserved cysteine of TSR domain at position 395 to alanine, without losing the binding activity of the protein (30). A similar mechanism has been reported in thrombospondin-related anonymous protein (TRAP), another sporozoite protein, where this domain could be eliminated without affecting the ability of sporozoites to infect mice (40). Our results necessarily alter our vision of sporozoite entry into the liver cells. We propose that, in CS, involvement of TSR domain could be a part of a multistage process of interaction where TSR domain could be acting as a low affinity molecular anchor for the parasite, whereas amino terminus mediates a high affinity and specific cell surface binding. A similar mechanism exists in protease nexin 1, where retention of the protein complex and cell surface binding is mediated by two different regions of the protein (41). Presence of both the motifs in the protein could reflect an optimal equilibrium between the two activities.

**Acknowledgments**—We thank Carl Hammer for mass spectrometry analysis and Mark Garfield for amino terminus sequencing of the recombinant proteins.

**REFERENCES**


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doi: 10.1074/jbc.M106862200 originally published online December 20, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106862200

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