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A Mutant of Melanoma Growth Stimulating Activity Does Not Activate Neutrophils but Blocks Erythrocyte Invasion by Malaria*

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Alanine scanning mutagenesis of the charged amino acids of melanoma growth stimulating activity (MGSA) was used to identify specific residues that are involved in binding to the human erythrocyte Duffy antigen/chemokine receptor (DARC) and to the type B interleukin-8 receptor (IL-8RB) on neutrophils. Receptor binding and biological studies with the alanine scan mutants of MGSA demonstrate that MGSA binds to DARC and the IL-8RB through distinct binding regions. One of the MGSA mutants, E6A, binds to human erythrocytes and is able to inhibit malaria invasion as efficiently as wild type MGSA but has a severely reduced ability to bind to or signal through the IL-8RB. Mutant chemokines like E6A could prove to be useful therapeutically for the design of receptor blocking drugs that inhibit erythrocyte invasion by *Plasmodium vivax* malaria.

The Duffy blood group antigen is required for the invasion of human erythrocytes by the human malarial parasite, *Plasmodium vivax* (1) and the related monkey malarial parasite, *Plasmodium knowlesi* (2). Recently the Duffy antigen was cloned (3) and shown to be a receptor for a family of chemotactic molecules known as chemokines (4, 5). The chemokines, which include Interleukin-8 (IL-8)[1], melanoma growth stimulating activity (MGSA), and monocyte chemotactic protein-1 (MCP-1) are potent chemoattractants and activators of blood leukocytes (6, 7). In a previous report we have shown that MGSA can block the invasion of human erythrocytes by the malarial parasite *P. knowlesi*, suggesting the possibility of receptor blockade for anti-malarial therapy (4). However, in addition to displaying high affinity binding to the human erythrocyte Duffy antigen/chemokine receptor (DARC) (8, 9) MGSA also binds with high affinity and activates neutrophils through the type B IL-8 receptor (IL-8RB) (9, 10). Thus, drugs modeled on the structure of MGSA (11) will be useful therapeutically only if they display strict specificity for binding to DARC and do not cross-react with and activate neutrophils via the IL-8RB.

In this study we have used an adaptation (12) of the alanine scanning mutagenesis technique of Cunningham and Wells (13) to evaluate the contribution of the charged residues of MGSA in binding to DARC and the IL-8RB. The charged amino acids were chosen for mutagenesis for the following reasons. First, charged residues are usually exposed on the surface of the protein and could therefore contribute to receptor binding. Second, mutagenesis of surface residues is less likely to disrupt the tertiary structure of the molecule. In addition, selected noncharged amino acids of MGSA were also substituted as has been described for IL-8 (14). Through receptor binding and biological studies with the alanine scan mutants of MGSA we show that MGSA binds to DARC and the IL-8RB through distinct and overlapping binding regions. In addition we have identified a specific mutant of MGSA which is able to inhibit invasion of human erythrocytes by *P. knowlesi* as efficiently as native MGSA but has a severely impaired ability to bind to or signal through the IL-8RB.

EXPERIMENTAL PROCEDURES

Materials—^{125}I-MGSA (specific activity 2,200 Ci/mmol) was from DuPont NEN. Unlabeled MGSA was purified as described previously (15). Reagents for electrophoresis were from Novex. HEPES and all other reagent grade chemicals were from Sigma.

Plasmid Construction—The expression plasmids for the alanine mutants were derived from the MGSA *Escherichia coli* secretion vector pMG34 (15). The mutation was incorporated into the original pMG34 vector by replacing an *Eco*RI-*Bsa*JI fragment in that vector with synthetic DNA containing the respective codon changes. The mutant plasmids were then sequenced over the entire region replaced by synthetic DNA to verify that the planned changes were correct.

Induction Cultures—The plasmid containing the alanine mutations were used to transform the *E. coli* strain *27C7 (tonAΔptr3 phoAΔE15Δ(argF-lac)169ompTΔdegP41)* by the $CaCl_2$/heat shock method (16). A freshly transformed culture was grown in LB (17) containing 50 μg/ml carbenicillin for 3–4 h at 37 °C. The LB culture was then diluted 100-fold into 1 liter of a low phosphate minimal medium (18) containing 50 μg/ml carbenicillin to induce the alkaline phosphatase promoter. After shaking for 21 h at 30 °C, the cells were harvested by centrifugation, washed once with 30 ml of 50 mM Tris-HCl, pH 8, 5 mM EDTA, 50 mM NaCl, and frozen until the MGSA protein could be extracted and purified. Small aliquots of the cells from induced cultures were analyzed by SDS-polyacrylamide gel electrophoresis (18% Tris-Gly, Novex, San Diego, CA) to verify the expression of the mutant MGSA protein.

Purification—The MGSA mutants were extracted from *E. coli* cell pastes by treatment in 8 M urea, 10 mM Tris, pH 7.8, containing 1% polyethyleneimine. The cell pastes were extracted in an end-over-end rotator for 10 min at room temperature and centrifuged at 20,000 × *g* for 10 min. The supernatant containing the MGSA was removed and purified by a combination of S-Sepharose chromatography and reverse phase C_4 HPLC as described previously (15). The mutants were analyzed for purity by SDS-polyacrylamide gel electrophoresis on 18% Tris-Gly gels (Novex) followed by silver staining; all mutants were 99% pure after HPLC. HPLC fractions were pooled based on silver staining and mass spectral data. Mass spectroscopy of tryptic-digested and sin-

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[1] The abbreviations used are: IL-8, interleukin-8; MGSA, melanoma growth stimulating activity; MCP-1, monocyte chemotactic protein-1; DARC, Duffy antigen/chemokine receptor; IL-8RB, type B interleukin-8 receptor; HPLC, high performance liquid chromatography; NAP-2, neutrophil-activating peptide-2; PF4, platelet factor-4; IP10, interferon-inducible 10-kDa protein; MIP, macrophage inflammatory protein.

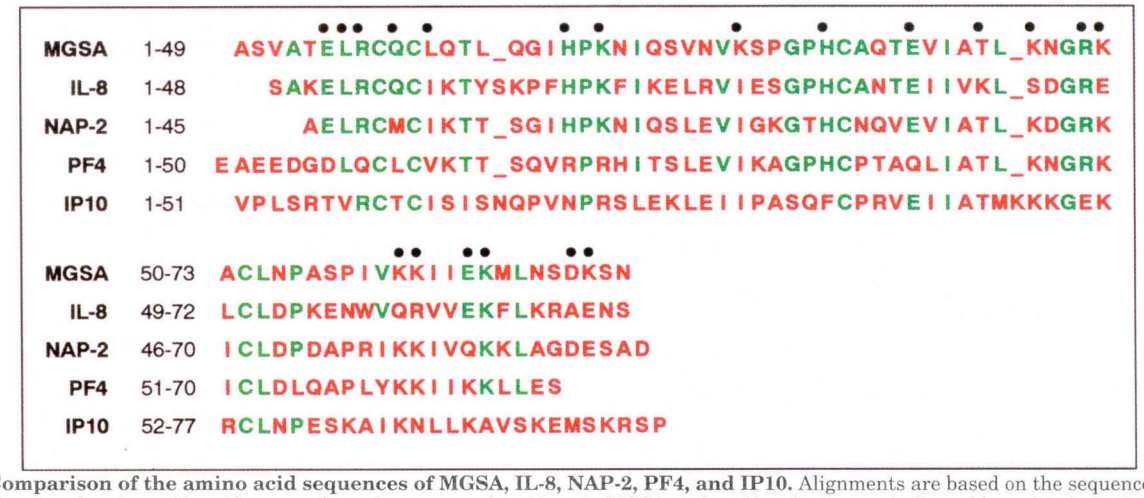

FIG. 1. **Comparison of the amino acid sequences of MGSA, IL-8, NAP-2, PF4, and IP10.** Alignments are based on the sequence of MGSA and IL-8. Conserved amino acid residues are shown in *green*, and residues in MGSA replaced by alanine are indicated by a *dot* above the sequence.

gle cycle Edman degradation peptides of the E6A mutant gave mass results with a 1→3 and 2→4-linked disulfide bond arrangement consistent with those of MGSA (11, 15). The concentrations of the MGSA and MGSA mutants were established by amino acid analysis.

Mass Spectrometry—Micromolar solutions (1–10 pmol/μl) of the HPLC-purified proteins were infused at 1.5 μl/min into a PE-SCIEX API III (Thornhill, Ontario) triple quadrupole mass spectrometer with an articulated, pneumatically assisted electrospray ionization source. These polypeptides eluted in approximately 39–42% aqueous acetonitrile with 0.1% trifluoroacetic acid. The mass spectrometer was operated with the Ionspray (PE-SCIEX) articulated nebulizer at 4,600 V, the interface plate at 600 V, and the orifice at 80–100 V. Data were collected every 0.2 atomic mass unit with the quadrupole typically scanning from 600 to 2,400 atomic mass units in 10 s.

Cell Culture—Human kidney 293 cells stably expressing the IL-8RB were obtained as described previously (10) and maintained in F-12/low glucose Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal calf serum. The cells were passaged weekly, and the medium was changed two additional times weekly. Cell number was determined by counting the cells in a hemacytometer, after trypan blue exclusion.

Receptor Binding Assays—Human kidney 293 cells stably expressing the IL-8RB (2×10^6 cells/ml) and human erythrocytes (4×10^6 cells/ml) were incubated with ^{125}I-MGSA (0.2 nM) and varying concentrations of unlabeled ligands at 4 °C for 1 h. The incubation was stopped by removing aliquots from the cell suspension and separating cells from buffer by centrifugation through a silicone/paraffin oil mixture as described previously (19). Nonspecific binding was determined in the presence of 1 μM unlabeled ligand. The binding data were curve fit with the computer program LIGAND (20) to determine the affinity (K_d), number of sites, and nonspecific binding.

RESULTS AND DISCUSSION

Fig. 1 shows an alignment of the primary sequences of the C-*X*-C chemokines MGSA, IL-8, neutrophil-activating peptide-2 (NAP-2), platelet factor-4 (PF4), and interferon-inducible 10-kDa protein (IP10); residues that were substituted in MGSA by alanine are indicated by *dots* above the amino acids. All of the expression plasmids for the alanine mutants were derived from the MGSA *E. coli* secretion vector pMG34 (15). Mutations were incorporated into the original pMG34 vector by replacing various restriction endonuclease fragments in that vector with synthetic DNA containing the respective codon(s) change. All mutant plasmids were then sequenced over the entire region replaced by synthetic DNA to verify that the planned changes were correct. Plasmids containing the alanine mutations were transfected into *E. coli* strain 27C7 as described previously (15). Under the conditions of growth described here, the major portion of the expressed MGSA mutant remained cell bound, and proteins were extracted from the *E. coli* cell pastes and purified by a combination of S-Sepharose fast flow and reverse phase chromatography. The purity and identity of the mutants were determined by electrospray ionization mass spectrometry (21) and by SDS-polyacrylamide gel electrophoresis followed by silver staining. Mass spectral analysis of tryptic digested and Edman degraded peptides of E6A showed a 1→3 and 2→4 disulfide bond arrangement (data not shown). The concentration of each purified mutant was established by determination of amino acid composition.

The alanine scan mutants were tested for their ability to displace bound ^{125}I-MGSA from human Duffy positive erythrocytes that express DARC and from human kidney 293 cells transfected with the IL-8RB. The binding constants for the mutants were determined by Scatchard analysis of competition binding studies. Fig. 2 shows the results of the competition binding with the mutants, E6A, L7A, and R8A, for DARC (Fig. 2*A*) and the IL-8RB (Fig. 2*B*) as well as a summary of the binding data for all 20 mutants tested (Fig. 2*C*). The binding analysis reveals that substitution of Glu^6, Leu^7, or Arg^8 in MGSA severely decreases binding affinity for the IL-8RB (Fig. 2, *B* and *C*). In addition the E6A, L7A, and R8A mutants were ineffective in stimulating elastase release from neutrophils even at a ligand concentration of up to 1 μM, and they have EC_{50} values greater than 10 μM (Fig. 2*D*). Using site-directed mutagenesis, Hébert *et al.* (14) have shown that IL-8-mediated receptor binding and neutrophil activation are abrogated when residues within the amino-terminal sequence Glu-Leu-Arg are replaced with alanine (14). All three residues, arginine in particular, are highly sensitive to modification. The same conclusions were drawn from later studies that showed that IL-8 activity is lost in chemically synthesized analogs of IL-8 in which the ELR motif is deleted (22, 23). In contrast, the binding affinities of the MGSA mutants E6A and L7A for DARC are only about 2-fold and 10-fold less than that of wild type MGSA, suggesting that these residues are not of major importance in determining binding to DARC. The binding affinity of the MGSA mutant R8A, however, is approximately 240-fold lower than wild type MGSA, indicating that a positive charge may be required in this region of the protein for binding to DARC. Interestingly, some members of the C-C chemokines (which all lack this positive charge as well as the Glu and Leu residues) such as RANTES and MCP-1 bind to DARC, whereas others such as the macrophage inflammatory proteins (MIP-1α, MIP-1β) do not bind (24). Based on analysis of the solution structure of

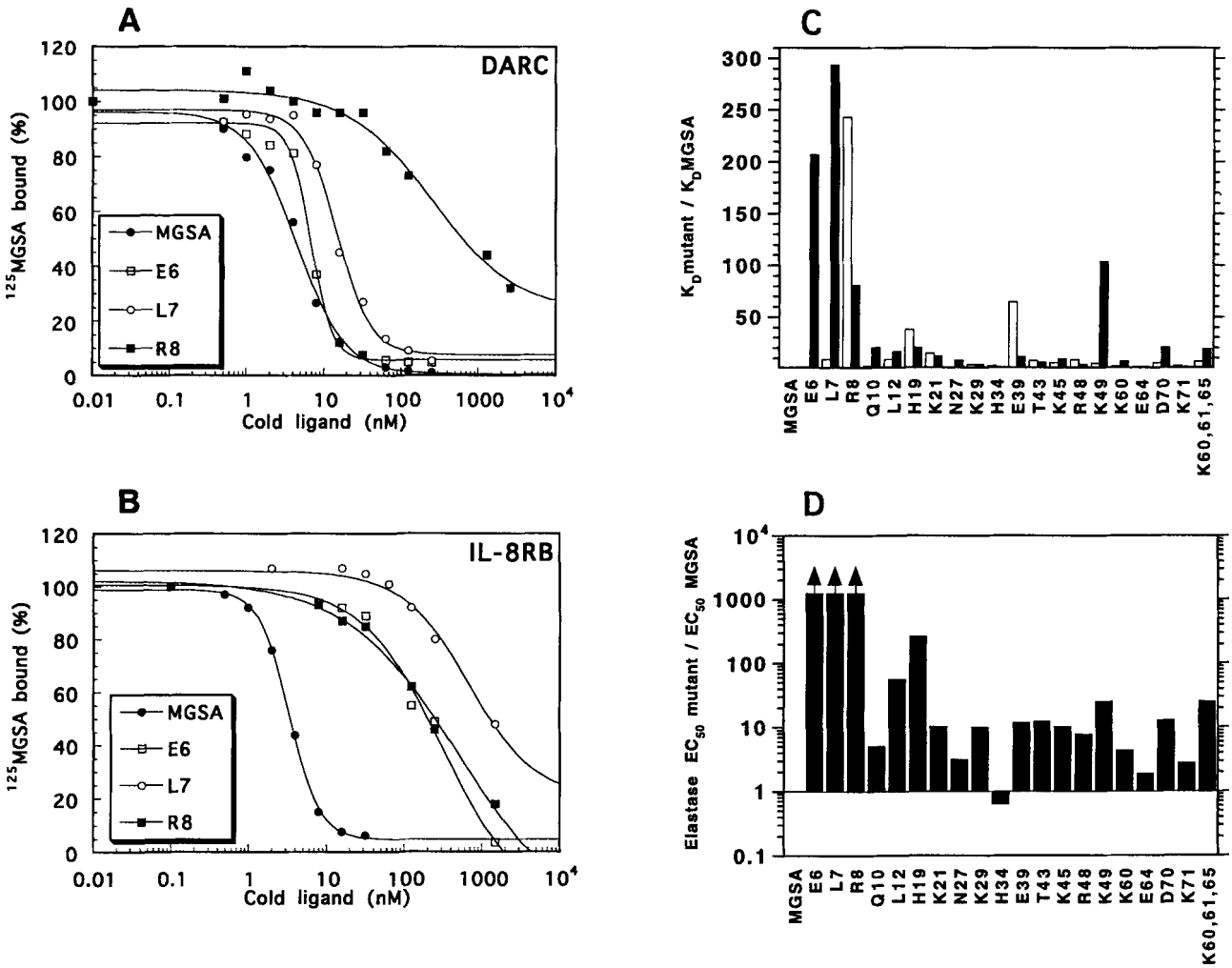

FIG. 2. **Receptor binding and biological activity of MGSA mutants.** Competition binding was between ^{125}I-MGSA and increasing concentrations of unlabeled MGSA mutants E6A, L7A, and R8A for human erythrocytes (*panel A*) and human kidney cells transfected with IL-8RB (*panel B*). Cells were incubated for 1 h at 4 °C with ^{125}I-MGSA in the presence of increasing amounts of the unlabeled MGSA mutants. The binding reactions were terminated by centrifugation of cells through a silicone/paraffin oil mixture as described previously (19). Nonspecific binding was determined in the presence of 100 nM unlabeled MGSA and was less than 10% of total binding. Typical binding assay, $n = 4$. *Panel C*, dissociation constant ratios for mutant MGSA relative to wild type MGSA for binding to DARC (*open boxes*) and the IL-8RB (*solid boxes*) plotted for all mutants, $n = 4$ or greater. MGSA IL-8RB K_d = 2.3 nM and DARC K_d = 3.5 nM. *Panel D*, elastase release assay. Data are given as the EC_{50} ratios for mutant MGSA relative to wild type MGSA. The EC_{50} value is defined as the concentration of MGSA or mutant required for half-maximal release of elastase from neutrophils. Assays were performed as described previously (29). *Arrows* indicate EC_{50} ratio values greater than 1,250, $n = 2$ or greater. MGSA EC_{50} = 8 nM.

RANTES[2] and the amino acid sequence of the C-C chemokines, we propose that the positively charged lysine residue adjacent to the third cysteine residue, at positions 33 in RANTES and 35 in MCP-1, may fill the role for the absence of the arginine at position 8. The chemokines MIP-1α and MIP-1β lack a positively charged residue at both positions.

Replacement of charged residues in MGSA by alanine has uncovered several other important regions of MGSA for receptor binding and activation, including residues His^{19}, Glu^{39}, and Lys^{49}. Substitution of residue His^{19} by alanine reduces binding to DARC by 37-fold and binding to IL-8RB by only 13-fold. This implicates His^{19} as part of a second binding region for DARC. Interestingly, based on elastase release data (Fig. 2*D*) and on its decreased ability to stimulate chemotaxis (data not shown), the H19A mutant appears to be a partial antagonist for the type B IL-8 receptor.

The MGSA mutant E39A has a moderately reduced binding affinity for DARC, down 64-fold, but reasonably high affinity for the IL-8RB, down 12-fold (Fig. 2*C*). From these data alone it is possible that Glu^{39} plays a role in the binding of MGSA to DARC. Glu^{39} is conserved in the C-*X*-C chemokines IL-8, NAP-2, and IP10, although PF4 contains a Gln at this position (Fig. 1). The NMR-derived solution structure of MGSA reveals that Glu^{39} is almost totally inaccessible to the solvent; the conformation of this residue is, however, not well defined due mainly to the lack of distance and dihedral restraints resulting from chemical shift degeneracy in the NMR spectra of MGSA (11).

Analysis of the structures of IL-8 (25, 26) and bovine PF4 (bPF4) (27), however, shows the equivalent residues (Glu^{38} in IL-8 and Gln^{55} in bPF4) are also buried and hydrogen bonded to the backbone amides of Gln^{8} and Cys^{9}, and Val^{26} and Cys^{27}, in IL-8 and PF4, respectively. Existence of similar hydrogen bonds is therefore expected in MGSA (*i.e.* between Glu^{39} and Gln^{10}-Cys^{11}), despite the relatively poor definition of Glu^{39} in the NMR solution structure. Thus, substitution of Glu^{39} by alanine will eliminate the possible hydrogen bond interactions

[2] Skelton, N. J., Aspiras, F., Ogez, J., and Schall, T. J. (1995) *Biochemistry*, in press.

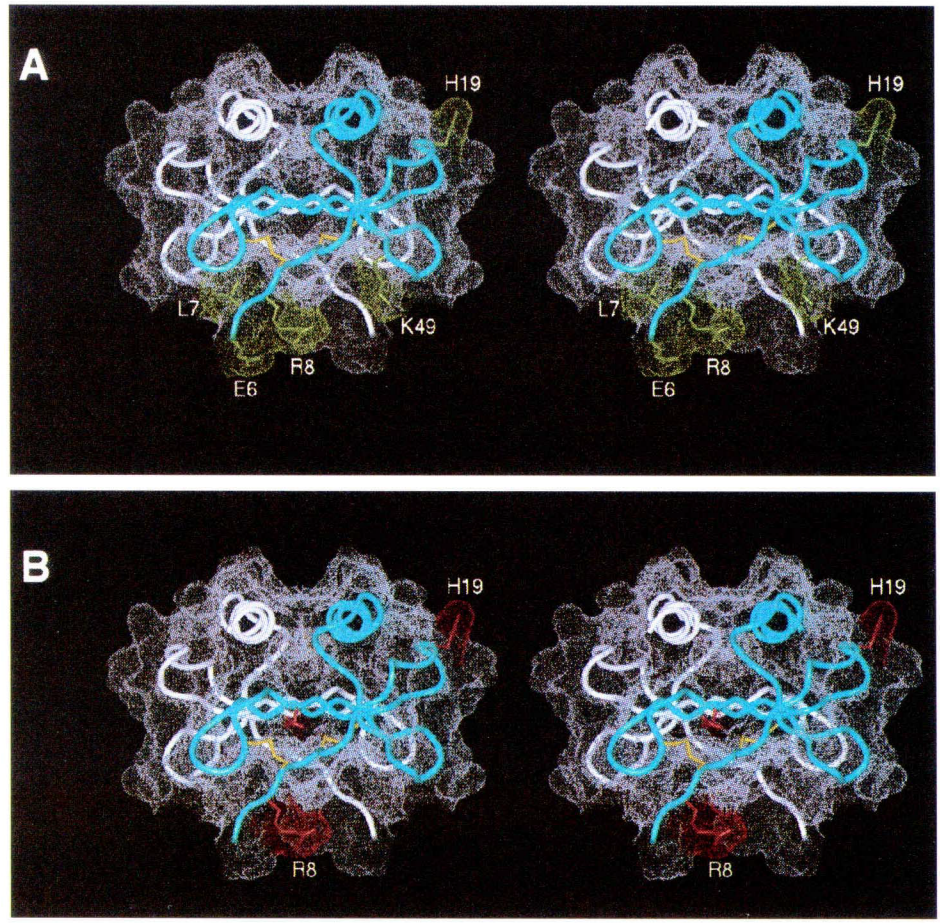

FIG. 3. **Stereo view of the proposed binding regions of MGSA for the IL-8RB (*panel A*) and DARC (*panel B*).** The disulfides are shown in *yellow*. Residues in *green* are Glu^{6}-Leu^{7}-Arg^{8}, His^{19}, and Lys^{49} (IL8RB binding regions). Residues in *red* are Arg^{8}, His^{19}, and Glu^{39} (DARC binding regions). The coordinates used are from the solution structure of MGSA (11). The *gray* surface represents the solvent accessible surface, calculated using a probe radius of 1.4 Å.

and might be expected to perturb the conformation of the amino-terminal region, Arg^{8} in particular. Therefore, the reduction of binding affinity of E39A to DARC, relative to wild type MGSA, is most likely due to a small but significant conformational change in the amino-terminal region of the protein. This change is specific for binding to DARC and not the IL-8RB, since only a slight decrease in binding affinity was seen for E39A on the IL-8RB. By contrast, the mutant K49A undergoes a 100-fold decrease in binding affinity for IL-8RB, which is accompanied by a reduced ability to stimulate elastase release from neutrophils but binds to DARC with high affinity (a 5-fold decrease compared with wild type).

These data suggest that MGSA has at least two distinct but overlapping binding regions for DARC and the IL-8RB as indicated in Fig. 3. One epitope for binding IL-8RB involves the ELR region of MGSA since mutation of these residues severely reduces binding. The importance of the ELR motif for binding to the neutrophil IL-8 receptors was recently demonstrated by the finding that when it is engineered into PF4, which is a chemokine that does not bind to these receptors, it converts PF4 into a high affinity ligand (28). However, insertion of the ELR motif into IP10, another chemokine that does not bind to the neutrophil IL-8 receptors, does not render it capable of binding to these receptors (28). Thus, the ELR motif is necessary but not sufficient for high affinity IL-8 receptor binding. These data are consistent with our findings for MGSA. In the case of MGSA we have found that the Lys^{49} and His^{19} residues may be part of an additional binding region(s) for binding to the IL-8RB. Although the Glu^{6} and Leu^{7} residues are not important for binding to DARC, replacement of the Arg^{8} residue severely reduces receptor binding affinity, and thus it appears to be part of a binding region for DARC.

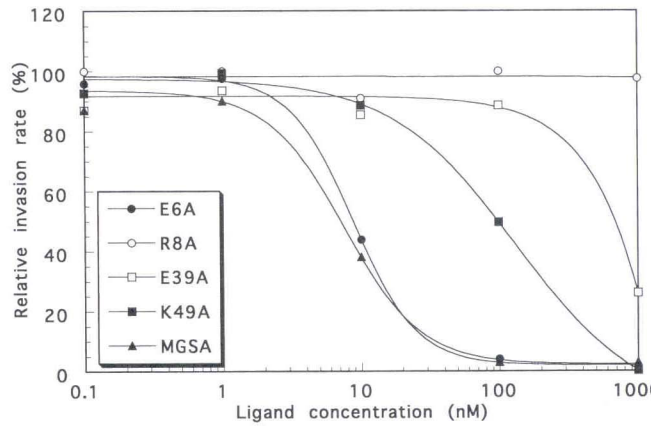

FIG. 4. **Inhibition of erythrocyte invasion by *P. knowlesi* by MGSA and MGSA mutants.** To study the effect of MGSA and its mutants on invasion, we preincubated erythrocytes (2×10^{7} in a volume of 870 μl of RPMI containing 22 mM glucose, 29 mM HEPES, pH 7.4, and 10% fetal calf serum, per invasion) with increasing concentrations of these chemokines for 1 h at room temperature. Percoll-purified *P. knowlesi* schizont-infected erythrocytes (2×10^{6} in 100 μl) and 6 μl of 7.5% sodium bicarbonate were added and incubated for 6–7 h at 37 °C during which time the infected erythrocytes ruptured, releasing merozoites that were able to invade other erythrocytes. The cells were centrifuged through Percoll to separate the ring-infected and normal erythrocytes. A thin smear of the resuspended erythrocytes was stained with Giemsa, and the percentage of erythrocytes infected with ring-stage parasites was determined. The invasion rates are expressed as a percentage of the rate of invasion in the absence of chemokines. Inhibition of invasion EC_{50}: MGSA = 7 nM, E6A = 8.6 nM, R8A = >1 μM, E39A = 710 nM, K49A = 96 nM.

We examined the ability of selected MGSA mutants to inhibit the invasion of human Duffy positive erythrocytes by *P. knowlesi* (Fig. 4). The mutants inhibited parasite invasion at ligand concentrations that were consistent with their receptor binding affinities for DARC. For example, the mutant E6A was almost as effective as MGSA with an EC_{50} of inhibition of invasion of 8.6 nM compared with 7 nM for wild type MGSA. However, mutant R8A, which binds poorly to DARC, K_d = 851 nM, did not inhibit parasite invasion at concentrations up to 1 μM. These data could suggest that the binding epitopes for the parasite ligand and the MGSA mutant R8A on DARC may be distinct. The inhibition curves for the mutants E39A, K49A, and R8A were rightward shifted consistent with their relative receptor binding affinities. The mutant E6A binds to DARC with high affinity and efficiently blocks parasite invasion (Fig. 4) but binds to the IL-8RB poorly and does not activate neutrophils (Fig. 2, *C* and *D*). Analogs of MGSA, like E6A, could be useful therapeutically in the design of small molecules that inhibit erythrocyte invasion by *P. vivax* but have no effect on neutrophils. With the increasing incidence of chloroquine-resistant strains of malaria, new approaches to combat this disease are required, and therapies based on chemokine analogs constitute a new and novel approach in the fight against *P. vivax*-induced malaria.

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