Malaria circumsporozoite protein inhibits protein synthesis in mammalian cells

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Native *Plasmodium* circumsporozoite (CS) protein, translocated by sporozoites into the cytosol of host cells, as well as recombinant CS constructs introduced into the cytoplasm by liposome fusion or transient transfection, all lead to inhibition of protein synthesis in mammalian cells. The following findings suggest that this inhibition of translation is caused by a binding of the CS protein to ribosomes. (i) The distribution of native CS protein translocated by sporozoites into the cytoplasm as well as microinjected recombinant CS protein suggests association with ribosomes. (ii) Recombinant CS protein binds to RNase-sensitive sites on rough microsomes. (iii) Synthetic peptides representing the conserved regions I and II-plus of the *P.falciparum* CS protein displace recombinant CS protein from rough microsomes with dissociation constants in the nanomolar range. (iv) Synthetic peptides representing region I from the *P.falciparum* CS protein and region II-plus from the *P.falciparum*, *P.berghei* or *P.vivax* CS protein inhibit *in vitro* translation. We propose that *Plasmodium* manipulates hepatocyte protein synthesis to meet the requirements of a rapidly developing schizont. Since macrophages appear to be particularly sensitive to the presence of CS protein in the cytosol, inhibition of translation may represent a novel immune evasion mechanism of *Plasmodium*.

Keywords: circumsporozoite protein/immune evasion/membrane translocation/*Plasmodium*/protein synthesis

Introduction

After the discovery that circumsporozoite (CS) protein is the most abundant protein on the surface of malaria sporozoites in the early 1980s (reviewed in Nussenzweig and Nussenzweig, 1985), the function of CS protein remained unknown for many years. Recent evidence suggests that the CS protein is a multifunctional molecule that plays a crucial role at various points of the malaria life cycle. CS protein is translocated continuously from the anterior to the posterior cell pole and has, therefore, been implicated in sporozoite gliding motility (Stewart and Vanderberg, 1991). The finding that malaria sporozoites invade the liver of the vertebrate host within minutes after

transmission by an infected mosquito (Shin *et al.*, 1982) suggested a receptor-mediated clearance mechanism. We and others have shown that the CS protein binds to highly sulfated, heparin-like oligosaccharides in heparan sulfate on the basolateral membrane of hepatocytes in the space of Disse (Cerami *et al.*, 1992; Pancake *et al.*, 1992; Frevert *et al.*, 1993; Ying *et al.*, 1997), and this interaction is held responsible for the rapid and selective targeting of the sporozoites to the liver sinusoid (Cerami *et al.*, 1994; Sinnis *et al.*, 1994, 1996). *In vitro*, the CS protein binds with high affinity to the low density lipoprotein receptor-related protein (LRP), and the dual interaction with heparan sulfate and LRP plays a dominant role in sporozoite invasion (Shakibaei and Frevert, 1996). Most recently, CS protein knock-out studies have demonstrated the crucial involvement of the CS protein in the formation of sporozoites in the mosquito midgut (Ménard *et al.*, 1997), and the presence of receptors on salivary glands has suggested a role for the CS protein in sporozoite adhesion to this organ of the mosquito (Sidjanski *et al.*, 1997).

We present data here which show that the CS protein may also have a function within cells of the vertebrate host. Upon cell contact, *Plasmodium berghei* and *P.yoelii* sporozoites actively translocate CS protein across the cell membrane into the cytoplasm of mammalian cells *in vitro* (Hügel *et al.*, 1996). This translocation occurs in the absence of parasite invasion by an as yet unknown mechanism. It requires neither the metabolic nor the endocytic machinery of the mammalian cell. The CS protein spreads throughout the entire cytosol of the affected cell and binds to cytosolic and endoplasmic reticulum-associated ribosomes (Hügel *et al.*, 1996). Native CS protein, when translocated by *P.berghei* or *P.yoelii* sporozoites, as well as recombinant *P.falciparum* CS protein, when microinjected into the cytosol of mammalian cells, also co-localizes with markers for the rough endoplasmic reticulum. Furthermore, we have shown that recombinant CS protein binds to RNase-sensitive sites on purified rough microsomes from rat liver. Based on these observations, we hypothesized that malaria sporozoites are able to control the protein synthesis machinery in cells of the vertebrate host. The data presented here also suggest that malaria sporozoites are able to eliminate non-invaded bystander cells, in particular macrophages, by means of the ribotoxic properties of the CS protein. Ordinarily, encounters with professional phagocytes and other antigen-presenting cells would be expected to lead to an immune response. Surprisingly, however, in endemic populations, immunity against natural infection with *Plasmodium* is generated slowly and is rarely, if ever, complete. The observed action of CS protein reported here offers an explanation for this discrepancy in that it would limit the presentation of parasitic antigens and, consequently, the generation of an effective humoral and cytotoxic T-cell response.

Results

Native *P.berghei* sporozoite-translocated CS protein inhibits protein synthesis in mammalian cells

After short co-cultivation periods, a large number of non-invaded HepG2 cells contained CS protein in the cytoplasm, translocated across the cell membrane by extracellularly attached sporozoites (Figure 1A). Other cells contained both cytoplasmic CS protein and a sporozoite (Figure 1C). Cells that contained CS protein in their cytoplasm, but no parasite, had not incorporated [³H]leucine, glycine or serine (Figure 1B), suggesting a complete arrest of protein synthesis. Sporozoite-infected cells were autoradiography-negative only early after parasite entry (30 min of co-cultivation); at later time points, invaded cells were radiolabeled normally despite the presence of CS protein in the cytoplasm surrounding the sporozoite (Figure 1D). Similar results were obtained for CHO-K1 cells (data not shown). Cells that showed no protein synthesis by microautoradiography were never found in cell cultures that had not been exposed to parasites, thus excluding the possibility that the CS protein binds preferentially to dead cells.

Malaria sporozoites inhibit protein synthesis in phagocytic cells

Since encounters with macrophages can be expected to have detrimental consequences for parasite survival *in vivo*, we asked if sporozoites are able to translocate cytotoxic amounts of CS protein into phagocytic cells thereby killing them. In striking contrast to the non-phagocytic HepG2 or CHO cells, the number of invaded macrophages was negligible even after 2 h of co-cultivation. However, a large number of the phagocytes contained CS protein in their cytoplasm, as indicated by the intense immunofluorescence labeling with monoclonal antibody (mAb) 3D11 (Figure 1E). Compared with unaffected neighboring macrophages, the CS protein-containing cells were small and rounded and were apparently dying (Figure 1F). Support for this notion was obtained in another set of experiments, in which the cultures were first co-cultivated with the sporozoites and then metabolically labeled with [³H]leucine or [³H]glycine. Combined immunofluorescence and microautoradiography studies of these cultures revealed that, in contrast to the neighboring control cells, the CS protein-containing macrophages had not incorporated any radiolabel (Figure 1G). These morphologic and metabolic changes in the macrophages had to be caused by the sporozoites, since neither small and rounded nor autoradiography-negative cells were found in uninfected cultures (data not shown). Thus, sporozoites are able to inhibit protein synthesis efficiently in phagocytic cells.

Expression of CS protein in the cytoplasm terminates protein synthesis in CHO cells

To elucidate whether CS protein alone is sufficient to inhibit protein synthesis in mammalian cells, a pCRTM3 plasmid coding for the central portion of the *P.falciparum* (amino acids 28–461) or the *P.berghei* (amino acids 34–308) CS protein, or coding for chloramphenicol acetyl transferase (CAT) was transiently transfected

into CHO pgsA cells using the transfection agent lipofectamine. The population of transfected cells consisted of flatly spread cells whose morphology, compared with the non-transfected cells, was unaltered. These cells contained CS protein in their entire cytoplasm in a distribution very similar to native, parasite-translocated CS protein (Figure 2A). Some transfected cells appeared to be severely damaged and exhibited only a narrow rim of CS protein-labeled cytoplasm around the nucleus (Figure 2C). Small rounded cells were not found in control cultures that had been transfected with CAT or incubated with lipofectamine alone in the absence of DNA or with DNA alone in the absence of lipofectamine (data not shown) indicating that cessation of protein synthesis was not caused merely by the transfection process.

To determine the effect of CS protein expression on protein synthesis, CHO pgsA cells were transfected with plasmids containing the *P.falciparum* or *P.berghei* CS constructs or with CAT under identical conditions. Some cells were faintly positive for the *P.falciparum* CS protein by immunofluorescence (Figure 2A), suggesting that expression of CS protein in these normally spread cells had just started. These cells consistently exhibited weak radiolabeling as determined by microautoradiography (Figure 2B). In cells that showed a strongly positive CS protein reaction, incorporation of tritiated leucine was never observed. Small degenerate cells were also always negative by microautoradiography (Figure 2D). In contrast, CHO pgsA cells expressing CAT were able to incorporate tritiated leucine like non-transfected control cells (Figure 2E and F). Autoradiography-negative cells were not found in control cultures that had been exposed to lipofectamine alone (data not shown). These findings suggest a complete arrest of protein synthesis in the transfected cells shortly after the cytoplasmic appearance of expressed CS constructs, but not of the control protein CAT.

Recombinant liposome-encapsulated *P.falciparum* CS protein inhibits protein synthesis in HepG2 cells

Subconfluent HepG2 cell cultures were incubated with fusogenic unilamellar liposomes containing in their lumen either the *P.falciparum* CS constructs CSFZ(Cys) or CS27IC, fluorescein isothiocyanate (FITC)-dextran or buffer. The CS protein-containing liposomes inhibited the incorporation of radiolabel into cellular proteins in a concentration-dependent fashion by a maximum of 45.3–76.5% at 4 h, whereas the control liposomes (containing no CS protein) had no effect (Figure 3A and B, Table 1A). The incorporation of radiolabel into the secreted hepatocyte proteins transferrin and albumin was monitored by immunoprecipitation. CS protein-containing liposomes inhibited the incorporation of radiolabel into these proteins with a maximum of 65.3 and 58.8%, respectively (Figure 3C and Table 1B). Control liposomes had no effect. Corresponding results were obtained for liposomes containing another CS construct (CS27IC) and another type of control liposomes (containing FITC-dextran; data not shown). Under all experimental conditions, the percentage of dead cells

Fig. 1. Protein synthesis in HepG2 cells and macrophages harboring native *P. berghei* CS protein is inhibited. (A) Double immunofluorescence labeling of a *P. berghei*-infected HepG2 cell culture shows two cells that contain Texas red-labeled CS protein in the cytoplasm (red). An extracellular sporozoite (arrow) is labeled with FITC (green), indicating its attachment to the outer surface of the HepG2 cell. (B) Autoradiographic exposure of the same field shows that in contrast to the neighboring control cells, the two cells that contain CS protein (red) have not incorporated [^3H]leucine as demonstrated by the lack of silver grains (green) in the photographic emulsion. (C) A sporozoite (arrow) has invaded a HepG2 cell and released CS protein (red Texas red label) into the cytoplasm of the cell. A neighboring cell contains CS protein, but no parasite (arrowhead). (D) Examination of the incorporation of radiolabel into these cells reveals that only the non-infected cell (arrowhead) is microautoradiography-negative, whereas the infected cell is covered by the same amount of silver grains as the neighboring control cells. (E) A 2 h co-cultivation period of *P. berghei* sporozoites with peritoneal macrophages causes the occurrence of large numbers of small degenerate cells whose nucleus is surrounded by a narrow rim of cytoplasm strongly stained for CS protein (green immunofluorescence labeling with mAb 2A10 and GAM-FITC). The cells appear red due to the counterstaining with Evans blue. (F and G) The combined immunofluorescence/microautoradiography assay reveals that the degenerate CS protein-positive macrophages (shown in green in F, arrow) do not exhibit any incorporation of tritiated leucine as shown by the missing silver grains (green) in the photographic emulsion, whereas the surrounding unaffected macrophages are strongly microautoradiography-positive. Bars in μm as indicated.

remained below 1% over the entire 6 h observation period (and up to 72 h) by Trypan Blue exclusion (data not shown), excluding liposome-mediated cell death as

a cause for the observed inhibition of incorporation of radiolabel into proteins. As judged by immunofluorescence, only a very small amount of CS protein had

Fig. 2. CHO cells expressing *Pfalciparum* CS protein in their cytoplasm are inhibited in protein synthesis. The cells were cultivated for 24 h after transfection and metabolically labeled with tritiated leucine. The cells were immunofluorescently labeled with mAb 2A10 and GAM-FITC (shown in green in A, C and D), counterstained with Evans blue (shown in red in all images) and exposed for microautoradiography. The distribution of the silver grains in the photographic emulsion for the corresponding fields is shown in green in (B), (D) and (F). The CS protein-positive cell in (A) represents the population of cells exhibiting a normal morphology, whereas the cell in (C) is obviously degenerate. The transfected cell in (A) has expressed only a low amount of CS protein and is covered by a small number of silver grains (B). The small degenerate cell (C), whose nucleus is surrounded only by a narrow rim of strongly CS protein-positive cytoplasm, has not incorporated any tritiated leucine, as shown by the lack of silver grains in the photographic emulsion (D). Cells expressing CAT (E) exhibit a normal incorporation of radiolabel (F). Bars in μm as indicated.

been translocated into the cells by the liposomes compared with the amount introduced by sporozoites or by transient transfection (data not shown). Thus,

inhibition of protein synthesis was incomplete under these experimental conditions, and cell death did not occur.

Recombinant CS protein binds with high affinity and specificity to RNase-sensitive sites on purified rat liver microsomes

To analyze the mode of interaction of the CS protein with the rough endoplasmic reticulum in more detail, we examined the binding of the iodinated recombinant CS construct CS27IVC to purified microsomes from rat liver. Pre-treatment of the microsomes with RNase A inhibited the binding of CS27IVC in a concentration-dependent manner with a maximum of 89.9% at 100 U/ml. In contrast, DNase pre-treatment had no effect on the interaction at 100 U/ml. Likewise, heparinase pre-treatment, which abolishes the binding of CS protein to heparan sulfate proteoglycans (Frevert *et al.*, 1993), was ineffective at 10 U/ml (Figure 4A). No binding of CS27IVC occurs to bovine serum albumin (BSA)-coated wells under these experimental conditions (Shakibaei and Frevert, 1996). To obtain insight into the specificity of the interaction between the CS protein and rough microsomes, we analyzed the inhibitory effect of CS peptides on the binding of the CS construct CS27IVC. In three independent experiments, a peptide representing the conserved region II-plus from the *P.falciparum* CS protein (PCSVTCGNGIQVRIKPGSAN) inhibited the binding of CS27IVC in a concentration-dependent fashion with a maximum of 59.6% at 100 µg/ml (50 µM), whereas a region II-control peptide (PCSVTCGNGIQVEIEPGSAN), in which the basic amino acids had been replaced by glutamic acid, was inactive. A peptide containing the conserved region I (GNEDNEKLRLKPKHKKLKQP) inhibited the binding by 51.6% at 100 µg/ml (42 µM) (Figure 4B). CS27IVC was displaced by the region II-plus and region I peptides with a K_D of 7.3–8.4 nM and 105.5–189.6 nM, respectively, suggesting affinities of $1.2\text{--}1.4 \times 10^8/\text{M}$ and $5.3\text{--}9.5 \times 10^6/\text{M}$, respectively. It appears, therefore, that the binding of CS27IVC occurs with high affinity and specificity.

Synthetic CS peptides inhibit *in vitro* translation

The *P.falciparum* region I and the region II-plus peptide inhibited *in vitro* translation in a concentration-dependent fashion by a maximum of 41.7–63.4% and 58.6–71.5%, respectively, at a concentration of 400 µg/ml (Table II). Similarly, the *P.berghei* and the *P.vivax* region II-plus peptides inhibited protein synthesis in a cell-free system by a maximum of 93.2 and 62.7%, respectively (see representative experiments in Table II). The *P.falciparum* peptide representing the repeats (NANPNANPNANP) and the *P.falciparum* peptide representing a sequence

Fig. 3. Effect of liposome-introduced recombinant *P.falciparum* CS protein on protein synthesis in HepG2 cells. (A) CSFZ(Cys)-containing liposomes inhibit the incorporation of [^{35}S]methionine/cysteine into proteins in a concentration-dependent fashion, whereas the control liposomes are inactive. (B) CSFZ(Cys)-containing liposomes inhibit the incorporation of [^{35}S]methionine/cysteine into proteins significantly more strongly than control liposomes. For both (A) and (B), 5 µl of each of the lysates were subjected to TCA precipitation. Each value represents the mean of duplicate determinations $\pm$ SD. (C) CSFZ(Cys)-containing liposomes inhibit the incorporation of [^{35}S]methionine/cysteine into transferrin strongly over time, whereas control liposomes have no effect. Liposomes were used at a final total lipid concentration of 0.1 µg/ml. Transferrin was immunoprecipitated from the cell lysates using a polyclonal antiserum. The figure shows one representative of two independent experiments.

Table I. Effect of CS protein-containing liposomes on protein synthesis

	Liposomes	Time (h)	Total lipid (mg/ml)	Incorporated c.p.m. (mean $\pm$ SD)	Percent inhibition	P-value
A						
Exp. 1	none	0	0.1	1868.0 $\pm$ 127.8	0	
	+CS	4	0.1	610.9 $\pm$ 150.3	67.3	0.0121
	-CS	4	0.1	1548.1 $\pm$ 133.9	17.1	0.1344
Exp. 2	none	0	1	6502.0 $\pm$ 1949.7	0	
	+CS	4	1	1525.6 $\pm$ 3.5	76.5	0.0689
	-CS	4	1	4683.6 $\pm$ 315.2	28.0	0.3227
Exp. 3	none	0	1	2149.6 $\pm$ 155.7	0	
	+CS	4	1	1176.3 $\pm$ 94.2	45.3	0.0034
	-CS	4	1	1673.8 $\pm$ 450.7	22.1	0.1046
B						
Albumin	none	0	1	298.7 $\pm$ 13.4	0	
	+CS	4	1	103.7 $\pm$ 14.1	65.3	0.0049
	-CS	4	1	215.4 $\pm$ 104.4	27.9	0.3795
Transferrin	none	0	1	364.5 $\pm$ 72.6	0	
	+CS	4	1	150.1 $\pm$ 16.2	58.8	0.0553
	-CS	4	1	306.1 $\pm$ 101.5	16.0	0.5766

The CS construct CSFZ(Cys) or no protein was encapsulated into the lumen of unilamellar fusogenic liposomes. Subconfluent HepG2 cell cultures were exposed to the liposomes at concentrations of 0–1 mg/ml total lipid for 4 h. The cells were then metabolically labeled with [³⁵S]methionine/cysteine and lysed in 1 ml lysis buffer. (A) The incorporation of radiolabel into total protein was measured by subjecting 5 μ l aliquots of the lysates to TCA precipitation. (B) The incorporation of radiolabel into albumin or transferrin was determined by immunoprecipitation from 500 μ l of the lysates. Shown are representative of six (A) and two (B) independent experiments. The values represent the mean of duplicate determinations $\pm$ SD.

C-terminal from the repeats (VDENANANNAVKNNN-NEEPS) had no effect under the same conditions (Table II). Taken together, these results suggest a direct and specific action of the two conserved regions of the CS protein on the translation machinery of mammalian cells.

Recombinant CS constructs yielded variable effects on *in vitro* translation. For example, one batch of CS27IVC inhibited protein synthesis in a concentration-dependent fashion by a maximum of 68.3% at 200 nM, whereas another preparation inhibited *in vitro* translation by a maximum of 39.3% at 2 μ M. Similar results were obtained for CSFZ(Cys) (data not shown). Aldolase, which was chosen as a negative control protein because of its natural occurrence in the cytosol and its similar mol. wt (39.2 kDa), compared with that of CS27IVC (35.4 kDa), consistently was inactive at concentrations up to 13.8 μ M.

Discussion

The generalized association of the CS protein with ribosomes reported by Hügel *et al.* (1996) suggested a possible effect on translation in mammalian cells. The data presented here indicate that CS protein translocated by malaria sporozoites across cell membranes into the cytosol of mammalian cells leads to inhibition of protein synthesis in the target cells. The data also suggest that the mechanism involves binding of CS protein to host cell ribosomes. The implication is that the parasite uses this strategy to limit the host's ability to present parasite antigens, thus attenuating immune responsiveness.

Native CS protein translocated by *P.berghei* sporozoites into HepG2 cells, CHO cells or peritoneal macrophages completely blocked incorporation of tritiated amino acids into proteins and apparently led to cell death. Significantly, macrophages appeared to react more sensitively to the presence of intracellular CS protein than non-phagocytic cells and appeared to undergo rapid degeneration after translocation of CS protein into their cytoplasm. The

introduction of recombinant CS protein into HepG2 cells by liposome fusion inhibited incorporation of [³⁵S]methionine/cysteine into proteins. Transient transfection causing expression of *P.falciparum* or *P.berghei* CS protein in the cytoplasm of CHO cells completely blocked the incorporation of both tritiated leucine and serine and rendered the transfected cells moribund. Thus, the CS protein causes arrest of protein synthesis in mammalian cells ultimately leading to cell death, and the likely mechanism appears to be binding to ribosomes.

In agreement with the findings with native CS protein, recombinant CS protein binds with high affinity to RNase-sensitive sites on purified rat liver microsomes. Synthetic peptides representing the conserved regions I and II-plus of the CS protein competed with this binding, thus demonstrating specificity. These synthetic peptides also inhibited protein synthesis in a cell-free system.

So far, our observations only establish the biological effects of CS protein on translation, and further studies are required to elucidate the molecular details of the RNA-binding domains. However, we present here preliminary information on the molecular interaction between the CS protein and the rough endoplasmic reticulum. The conserved regions I and II-plus were both involved in the binding of the CS protein to rough microsomes and in the inhibition of *in vitro* translation, although the region II-plus peptide appeared to have a stronger effect than region I (Figure 4B and Table II). Binding of the CS protein to rough microsomes was specific and not simply mediated by charge since: (i) the *P.falciparum* region I peptide with a net charge of +4 was less active than the *P.falciparum* region II-plus peptide with a net charge of +2; (ii) the *P.berghei* and the *P.vivax* region II-plus peptides both possess an overall net charge of +5, but the *P.berghei* peptide was a significantly stronger inhibitor of *in vitro* translation (90–93%) than the *P.vivax* peptide (59–63%) at similar molar concentrations; and (iii) aldolase, which had been chosen as a negative control protein because of

Fig. 4. Recombinant CS protein binds with high specificity to RNase-sensitive sites on purified rough rat liver microsomes. (A) Pre-treatment of the microsomes with increasing concentrations of RNase A inhibits the subsequent binding of [125 I]CS27IVC in a concentration-dependent fashion, whereas heparinase and DNase I are inactive. (B) Peptides encompassing the conserved regions I and II-plus of the *Pfalciparum* CS protein inhibit the binding of iodinated CS27IVC to the microsomes, whereas a region II-plus control peptide has no effect. Bound c.p.m. were measured by liquid scintillation counting. Each point represents the mean $\pm$ SD of duplicate determinations.

its more basic pI of 8.4 compared with CS27IVC (pI = 5.9), had no effect on *in vitro* translation.

Inhibition of *in vitro* translation by the recombinant CS construct CS27IVC occurred at nanomolar concentrations (under apparently optimal conditions), but with high variability. However, recombinant CS constructs were also active in intact cells when introduced via liposomes, even at concentrations close to the detection limit of the immunofluorescence assay. As the histidine-tagged CS constructs are purified routinely by chelation to Ni-NTA agarose, which involves complete denaturation and sub-

sequent refolding of the proteins on the matrix using a linear urea gradient (Stüber *et al.*, 1990), we suspect that these processes altered the tertiary structure and caused the observed variable effects of the recombinant constructs on *in vitro* translation. In contrast to the displacement of recombinant CS protein by nanomolar concentrations of the region I and II-plus peptides, micromolar amounts were required for inhibition of *in vitro* translation. Although other potential cytotoxic effects of the CS protein have not been excluded ultimately to explain these discrepancies, our data suggest that more than the short amino acid sequences represented by the peptides may be involved in the arrest of protein synthesis. The peptides may be sufficient to disrupt the binding of recombinant CS protein to the microsomes, but the actual inhibitory principle may require additional structural elements of a correctly folded protein. Nevertheless, even the micromolar concentrations of the peptides reflect an interaction of high specificity, since for comparison, 7-methylguanosine-5'-monophosphate, a selective inhibitor of initiation of translation, is used commonly at a concentration of 2 mM (Walter and Blobel, 1981).

The sequences in and N-terminal from region I of the CS proteins from all mammalian *Plasmodium* species, as well as in the C-terminal portion of region II-plus (Table III), exhibit a striking similarity to the arginine-rich motif (ARM). ARM is one of the conserved nucleic acid-specific motifs which have been identified in RNA-binding proteins from eukaryotic cells, viruses and bacteriophages, consisting of short sequences rich in arginine and lysine residues (Lee *et al.*, 1993; Ellington *et al.*, 1995; Tan and Frankel, 1995; Hemmerich *et al.*, 1997; reviewed in Burd and Dreyfuss, 1994). Because hydrophobic residues play an important role in other RNA-binding motifs (Kenan *et al.*, 1991; Burd and Dreyfuss, 1994; Nagai, 1996), it is possible that the ARM-like basic amino acids of the CS protein act in concert with hydrophobic residues. Interestingly, hydrophobic amino acids within region II-plus were shown to be crucial for the interaction between the *Pfalciparum* CS protein and heparan sulfate (Sinnis *et al.*, 1994).

The mode of the ribotoxic action of the CS protein is not completely understood at this time. A large variety of cytotoxic plant and bacterial proteins have been shown to inhibit protein synthesis enzymatically, and the mechanisms these toxins use to kill cells include RNA *N*-glycosidase, RNase and ADP-ribosylase activity (Lee and Iglewski, 1984; O'Brien *et al.*, 1992; Wool *et al.*, 1992; reviewed in Stirpe *et al.*, 1992; Barbieri *et al.*, 1993). Due to the enzymatic mode of action, one ribotoxin molecule could be sufficient to kill a cell. In contrast, ~50–100 μ M of the region I and II-plus peptides were required to inhibit micromolar concentrations of ribosomes. A rabbit reticulocyte lysate typically contains ~25 μ M ribosomes, about half of which are active under optimal conditions (Jackson and Hunt, 1983). These roughly equimolar concentrations of peptides and ribosomes would suggest a stoichiometric mode of action. From the point of view of a *Plasmodium* sporozoite which releases CS protein also into invaded host cells (Hügel *et al.*, 1996), this mechanism may bear advantages because it may be reversible. However, much lower concentrations of the whole CS protein inhibited *in vitro* translation under optimal conditions. The

Table II. Effect of CS protein-derived peptides on *in vitro* translation in an untreated rabbit reticulocyte lysate

Exp.	Peptide	Concentration		Incorporated c.p.m. mean $\pm$ SD	Percent inhibition	P-value
		μ g/ml	μ M			
1	Pf R I	400	167	4253.9 $\pm$ 92.7	63.4	0.0068
		200	83	7544.6 $\pm$ 347.1	35.0	0.0245
		100	42	10 559.8 $\pm$ 384.6	9.1	0.2427
	Pf R II-plus Pf repeats control	400	200	33 10.3 $\pm$ 1330.4	71.5	0.0177
		400	331	10 657.5 $\pm$ 2923.5	8.2	0.7001
2	Pf R I	400	167	22 190.8 $\pm$ 875.0	41.7	0.0114
		0	0	38 098.1 $\pm$ 2259.1		
		400	200	9904.9 $\pm$ 217.2	58.6	0.0008
	Pf R II-plus	200	100	13 660.4 $\pm$ 2324.1	42.8	0.0260
		100	50	18 987.5 $\pm$ 57.3	20.5	0.0056
		400	331	21 878.2 $\pm$ 869.6	8.5	0.1057
	Pf repeats control	0	0	23 899.8 $\pm$ 516.9		
3	Pb R II-plus	400	146	20 490.0 $\pm$ 161.2	93.2	0.0037
		200	73	12 641.5 $\pm$ 1361.2	58.2	0.0123
		100	37	23 615.5 $\pm$ 118.1	22.0	0.0614
	control	0	0	30 268.5 $\pm$ 2441.6		
4	Pv R II-plus	400	186	33 303.5 $\pm$ 1219.8	62.7	0.0033
		200	93	37 648.0 $\pm$ 2008.2	57.9	0.0043
		100	47	43 636.5 $\pm$ 2126.3	51.1	0.0056
	control	0	0	89 341.0 $\pm$ 4384.1		
5	Pf control	400	197	28 366.8 $\pm$ 366.6	0.3	0.9205
	control	0	0	28 445.5 $\pm$ 916.5		

Incorporation of [35 S]methionine into reticulocyte proteins was measured by TCA precipitation in the presence of the peptides at the indicated concentrations after a 60 min incubation period at room temperature. The indicated values represent the mean of duplicate determinations $\pm$ SD. Pf, *Pfalciparum*; Pb, *P. berghei*; Pv, *P. vivax*.

Table III. Sequences of RNA-binding proteins containing an arginine-rich motif (ARM) in comparison with the conserved regions of CS proteins and aldolase

Ribosomal protein	
RP L7	MEGVEEKKEVPAPVETLKKRRNFAELIKIKRLRKKFAQKMLRKARRKLIYEKAKHYHKEYRQMYRTEIRMARMARKAGNFYVP
Bacteriophage N proteins	
λ N	MCQSRGVFVDYNDHTTPPKLTDRRIQMDAQTRRRERRAEKQAQWKAANPLLVGVSAKPVNRPILSLNRKPKSRVESALNPIDLIT
Φ 21 N	MVTIVWKESKGTAKTRYKARRAEELIAERRSNEALARKIALKLSGCVRADKAASLGSLRCKKAEVEVERKQNRRIYYSKPRSEMGVT
P22 N	MTVITYGKSTFAGNAKTRRHERRRKLAIERDTCINIIIDISIFGCDAPDASQEVKAKRIDRVTKAISLAGTRQKEVEGGSVLLPGV
Viral anti-terminator proteins	
HIV Rev	MAGRSGDSEDELLKAVRLIKFLYQSNPPNPPEGTROARRNRRRWRERQRQIHSISERILSTYLGRSAEPVPLQLPPLERLTLD
HIV Tat	MEPVDPNLEPNQPGSQPQTACNNCYCKCCFHCQVCFSSKGLGISYGRKKRRQRRTGTPQDRKDHQNPVPRQPLPTTRGNPTGP
HTLV-1 Rex	MPKTRRRPRRSQKRPPPTPWPTSQGLDRVFFSDTQSTCLETVYKATGAPSLGDYVRPAYIVTPYWPVQSIIRSPGTPSMDALSA
HDag	EGAPPAKARTDRMEVDSGPRKRPLRGFTDKERQDHRRRKALENKKQLGAGGKNLSREEEELRLRTEEDERRERRVAGPPP
Circumsporozoite proteins	
<i>P. berghei</i>	GNDNRLYHVLNSKNGKIYNRTNVNRLADAEPEGKKNEKKNEKIERNNKLKQP...EWSQCNVTCGSGIRVKKRGSNKKAED
<i>P. yoelii</i>	EKKDDPPKEEKKDDLPEKEEKKDDAPKDGNDAPKEEKKADPPKEAQNKLKQP...EWSQCSVTCGSGVVRKKRKNVKNQFEN
<i>P. vivax</i>	ASSLGAHVQGSASRGRGLGENPDDEEGDAKKKKDGKKAEPKNPRENKLKQP...EWTPCSVTCGVGVVRVRSRVNAANKKPE
<i>P. simium</i>	ASSLGAANVGQSASRGRGLGENPDDEEGDAKKKKDGKKAEPKNPRENKLKQP...EWTPCSVTCGVGVVRVRRVGAKNKKPA
<i>P. knowlesi</i>	SLGAQVVRNSASRGRGLGENPKKEGADKEKKKEGKEKEEPEKKNENKLKQP...EWTPCSVTCGNGVRIIRKKGHAGNKKQAQ
<i>P. cynomolgi</i>	SSHGAEQVRQSASRGRGLGENPKDEEGADKPKKKDEKQVEPKPKPRENKLKQP...EWSPCSVTCGKGVMRKRVSAANKKPE
<i>P. brasiliense</i>	LEVRYSTNQDHFYNYNKTIRLLNENNNEKDGNTVNERKKKPKTKAVENKLKQP...EWSPCSVTCGSGIRARRKVDKANKKPA
<i>P. malariae</i>	LEVRYSTNQDHFYNYNKTIRLLNENNNEKDGNTVNERKKKPKTKAVENKLKQP...EWSPCSVTCGSGIRARRKVGAKNKKPA
<i>P. falciparum</i>	LKKNRSRLGENDGNNNNGDNGREGKDEKRRDGNNEKNEKLKPKKKLKQP...EWSPCSVTCGNGIQVRIKPGSANKPKD
Negative control protein	
aldolase	PHSHPALTPPEQKELSDIAHRIVAPGKGLAADESTGSIARLQSIGTENTENRRFYRQLLLTADDRVNPCIGGVILFHETLY QKADDGRFPFQVIKSKGGVVGIVKVDKGVVPLAGTNGETTTQGLDGLSEKCAQYKKDGADFAKWRCLKIGEHTPSALAIMENAN VLARYASICQNGIVPIVEPEILPDGDHDLKRCQYVTEKVLAAVYKALSDHHIYLEGTLKPNMVTGPHACTQKYSHEEIAMAT VTALRRTPVPAVTGVTFLSGGQSEEEASINLNAINKCPLLKPWALTFSYGRALQASALKAWGGKKNLKAQEEYVKRALANSL ACQGYKTPSGQAGAAASELSFINHAY

The peptides in the ARM proteins that have been shown to be essential for specific RNA binding are underlined. Arginines and lysines are shown in bold. The CS protein-derived peptides active in the *in vitro* translation assay are underlined. Aldolase, which was used as a negative control protein for *in vitro* translation assays, contains no clusters of basic amino acids.

exact mode of the ribotoxic action of the CS protein remains to be elucidated in future studies.

The mechanism by which the malaria CS protein gains access to the cytoplasm of mammalian cells is unknown.

In contrast to bacterial or plant ribotoxins which possess a translocating domain that mediates entry of the enzymatically active domain into the cytosol (Pelham *et al.*, 1992; Sandvig *et al.*, 1992), endocytosed soluble recombinant CS

constructs remained restricted to the endocytic compartment of the cell and did not exert any adverse effect on cell proliferation (Shakibaei and Frevert, 1996; U.Frevert, unpublished data). The translocating principle of the CS protein may reside in its N- or C-terminus, both of which are missing in the recombinant CS constructs used in our studies. Two interesting parallel phenomena are worth mentioning in this context. First, two other proteins which, like the CS protein, use LRP as an endocytosis receptor are also able to translocate into the cytosol. Apolipoprotein E3, which is thought to play a preventative role against Alzheimer's disease due to its involvement in myelin repair and cholesterol transport (Weisgraber and Mahley, 1996), enters the cytosol of cortical neurons and glial cells (Han *et al.*, 1994). Similarly, lactoferrin enters human myelogenous leukemia cells and is transported into the nucleus (Garré *et al.*, 1992) where it acts as a transcriptional activator (He and Furmanski, 1995). The second example is a defense mechanism that pathogenic *Yersinia* species use against macrophage respiratory burst and phagocytosis (Rosqvist *et al.*, 1988; Hartland *et al.*, 1994). Cell contact with macrophages induces these bacteria to translocate the tyrosine phosphatase YopH and the actin-disrupting cytotoxin YopE into the cytoplasm of challenging macrophages, thereby rendering the phagocytes paralyzed. Amphiphilic molecules such as YopN have been implicated in this highly polarized transfer (Rosqvist *et al.*, 1994; Persson *et al.*, 1995) which requires neither a signal sequence nor membrane translocase activity (Salmond and Reeves, 1993). Future studies may reveal if malaria sporozoites possess a translocation apparatus for the intracytoplasmic deposition of CS protein similar to *Yersinia*, if the CS protein inherently exhibits properties that allow the utilization of an existing translocation system of the host, or if the CS protein has membrane-destabilizing properties that allow spontaneous translocation.

The finding that malaria sporozoites are able to inhibit protein synthesis in cells of phagocytic origin is of great significance, since it offers an explanation for why entry of *P.berghei* or *P.yoelii* sporozoites into the parasitophorous vacuole of peritoneal macrophages or Kupffer cells does not affect the parasites negatively, but instead morphologically alters and finally destroys the phagocytes (Danforth *et al.*, 1980). Inhibition of translation may also explain why mature *P.yoelii* sporozoites are able to evade the respiratory burst of peritoneal macrophages (Smith and Alexander, 1986) and why *P.berghei* sporozoites frequently cause the subsequent death of macrophages they passaged through *in vitro* (Vanderberg and Stewart, 1990). Since the majority of the transmitted sporozoites conceivably do not enter the liver circulation during their first pass through the vascular system of the vertebrate host, encounters with professional phagocytes and antigen-presenting cells, in particular in the spleen, are very likely to occur. These contacts should result in processing and presentation of *Plasmodium* antigens and, thus, initiate an immune response against the parasite. Inhibition of host protein synthesis by the CS protein would block this initial step, thus providing a mechanism of immune evasion.

Materials and methods

Recombinant proteins, peptides and antibodies

Escherichia coli-derived *P.falciparum* CS proteins CS27IVC-His₆ (amino acids 27–123[NANPNVDP]₃[NANP]₂₁300–411), which contains region

I and II-plus as well as the complete repeat region, and CSFZ(Cys)-His₅ (amino acids 27–123[NANP]300–411) and CS27IC-His₆ (amino acids 27–123[NANP]300–411), which also contain region I and region II-plus, but only one repeat (Cerami *et al.*, 1992), as well as plasmids encoding these constructs were kindly provided by Drs Bela Takacs and Dietrich Stüber (F.Hoffmann-La Roche Ltd., Basel, Switzerland). The following synthetic peptides were used in this study: a *P.falciparum* CS protein-derived region I peptide (GNNEDNEKLKPKHKKLKQP) which contains the conserved motif KLKQPG and is N-terminally extended by 14 amino acids; a *P.falciparum* CS protein-derived region II-plus peptide (PCSVTCGNGIQVRIKPGSAN), a *P.berghei* region II-plus peptide (QCNVTCGSGIRVRKRKGSNKKAEDL) and a *P.vivax* region II-plus peptide (CSVTCGVGVVRVRSRVNAANK), which represent the conserved region II-plus motifs from these CS proteins; a *P.falciparum* region II-plus control peptide (PCSVTCGNGIQVEIEPG SAN), in which the basic amino acids have been replaced by glutamic acid; a *P.falciparum* control peptide (VDENANANNAVKNNNEEPS) representing a sequence C-terminal from the repeats; and a *P.falciparum* repeat peptide (NANPNANPNANP). *P.falciparum* CS constructs were detected by mAb 2A10 (Nardin *et al.*, 1982), and *P.berghei* CS protein by the mAb 3D11 (Yoshida *et al.*, 1980).

Solid phase binding assay

Rough microsomes were isolated from rat livers (Gaetani *et al.*, 1983), and the purity of the preparation was confirmed by electron microscopy (data not shown). A previously developed solid phase binding assay (Shakibaei and Frevert, 1996) was adapted to this system. Briefly, Removawell microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated overnight at 4°C with 100 µl per well of the microsomes diluted in Tris-buffered saline (TBS; 50 mM Tris, pH 7.4, containing 150 mM NaCl and 5 mM CaCl₂). The wells were then blocked for 1 h at room temperature with 10 mg/ml BSA (fraction V; Sigma) in TBS. CS27IVC was iodinated (Sinnis *et al.*, 1994) and added to the wells at a final concentration of 2 nM in TBS. Other wells were pre-treated for 1 h at room temperature with 0–100 U/ml DNase I or RNase A (EC 3.2.2.1 and EC 3.1.27.5, respectively; both from Boehringer, Indianapolis, IN) or with 0–10 U/ml heparinase (EC 4.2.2.7; Seikagaku, Rockville, MD) before addition of the iodinated CS protein. After an overnight incubation at 4°C, the wells were washed and the amount of bound [¹²⁵I]CS27IVC per well measured by liquid scintillation counting as described (Shakibaei and Frevert, 1996). In another set of experiments, the binding of CS protein was examined in the presence of 0–100 µg/ml of (i) the *P.falciparum* region I peptide, (ii) the *P.falciparum* region II-plus peptide or (iii) the *P.falciparum* region II-plus control peptide. The data were analyzed with the computer program LIGAND (Munson and Rodbard, 1980).

Sporozoite invasion assay (modified from Sinden *et al.*, 1990)

Human hepatoma (HepG2; ATCC # HB8065, Rockville, MD) or Chinese hamster ovary cells (CHO-K1; ATCC # CCL-61) were cultivated to sub-confluency (Shakibaei and Frevert, 1996). Rat peritoneal macrophages were harvested from Brown Norway rats and cultivated for 1 day. The *P.berghei* sporozoites were prepared from infected mosquito salivary glands and co-cultivated with the cells for 30 min to 6 h. Extra- and intracellular sporozoites were distinguished by double labeling (Frevert *et al.*, 1996). Briefly, extracellular sporozoites were labeled with mAb 3D11 in combination with goat anti-mouse IgG conjugated to FITC (GAM-FITC; Boehringer Mannheim). The cells were then permeabilized with 0.01% saponin, and invaded parasites and intracellular CS protein were detected with the same mAb followed by goat anti-mouse IgG conjugated to Texas red (GAM-TX; Accurate, Westbury, NY). Specimens with a single FITC-label were counterstained with Evans blue (Hügel *et al.*, 1996). For the autoradiography studies, the cell cultures were starved for 30 min in deficient medium (see below) after co-cultivation with the parasites. Subsequently, the cultures were metabolically labeled for 30 min either with 50 µCi/ml [³H]leucine in serum-free nutrient mixture F-12 Ham (Sigma), 50 µCi/ml [³H]glycine in serum-free basal medium Eagle (BME; Sigma) or 50 µCi/ml [³H]serine in serum-free BME, chased for 15 min with complete medium, rinsed and fixed. Distributions of the fluorescence labels as well as the silver grains in the photographic emulsion were determined by laser scanning confocal microscopy.

Confocal microscopy

Specimens were examined with an argon/krypton laser using a Nikon Optiphot II microscope equipped with a Sarastro 2000 laser scanning system (Molecular Dynamics, Sunnyvale, CA). Images were processed

using Adobe Photoshop (Adobe, San Jose, CA) and QuarkXPress (Quark, Denver, CO) software and printed with a Fujix Pictography 3000 printer.

Microautoradiography

Fixed specimens were immunofluorescence labeled, air-dried and coated with Hypercoat EM-1 photographic emulsion (Amersham). The silver grains in the photographic emulsion were visualized by transmission confocal microscopy (Shakibaei and Frevert, 1996).

Plasmids and transient transfection

Plasmids containing the gene sequences encoding the hydrophilic portion of the *P.falciparum* CS protein (bases 55–1248; Dame *et al.*, 1984) or the *P.berghei* CS protein (bases 157–1046; Eichinger *et al.*, 1986) were designed using primer pairs hybridizing to the bases 55–76 and 1228–1248 of the *P.falciparum* CS protein gene and to the bases 157–180 and 1023–1046 of the *P.berghei* CS protein gene. Twenty base pairs of upstream sequence and a start codon were added to the 5' end of the upstream primer, and the bases coding for the signal peptide and the lipophilic C-terminus were omitted to allow translation of the CS proteins in the cytosol. The corresponding CS protein gene products were amplified from genomic DNA using PCR, ligated into the multiple cloning site of a pCRTM3 vector, and transformed into XL1-Blue *E.coli* cells. The correct orientation of the cloned inserts was confirmed by restriction enzyme analysis, and the sequences of the correctly oriented inserts were confirmed (Sanger *et al.*, 1977). After amplification and purification (Qiagen, Santa Clara, CA), these plasmids were used for the transient expression of the CS proteins in CHO cells using lipofectamine (Life Technologies, Gaithersburg, MD). The subcellular distribution of the CS proteins at 24–72 h after transfection was determined by immunolabeling using the mAbs 2A10 and 3D11 (Yoshida *et al.*, 1980; Nardin *et al.*, 1982), which detect the repeat region of the *P.falciparum* and *P.berghei* CS protein, respectively, in combination with GAM-FITC. Other cultures were metabolically labeled with tritiated leucine 24 h after transfection, fixed, immunofluorescence-labeled and processed for microautoradiography.

Liposome fusion assay

Liposomes were prepared as described (Chander and Schreier, 1992). The lipid composition of the liposomes, an equimolar mixture of dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine, phosphatidylserine and sphingomyelin combined with cholesterol at a 1:1 (total lipid/cholesterol) molar ratio, resembles that of enveloped viruses and confers intrinsic fusogenic properties to the lipid membrane. Briefly, the lipids and cholesterol were combined in chloroform, dried via rotary evaporation and dispersed in 50 mM phosphate-buffered saline (PBS) containing the peptide or fluorescent marker, respectively, at a final lipid concentration of 10 mg/ml. Liposomes were allowed to hydrate at 4°C overnight. The hydrated dispersion underwent five freeze-thaw cycles to reduce the number of lipid layers and increase the encapsulated volume, and then were extruded through 200 nm polycarbonate filters. The CS constructs CSFZ(Cys) or CS27IC, dextran-FITC or buffer were encapsulated into the lumen of the liposomes, and unincorporated payload was removed via Sephadex G-75 chromatography. Subconfluent HepG2 cells were incubated for 4 h in 12-well plates (Falcon, Lincoln Park, NJ) with 500 µl per well of 0, 12.5, 25, 50 or 100 µl/ml of the liposome preparations in assay medium [Dulbecco's modified Eagle's medium (DMEM)/1% fetal calf serum (FCS)]. These dilutions correspond to total lipid concentrations of 0, 0.125, 0.25, 0.5 and 1.0 µg/ml. In other experiments, the cells were incubated in the presence or absence of 100 µl/ml of the liposomes for 0, 0.5, 1, 2, 4 or 6 h. The wells were then washed with DMEM (without methionine and cysteine, containing 0.1% dialyzed FCS) and starved for 30 min at 37°C in the same medium. The medium was replaced by fresh DMEM containing 10 µCi/ml [³⁵S]methionine/cysteine (Tran³⁵S-Label, ICN, Irvine, CA), and the cells were incubated for another 30 min at 37°C. After washing at 4°C, the cells were proteolytically detached (Shakibaei and Frevert, 1996), pelleted and lysed on ice in the presence of protease inhibitors [phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin and antipain; all from Boehringer Mannheim]. Aliquots (5 µl) of the lysates were subjected to trichloroacetic acid (TCA) precipitation to determine the overall incorporation of radiolabel. Other aliquots (500 µl) of the cell lysates were incubated with a rabbit antiserum against human albumin (Boehringer Mannheim), a rabbit antiserum against human transferrin (Boehringer Mannheim) or a normal rabbit serum. After immunoprecipitation with *Staphylococcus aureus* cells (Calbiochem, La Jolla, CA), aliquots were analyzed by liquid scintillation counting.

In vitro translation

Untreated rabbit reticulocyte lysates from Promega (Madison, WI), supplemented with hemin, creatine phosphate and creatine phosphokinase,

according to the manufacturer's instructions, were incubated for 60 min at room temperature with final concentrations of 0, 100, 200 or 400 µg/ml of the following peptides: (i) the *P.falciparum* region I peptide; (ii) the *P.falciparum*, *P.berghei* or *P.vivax* region II-plus peptide; or (iii) the two *P.falciparum* control peptides. In other experiments, 0–2 µM of the CS construct CS27IVC were added to the lysates. Aliquots were removed from the lysates at various time points of the incubation period to measure the incorporation of [³⁵S]methionine (*in vivo* cell labeling grade; Amersham) into reticulocyte proteins by TCA precipitation and the incorporation of radiolabel into globin by SDS-PAGE and autoradiography.

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