Cell Adhesion to a Motif Shared by the Malaria Circumsporozoite Protein and Thrombospondin Is Mediated by Its Glycosaminoglycan-binding Region and Not by CSVTCG*

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The malaria circumsporozoite protein (CS), thrombospondin (TSP), and several other proteins including the terminal complement peptides and the neural adhesion molecules F-spondin and Unc-5, share a cell adhesive sequence. In CS this sequence is designated as region II-plus (EWSPCSVTCGNGIQVRIK) and in TSP it is found in the type I repeats. Previous studies aimed at defining the amino acid residues required for cell adhesion have yielded discrepant results. Here we show in three different cell lines that the downstream basic residues are required for cell adhesion whereas the CS- VTCG sequence is not. Using mutant Chinese hamster ovary cells selected for deficiencies in proteoglycan synthesis, we show that in wild type cells, heparan sulfate proteoglycans are the binding sites for this motif. This finding is supported by additional experiments with two other cell lines demonstrating that treatment with heparitinase but not chondroitinase abolishes cell adhesion to peptides representing this motif. Using Chinese hamster ovary cell mutants deficient in heparan sulfates proteoglycans but possessing chondroitin sulfate proteoglycans, we show that cell surface chondroitin sulfate proteoglycans can also mediate binding to this motif although higher concentrations of peptides are required for adhesion. Chondroitinase, but not heparitinase, treatment of these cells destroys cell surface binding sites. Taken together, these results indicate that cell adhesion to this motif involves an interaction between the downstream positively-charged residues and the negatively charged glycosaminoglycan chains of heparan sulfate, or in some cases chondroitin sulfate, proteoglycans on the cell surface.

The major surface protein of malaria sporozoites, the circumsporozoite protein (CS),

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‡The abbreviations used are: CS, circumsporozoite protein; TSP, thrombospondin; GAG, glycosaminoglycan; CHO, Chinese hamster ovary; FCS, fetal calf serum; PBS, phosphate-buffered saline; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan.

malaria sporozoites. Thrombospondin (TSP), a 420-kDa glycoprotein that functions in the attachment, migration, and proliferation of many different cell types, has several cell adhesive domains (reviewed in Refs. 6–8). One of these lies within the type I repeats and has strong sequence similarity with region II-plus of CS (Fig. 1A). Another sporozoite surface protein, TRAP/SSP2 (9–11), the terminal complement components (12), the complement protein properdin (12), and the neural adhesion molecules F-spondin (13) and Unc-5 (14) also contain similar sequences (Fig. 1A). The importance of this sequence has been demonstrated in several systems. In the life cycle of the malaria parasite, it is responsible for the rapid and specific homing of sporozoites to hepatocytes where the first stage of malaria infection is initiated (15, 16). In thrombospondin this sequence mediates cell adhesion (17), and peptides representing this sequence have anti-angiogenic activity (18) and inhibit platelet aggregation (19) and melanoma metastasis (19).

The shared adhesive motif includes two cysteines at the amino-terminal and a series of positively charged amino acids at the COOH-terminal (Fig. 1A). Studies aimed at the identification of the amino acids required for cell adhesion have yielded discrepant results. Several groups of investigators, using thrombospondin-derived sequences, have reached the conclusion that the CSVTCG segment of the motif is responsible for cell adhesive activity (2, 19). In contrast, our work in malaria with region II-plus peptides has demonstrated the importance of the downstream positively-charged amino acids (4). We have shown that these residues are required for CS binding to hepatocytes and that they interact with the negatively-charged glycosaminoglycan chains (GAGs) of proteoglycans on the cell membrane.

One important discrepancy between the two series of studies lies in the methodology used to detect the interaction between target cells, and peptides representing various stretches of the adhesion motif. The standard assay used by most investigators measures the binding of live cells to immobilized peptides, while in our previous studies, peptides were in solution and we measured their binding to fixed and immobilized target cells. Although similar peptide ligands were used in all of these studies, cell adhesion is a multivalent process and may involve a different set of receptors than a protein-ligand interaction such as CS binding to hepatocytes. In addition, different cell lines were used in these studies. Our experiments were performed with a hepatoma cell line, HepG2, whereas others used a variety of cell lines none of which was derived from hepatocytes.

In an attempt to resolve these discrepancies, we used a variety of cell lines in a standard cell adhesion assay to analyze the interaction between live cells and peptides representing this cell-adhesive motif from CS (PCSVTCGNGIQVRIK). We
tested modifications of both the peptide ligand and the cell surface-binding sites to determine the role of the downstream basic residues and the CSVTCG segment of this motif in cell adhesion.

**MATERIALS AND METHODS**

**Peptides**—Peptides were synthesized by Boc chemistry using the multiple peptide synthesis method described by Houghten (20). Cleavage from the resin was performed with hydrofluoric acid in two steps. A low concentration of hydrofluoric acid, used to remove protecting groups from the amino acid side chains, was followed by a high concentration of hydrofluoric acid which removed the peptide from the resin. Purity was verified by high performance liquid chromatography and amino acid analysis. CSVTCG peptides were synthesized in two ways, with blocked cysteines using acetylated groups and also with free sulfhydryls. These peptides were further purified on an FPLC C_{18} column using an acetonitrile gradient of 1 to 20% with 0.05% trifluoroacetic acid. CSVTCG without blocked cysteines was reduced with 50 mM dithiothreitol for 1 h before purification on the FPLC C_{18} column. The presence or absence of free sulfhydryl groups in CSVTCG peptides was confirmed with the Ellman reaction (21) before each experiment. The full-length region II-plus peptides used as soluble inhibitors in cell binding assays were partially oxidized and contained trimerers, trimers, dimers, and monomers by gel filtration chromatography and mass spectrometry (4).

**Cells**—Mutant and wild-type Chinese hamster ovary (CHO) cells were grown in Ham's F-12 (Life Technologies, Inc.) supplemented with 7.5% fetal calf serum (FCS) and 1 mM glutamine. The growth, isolation, and cryogenic storage of cells is described in Ref. 22. The mutants used in this study had the following characteristics: mutant pgA-745 lacks xylosyltransferase activity and produces less than 2% of wild-type levels of glycosaminoglycans (23). pgD-677 does not produce chondroitin sulfate due to a deficiency in the chain polymerization enzyme (23). pgsD-677 does not produce wild-type levels of glycosaminoglycans (23). pgsE-606 has diminished GlcNAc N-sulfotransferase activity, which results in the formation of heparan sulfate chains with less overall sulfation (25–27).

Human cell lines were obtained from the American Type Culture Collection (Rockville, MD). MG63 cells (CRL 1427) are a human osteosarcoma line and were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) with 10% FCS and 1 mM glutamine. The growth, isolation, and cryogenic storage of cells is described in Ref. 22. The cells were allowed to grow for 18–24 h, the medium was reduced back to complete medium with the indicated concentration of peptide. Incubations with peptide were for 30 min at 37 °C on an orbital rotator in sterile Eppendorf tubes that had been blocked with 5% bovine serum albumin for 2 h at 37 °C to avoid peptide and cell adhesion to the sides of the tubes. The tubes were then plated without being washed, in wells that had been coated with 1.25 μg/ml region II-plus peptide and blocked as above.

**Glycosaminoglycan Inhibition**—Cells were prepared as above and after 3 washes in incomplete medium were resuspended in incomplete medium with the indicated concentration of peptide. Incubations with peptide were for 30 min at 37 °C on an orbital rotator in sterile Eppendorf tubes that had been blocked with 5% bovine serum albumin for 2 h at 37 °C to avoid peptide and cell adhesion to the sides of the tubes. The cells were then plated without being washed, in wells that had been coated with 1.25 μg/ml region II-plus peptide and blocked as above.

**RESULTS**

In initial experiments we measured the adhesion of live cells to region II-plus peptides (PCSVTCGNGIQVRKPGSAN) immobilized onto wells of microtiter plates. For these studies we used two cell lines, one of which, K562 cells, had been previously shown to adhere to the CSVTCG portion of this motif (2).

The other cell line, MG63, is a standard cell line used in adhesion studies although the fine specificity of its interaction with this motif had not been previously studied. The full-length region II-plus peptide used in this study (Fig. 1B) contains a 5-amino acid extension, PGSAN, present for reasons extrane-
ous to the current study. We have previously shown that these additional amino acids do not interfere with or enhance binding activity of the peptide (4). As shown in Fig. 2, both MG63 and K562 cells adhere to region II-plus peptides in a dose-dependent manner. To determine which portion of this motif is required for cell adhesion, we tested the truncated and substituted peptides shown in Fig. 1B for cell adhesive activity. When the NH$_2$-terminal portion was truncated but the downstream portion with the basic and hydrophobic residues was intact (CGNGIQVRIKPGSAN), the cells bound to the peptide-coated wells with identical efficiency (Fig. 2). However, neither cell line adhered to peptides in which the downstream basic residues had been replaced by either negatively charged or neutral amino acids (PCSVTCGNGIQVEIEPGSAN and PCSVT CGNGIQVNIN), even though the entire NH$_2$-terminal CSVTCG sequence was present (Fig. 2).

The apparent lack of activity of CSVTCG is in contradiction with the results of Tuszynski et al. (19). In their studies, however, the short CSVTCG peptide was blocked with acetamide groups and another with free sulfhydryl groups (Fig. 1B). As shown in Fig. 2, however, neither cell line adhered to the wells coated with these peptides. To investigate whether these short peptides had indeed bound to the plastic, we performed experiments with a slightly longer and more hydrophobic, $^{125}$I-labeled YCSVTCG peptide. We found that, as is frequently the case for short peptides, YCSVTCG bound very poorly to plastic (data not shown) and therefore could not be reliably used as a substrate for cell adhesion. For this reason, we tested CSVTCG as a soluble inhibitor of cell adhesion to immobilized PCSVT CGNGIQVRIKPGSAN.

As shown in Fig. 3, adhesion of both cell lines is inhibited by soluble full-length peptide, whereas CSVTCG peptide, at high concentrations has no inhibitory activity. Because the oxidation state of the cysteines in CSVTCG could influence the cell adhesive activity of this sequence, we used CSVTCG peptides whose cysteines were blocked and peptides whose cysteines...
contained free sulfhydryls. Neither peptide inhibited cell adhesion to the full-length sequence.

The requirement of the downstream basic residues for cell adhesive activity suggested that an ionic interaction between this sequence and negatively-charged cell surface molecules was involved. Others have shown that this motif binds to sulfated glycoconjugates (17, 29–33) and our previous studies using fixed immobilized cells demonstrated that soluble region II-plus peptides bound to the heparan sulfate (HS) GAGs of cell surface HSPGs (4, 34). To investigate which sulfated glycoconjugate on the cell surface bound to this motif in live-cell adhesion assays, we used mutant CHO cells selected for deficiencies in GAG synthesis. As shown in Fig. 4, wild-type CHO cells, similarly to MG63 and K562 cells, adhere to the full-length region II-plus peptide (PCSVTCGNGIQVRIKPGSAN). Mutant CHO cell line pgsA, lacking proteoglycan GAGs, does not bind to the peptide. When GAGs are partially undersulfated, as in mutant cell lines pgsE and pgsF, there is decreased cell binding when compared with wild-type cells (Fig. 4). These results suggest that the GAGs of cell surface proteoglycans mediate binding to region II-plus peptides and that this is due, at least in part, to an ionic interaction between the basic residues of this cell adhesive motif and the negatively charged sulfate moieties of cell surface GAGs.

We then used GAG lyases to investigate the identity of the cell surface GAGs which mediate adhesion to this motif since these cell lines have chondroitin sulfate proteoglycans as well as HSPGs (Table I). As shown in Fig. 5, heparitinase treatment abolishes adhesion of MG63, K562, and wild-type CHO cells to this sequence whereas chondroitinase treatment does not significantly decrease cell binding, suggesting that HS and not chondroitin sulfate GAGs mediate binding to this motif. To confirm this finding, we performed studies with the CHO mutant pgsD, which has very low amounts of cell surface HS (<2% of wild-type cells) but increased amounts of chondroitin sulfate GAGs (24). To our surprise, we found that these cells bound to region II-plus peptides although with lower efficiency when compared with wild-type CHO cells (Fig. 6A). The role of chon-
Chondroitin sulfate GAGs in binding of the CHO pgsD cells to region II-plus peptides was confirmed with the demonstration that heparitinase treatment of these cells had little effect on cell adhesion whereas chondroitinase treatment completely abolished binding to region II-plus peptides (Fig. 6B). These results suggest that both HSPGs and chondroitin sulfate proteoglycans can mediate binding to this motif. However, HSPG-deficient pgsD cells require higher concentrations of peptide for binding when compared with wild-type cells, suggesting that HSPGs bind with higher avidity to this motif than chondroitin sulfate.

We then used soluble GAGs as competitive inhibitors of cell adhesion to compare the avidity of binding of chondroitin sulfate and heparan sulfate to this motif (Fig. 7). Binding of MG63 cells, K562 cells (data not shown), and wild-type CHO cells is completely inhibited in the presence of 1 μg/ml heparin. Higher concentrations of chondroitin sulfate B (10–100 μg/ml) can inhibit binding of these cells to region II-plus peptides, however, chondroitin sulfates A and C do not significantly inhibit cell adhesion. Binding of CHO mutant pgsD to region II-plus peptides is inhibited in the presence of 1 μg/ml heparin, similar to the wild-type cell line. However, 10-fold less chondroitin sulfate B is required to inhibit adhesion of these cells when compared with wild-type cells and chondroitin sulfates A and C also inhibit at lower concentrations. In these experiments, cell surface GAGs are competing with soluble GAGs for binding to immobilized region II-plus peptides. These results therefore demonstrate that higher concentrations of soluble chondroitin sulfate are required to compete with cell surface HS than with cell surface chondroitin sulfate for binding to this motif.

**DISCUSSION**

These studies confirm the findings of Prater et al. (17) demonstrating the cell adhesive properties of the conserved motif found within the type I repeats of TSP. Our fine mapping of the residues required for cell adhesion, however, are not in agreement with previous studies (2, 19). The present findings demonstrate that the downstream basic residues are required for cell adhesion whereas the CSVTCG sequence is not. If CSVTCG is present without the downstream basic residues, cell adhesive activity is lost whereas truncated peptides beginning with the second cysteine and including the downstream residues support cell adhesion with an efficiency equal to that of full-length peptide. Unlike other investigators, we could not demonstrate cell adhesive activity using the short CSVTCG peptide either when it was used to coat microtiter plate wells or when used as a soluble inhibitor.

More studies are required to explain the role of the CSVTCG portion of the motif. One possibility is that its role is structural. We have previously shown that only multimers of the downstream basic residues bind to cell surface HS (2). Consistent with this finding is the fact that most proteins containing this motif have two to six copies of this sequence (6, 12–14) so that folding of the protein could bring multiple GAG-binding sequences together. Of all the proteins containing this motif, only the malaria CS and TRAP/SSP2 proteins do not contain multiple copies of this sequence. In the case of CS, however, the requirement for aggregation is met by the high density of CS molecules, as well as the presence of disulfide-linked aggregates (4), on the surface of the parasite. CSVTCG may therefore contribute to the formation of a structure that aligns the downstream basic residues of these motifs from different regions of the same protein or, in the case of CS, from different CS monomers. This may be accomplished either via disulfide bond formation between cysteines, or via hydrophobic interactions between the side chains of the intervening amino acids, from different region II-plus sequences or type I TSP repeats. The importance of hydrophobic residues in the formation of structures in which the basic residues are aligned with one another has been demonstrated for other heparin-binding proteins whose crystal structures have been resolved or inferred (35, 36). In this study, the requirement for aggregation was met by immobilization of the peptides in microtiter plate wells. In this way, the peptides are within sufficient proximity of one another such that their heparin binding activity is intact. In the experiments where region II-plus peptides are used as soluble inhibitors they had been oxidized and contained tetramers, trimers, and dimers as well as monomers (4).

Alternately the CSVTCG portion of the motif may bind to a cell surface receptor after the downstream basic residues bind to proteoglycans. TSP is known to bind to CD36 (37–40) and some investigators have suggested that this binding is CSVTCG-dependent (41–43). However, in some of these studies the peptides used contained the downstream basic residues as well as CSVTCG so it is not clear which portion of the motif binds to CD36.

We provide additional evidence for the involvement of the downstream basic residues in cell adhesion with the demonstration that negatively-charged GAGs of cell surface proteoglycans serve as binding sites for this motif. Mutant cells lacking GAGs and cells whose GAGs have been digested with
enzymes do not bind to region II-plus peptides. In addition, experiments with mutant cell lines pgsE and pgsF show that altered sulfation of HS decreases cell adhesion to region II-plus peptides. These two mutant cell lines have different sulfotransferase deficiencies (25, 28). pgsE cells lack the enzyme responsible for removal of N-acetyl groups and the addition of N-sulfate groups to a subset of N-acetylglucosamine residues in HS. This enzyme acts first during polymer modification reactions and creates regions of modified residues interspersed with regions of unmodified residues. After N-deacetylation/N-sulfation, the adjacent glucuronic acid residues epimerize to iduronic acid, and sulfate groups are added to the hydroxyl groups at C2 of the uronic acids and C6 of the glucosamine residues. The modifications occur in blocks along the chains much like variably sized beads on a string. The defect in pgsE cells depresses N-sulfotransferase activity by about a factor of 2 to 3, causing a decrease in the frequency of modified blocks along the chain (25). Interestingly, the overall degree of modification within the blocks does not change significantly (26). The structure of HS from pgsF cells differs significantly from that of pgsE cells. These cells lack the 2-O-sulfotransferase that acts on the uronic acid residues within the blocks (28). A side effect of this mutation is that the chains are actually more highly N-sulfated, suggesting that the blocks of modified residues are somewhat longer or more frequent. Nevertheless, the lack of 2-O-sulfotransferase activity diminishes the degree of sulfation within the blocks. Both strains attach to region II-plus peptides to a lesser extent than wild-type cells, suggesting that both the degree of sulfation (pgsE) and the pattern of sulfation (pgsF) are important.

**FIG. 5. Adhesion of cells to region II-plus peptides after treatment with GAG lyases.** MG63 cells (panel A), K562 cells (panel B), or CHO wild-type cells (panel C) were preincubated with the indicated concentrations (units/ml) of chondroitinase ABC (open circles) or heparitinase (closed circles) for 3 h at 37 °C, then added to wells coated with 1.25 µg/ml region II-plus peptide (PCSVCNQGIVKPGSAN) and allowed to adhere for 1 h at 37 °C. Bound cells were quantified with crystal violet. Each point was performed in triplicate and shown are the means with standard deviations.
Here we demonstrate that in addition to HS, cell surface chondroitin sulfate can also serve as adhesion receptors for this motif. The mutant CHO cell line pgsD lacks the enzymes required for HS GAG chain polymerization and makes ~3-fold more chondroitin sulfate than wild-type cells so that the total amount of GAGs remains the same. This mutant binds to region II-plus peptides and binding is abolished with chondroitinase but not heparitinase treatment of the cells. These cells, however, require higher concentrations of peptide for adhesion and binding is more easily inhibited with sulfated compounds when compared with wild-type cells, suggesting that there is a lower avidity interaction between chondroitin sulfate and this cell adhesive motif.

Using some of the same mutant CHO cell lines, others have shown that adhesion to the entire TSP molecule is largely dependent upon cell surface HSPGs, although a mutant deficient in HS HSPGs but possessing chondroitin sulfate proteoglycans (pgsD) also binds to TSP (44, 45). In addition, a more recent study has shown that myoblasts bind to TSP via cell surface chondroitin sulfate and that this interaction is mediated in part by the type I repeats of TSP (46). Our demonstration that the motif of basic and hydrophobic residues found within the type I repeats can bind to chondroitin sulfate, suggests that these residues are involved in the adhesion of mutant CHO cells and myoblasts to TSP.

Interestingly, K562 cells have relatively more chondroitin sulfate than heparan sulfate proteoglycans on their surface and yet heparinase digestion of these cells abolishes binding to region II-plus peptides and, in contrast to the CHO pgsD cells, chondroitinase digestion does not. This may be due to differences in the type and structure of the chondroitin sulfate, to differences in the display of cell surface chondroitin sulfate or in the absolute amount of chondroitin sulfate chains possessed by these two cell lines. It has been previously shown for the syndecans that chondroitin sulfate chains are located near the transmembrane domain of the core protein and HS chains are
found more NH₂-terminal (47, 48). In the CHO pgsD mutant, chondroitin sulfate chains are substituted for HS chains and so at least a portion of them will be located N-terminal and may therefore be more accessible for binding to receptors.

Another region within the type I repeats of TSP has been shown to have heparin binding activity (49, 50). NH₂-terminal to the CSVTCG sequence is a conserved tryptophan-containing region, SHWSPWSS. The minimum sequence necessary for heparin binding activity is the pentapeptide WSPWS. However, not all of the proteins shown in Fig. 1 contain this heparin-binding sequence, including the malaria CS proteins. It is possible that WSPWS has a different GAG chain specificity than the downstream cluster of basic amino acids. Alternately, when both heparin-binding motifs are present, as in the case of TSP, the affinity of the cell surface receptor-ligand interaction may increase. In support of this, Guo et al. (49) have shown that when a cluster of positively charged residues was added to the SHWSPWS sequence, heparin binding activity of the peptide was enhanced 10-fold (49).

In some instances, therefore, the CSVTCG sequence is flanked on both sides by heparin- or GAG-binding domains. We previously discussed the possibility that CSVTCG performs a structural role, aligning the downstream basic residues from different region II-plus motifs, or type I TSP repeats, for multimeric binding to HSPGs. An alternate possibility is that, in some cases, it may be part of a structural motif that permits sequences flanking it on both sides to bind to HSPGs. It is also possible that binding of the downstream basic residues of the region II-plus motif, and/or the WSPWS sequence discussed above, to HSPGs is a prerequisite for the participation of the CSVTCG sequence in a subsequent binding event. Future work will hopefully elucidate the function of the CSVTCG portion of this motif which together with existing data will shed some light on the significance of this conserved sequence.

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2 L. Zhang and J. Esko, unpublished data.
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