Expression of the Duffy Antigen in K562 Cells

EVIDENCE THAT IT IS THE HUMAN ERYTHROCYTE CHEMOKINE RECEPTOR*

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The human malarial parasite Plasmodium vivax invades erythrocytes by binding to a cell surface protein identified as the Duffy blood group antigen. The molecular properties of the Duffy antigen, which was recently cloned, are very similar to those of a chemokine binding protein known as the human erythrocyte chemokine receptor. This has led to the suggestion that these two molecules are the same protein. To further investigate the suspected double identity of the Duffy antigen we have transfected it into a human erythrocylemic cell line, K562. Cells stably expressing the Duffy antigen were isolated and used to characterize the protein. K562 cells transfected with the Duffy antigen displayed specific 125I-melanoma growth-stimulating activity (MGSA) binding while mock transfected cells did not. Comparison of 125I-MGSA binding to the Duffy antigen and the human erythrocyte chemokine receptor showed that the specific 125I-MGSA binding to both proteins was displaced by excess unlabeled MGSA, interleukin-8, RANTES, monocyte chemotactic peptide-1, and platelet factor 4, but not by macrophage inflammatory protein-1α or -1β. Scatchard analysis of competition binding studies with these unlabeled chemokines revealed high affinity binding to the Duffy antigen with KD binding values of 24 ± 4.9, 20 ± 4.7, 41.9 ± 12.8, and 33.9 ± 7 nM for MGSA, interleukin-8, RANTES, and monocyte chemotactic peptide-1, respectively. A monoclonal antibody, Fy6, to the Duffy antigen inhibited 125I-MGSA binding to K562 cells expressing the Duffy antigen. Cell membranes from K562 cells permanently expressing the Duffy antigen were chemically cross-linked with 125I-MGSA. SDS-polyacrylamide gel electrophoresis analysis of the cross-linked products showed covalent incorporation of radiolabeled MGSA into a protein of molecular mass 47 kDa, and cross-linking was inhibited in the presence of unlabeled MGSA. These studies provide evidence that the Duffy blood group antigen is the same protein as the human erythrocyte chemokine receptor.

The invasion of the body by pathogenic organisms triggers a cellular response by the immune system that leads to the recruitment of leukocytes that seek out and destroy the foreign invaders. The initial migration of leukocytes toward the site of infection occurs by chemotaxis and is mediated by the chemokines, a group of small soluble polypeptides (1–3). So far, over 20 chemokines have been identified, and these have been classified into two separate groups dependent on whether the first two conserved cysteine residues are separated by an intervening amino acid (C-X-C) or whether they are adjacent (C-C) (1). The C-X-C class members include interleukin-8 (IL-8) and melanoma growth-stimulating activity (MGSA), while the C-C class includes RANTES and monocyte chemotactic peptide-1 (MCP-1).

The first step in chemokine action is binding to specific cell surface receptors, and chemokine receptors in neutrophils, monocytes and lymphocytes have been identified and cloned (4–6). In general these receptors are highly specific, and C-X-C and C-C chemokines do not cross-compete for binding (6, 7). However, the human erythrocyte chemokine (CK) receptor, originally postulated to be a “sink” for IL-8 (8), is an exception to this rule and binds chemokines of both classes with high affinity (7, 9). In addition to its role as a clearance receptor for proinflammatory chemokines the CK receptor may have another hitherto unsuspected identity. It appears to be the same molecule as the Duffy blood group antigen that is a receptor for the human malarial parasite Plasmodium vivax (10). These findings were based on three observations. First, erythrocytes from all individuals who failed to bind IL-8 also lacked the Duffy blood group antigen. Second, a monoclonal antibody that specifically binds to the Duffy blood group antigen (11) blocked binding of IL-8 and other chemokines to Duffy positive erythrocytes. Third, increasing concentrations of both MGSA and IL-8 dose dependently blocked the binding of the parasite ligand and the invasion of human erythrocytes by Plasmodium knowlesi, a related monkey malaria. However, direct evidence in support of the idea that the Duffy blood group antigen and the chemokine receptor are the same protein requires their molecular cloning.

Recently the Duffy blood group antigen was cloned (12). The protein consists of 338 amino acid residues with a theoretical molecular mass of around 36 kDa that agrees with the molecular mass of the deglycosylated Duffy antigen (13, 14). It is a highly hydrophobic protein, and 60% of its mass is made up of hydrophobic amino acids. The protein has an N-terminal stretch of 65 amino acids with two potential N-glycosylation sites on the extracellular side of the cell and a 24-amino acid C-terminal region on the cytoplasmic side of the cell. Around 72% of the molecule is buried in the membrane, and based on hydropathy plots it traverses the membrane nine times.

In this communication we have transfected DNA coding for the cloned Duffy blood group antigen into a human erythrocylemic cell line, K562. We show that cells expressing the recombinant Duffy antigen bind IL-8, MGSA, MCP-1, and RANTES with high affinity. Furthermore, this binding is inhibited by incubation with an antibody (Fy6) to the Duffy antigen. Finally membranes from cells expressing the Duffy blood group antigen can be specifically cross-linked with radiolabeled MGSA.

The abbreviations used are: IL-8, interleukin-8; MGSA, melanoma growth-stimulating activity; MCP-1, monocyte chemotactic peptide-1; CK, chemokine; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; PAGE, polyacrylamide gel electrophoresis; MIP, macrophage inflammatory protein.

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The molecular mass of the cross-linked protein is 47 kDa that is identical with the molecular mass of the 125I-IL-8 cross-linked CK receptor. These data unequivocally demonstrate that the Duffy blood group antigen is the human erythrocyte CK receptor.

**EXPERIMENTAL PROCEDURES**

**Materials.** 125I-IL-8, 125I-MCP-1, 125I-RANTES, and 125I-MGSA (specific activity, 2200 Ci/mmol) were from DuPont NEN. Unlabeled IL-8 and MGSA were purified as previously described (15, 16). Unlabeled RANTES and MCP-1 were from Peprotech. Reagents for electrophoresis were from Novex. Hepes and all other reagent grade chemicals were from Sigma.

**Cell Culture.** K562 cells were obtained from the American Type Culture Collection and were maintained in RPMI 1640 medium containing 10% fetal calf serum. Transfected K562 cells were maintained in RPMI 1640 medium containing 10% fetal calf serum and 400 μg/ml gentamicin. The cells were passaged weekly, and the medium was changed two additional times weekly. For binding assays the cells were collected, washed three times with RPMI 1640, and resuspended in binding buffer (RPMI 1640 containing 1% bovine serum albumin, 20 mM Hepes, pH 7.4). Cell viability was assessed by trypan blue exclusion, and the cell number was determined by counting the cells in a hemacytometer.

**Construction of Expression Plasmid and Transfection.** The full-length cDNA insert coding for the Duffy antigen was subcloned in pBluescript-SK vector (Stratagene). This was digested with PstI and double digested pcDNA 1 (Invitrogen). The human erythrocyte leukemic cell line K562 was co-transfected with 2 μg of pcDNA 1 plasmid DNA and 200 ng of a plasmid containing the neomycin gene and an Rous sarcoma virus promoter. Transfections were carried out by using 12 μg of Lipofectamine (Life Technologies Inc.) according to the manufacturer’s protocol. Stable transfectants expressing neomycin resistance were selected, and from these a stable cell line expressing the Duffy antigen was selected by growing the transfected cells in RPMI 1640 medium containing 10% fetal calf serum and 400 μg/ml gentamicin (Life Technologies Inc.). Mock transfected K562 cells were transfected with the same expression plasmid lacking the cDNA for the Duffy antigen. Mock transfected cells were transiently transfected.

**Flow Cytometry Analysis.** The expression of the Duffy antigen in K562 cells was assayed by staining the cells with a monoclonal antibody (Fy6) to the protein. Briefly, 1.5 × 10⁶ cells in 1.5 ml of FACS medium (RPMI containing 10% fetal calf serum) were incubated with antibody at 4 μg/ml for 1 h at room temperature. Cells were washed three times, 5 min each, with FACS medium. Washed cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Life Technologies Inc.) at 200-fold dilution in FACS medium for 1 h at room temperature in the dark. After incubation, cells were washed three times with PBS, resuspended in the same buffer at a density of 1 × 10⁶ cells/ml, and fluorescence was measured on a FACS scan flow cytometer (Becton Dickinson). Results are given as the mean intensity. Controls were set up by analyzing mock transfected K562 cells and by analyzing K562 cells transiently expressing the Duffy antigen except that the incubation step with the monoclonal antibody was omitted.

**Receptor Binding Assays.** K562 cells (1 × 10⁶ cells/ml) stably expressing the Duffy antigen were incubated with 125I-labeled ligands (0.2 nM) and varying concentrations of unlabeled ligand at 4 °C for 1 h. The incubation was terminated by removing aliquots from the cell suspension and separating cells from buffer by centrifugation through a silicone/paraffin oil mixture as described previously (17). Nonspecific binding was determined in the presence of 1 μM unlabeled ligand. The binding data were curve fit with the computer program LIGAND (18) to determine the affinity (Kd), number of sites, and nonspecific binding.

**Preparation of Cell Membranes.** Cell membranes were made from K562 cells stably expressing the Duffy antigen and from mock transfected cells. Briefly, cells were resuspended to a final concentration of 2 × 10⁷ cells/ml in 50 mM Tris-HCl buffer, pH 7.4, containing 5 μg/ml each of leupeptin and aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, 0.05 mM PMSF, 0.1 mM EDTA, and 1 mM EGTA (lysis buffer). The cells were placed in a nitrogen cavitation chamber under 500 psi of pressure at 4 °C for 30 min. After 30 min the lysed cells were removed and centrifuged at 500 g for 20 min. The cell pellet, which consisted of cell debris and nuclei, was discarded, and the supernatant was centrifuged at 48,000 g for 30 min. The cell pellet, which consisted of total cell membranes, was removed and resuspended to a final concentration of 1.5 mg/ml in lysis buffer and stored at −20 °C until further use.

**Cross-linking of 125I-MGSA to K562 Cell Membranes.** Cross-linking of 125I-MGSA to K562 cell membranes was performed as described (19). The cross-linked membranes were incubated in the presence of 5 nM 125I-MGSA, in the presence or absence of 1 μM unlabeled MGSA, for 1 h at 37 °C in PBS, pH 7.4. At the end of the incubation, the membranes were pelleted by centrifugation (100,000 × g for 15 min), made up to the original volume in PBS, and chemically cross-linked with EDC at a final concentration of 1 M for 1 h at room temperature. The membranes were then pelleted as described above and solubilized in SDS sample buffer in the presence of 50 mM dithiothreitol for 3 min at room temperature and then analyzed by 12% SDS gels.

**RESULTS AND DISCUSSION**

In a recent communication we suggested that the Duffy blood group antigen, a receptor for the human malarial parasite Plasmodium vivax, may be a human erythrocyte CK receptor (10). With the recent cloning of the Duffy blood group antigen (12) it is now possible to test the hypothesis that these two proteins share a common identity, and the present studies were initiated to aid in this determination. In this communication we describe the molecular analysis of the Duffy blood group antigen, which we have stably expressed into a mammalian cell line.

A full-length cDNA clone coding for the Duffy antigen was isolated from a human bone marrow cDNA library using a polymerase chain reaction-amplified DNA fragment encoding an internal peptide sequence of the protein (12). The cDNA clone was subcloned into an expression vector, shown in Fig. 1A, and used to transfect a human erythrocyte leukemic cell line, K562. Stable K562 transfectants expressing the Duffy antigen cDNA were isolated as described under “Experimental Procedures.” Expression of the Duffy blood group antigen in K562 cells was assessed by flow cytometry, using a monoclonal antibody (Fy6) directed against the Duffy antigen (Fig. 1B). Stable K562 transfectants expressing the Duffy antigen stained strongly with Fy6 and produced a strong shift specific mean channel in the FACS scan compared with the mock transfected K562 cells that are negative for staining with the antibody. The staining of the cells by Fy6 is specific since omission of the Fy6.
antibody reduces the fluorescence to levels observed with mock transfected K562 cells.

The CK receptor specifically binds a wide array of radiolabeled chemokines including IL-8 and MGSAs (7, 9). To determine whether recombinant Duffy antigen has a similar receptor binding profile to the human erythrocyte CK receptor, we carried out receptor binding studies in both cell types with $^{125}$-labeled MGSAs. Both the recombinant Duffy antigen and the human erythrocyte CK receptor bound radiolabeled MGSAs (Fig. 2). This binding was specific since addition of 1 µM unlabeled MGSAs reduced the binding to less than 15% (Fig. 2). In contrast, mock transfected K562 cells did not display any specific $^{125}$-labeled MGSAs binding. In addition the specific $^{125}$-labeled MGSAs binding to both the CK receptor and to the Duffy antigen was fully displaced by the addition of the unlabeled chemokines IL-8, MCP-1, RANTES, and PF4 but not by unlabeled IL-8, MCP-1, and MIP-1β (Fig. 2). Based on these data the Duffy antigen can bind radiolabeled MGSAs, and this binding can be displaced by the same range of chemokines that compete for MGSAs binding to the CK receptor.

To compare the receptor binding affinity of $^{125}$-labeled MGSAs for the CK receptor and the Duffy antigen, receptor competition curves were generated over a wide concentration range of unlabeled chemokines (MGSA, IL-8, RANTES, and MCP-1). The competition binding data were transformed by Scatchard analysis, and the resulting plots are shown (Fig. 3). The competition binding of $^{125}$-labeled MGSAs with unlabeled MGSAs, IL-8, RANTES, and MCP-1 gave linear plots consistent with a single class of sites with $K_D$ values of 24 ± 4.9, 20 ± 4.7, 41.9 ± 12.8, and 33.9 ± 7.6 nM, respectively, and a receptor density of 300,000 ± 30,000 sites/cell (Fig. 3). These binding constants for the Duffy antigen are a little higher than those previously obtained for chemokine binding to the CK receptor, which range from 5 to 10 nM with a receptor density of 5000 sites/cell (7, 9). The protein and lipid composition of the erythrocyte cell membrane is very different from that in most other cell types (19), and this difference in its membrane environment may play a role in the receptor binding affinity of the Duffy antigen/chemokine receptor. It is also possible that the higher affinity binding of chemokines to the CK receptor in erythrocytes could be due to an association of the CK receptor with another protein in erythrocytes that is not present in K562 cells and that this gives rise to the higher affinity binding observed in erythrocytes.

We have previously shown that a monoclonal antibody to the Duffy antigen, Fy6, dose responsively inhibited binding of IL-8 and other chemokines to Duffy positive erythrocytes (10). Here we show that Fy6 inhibits $^{125}$-labeled MGSAs binding to the Duffy antigen (Fig. 4). The $K_I$ for this inhibition is around 600 pm, which is similar to that for the human erythrocyte CK receptor (10).

To examine the biochemical properties of the Duffy antigen we prepared membranes from K562 cells stably expressing the protein. The membranes were incubated with $^{125}$-labeled MGSAs, in the presence and absence of 1 µM unlabeled MGSAs, and were covalently labeled with EDC (Fig. 5). Analysis by SDS-PAGE revealed the covalent labeling of a protein of approximately molecular mass of 47 kDa (Fig. 5, lane 1). The inclusion of unlabeled MGSAs decreased its intensity of labeling (Fig. 5, lane 2). In contrast, membranes prepared from mock transfected K562 cells did not cross-link with radiolabeled MGSAs (Fig. 5, lane 3). If MGSAs binds as a monomer (8 kDa) the molecular mass of the Duffy antigen from cross-linking studies with radiolabeled MGSAs is 39 kDa. This is identical to the molecular mass of the human erythrocyte CK receptor from cross-linking studies with radiolabeled IL-8 (7, 9) and is further proof that these two proteins are identical.

In summary, we have presented several lines of evidence that demonstrate that the recently cloned Duffy blood group antigen

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**Fig. 2. Inhibition of $^{125}$-labeled MGSAs binding to human erythrocytes (A) and K562 cells (B) stably expressing the Duffy antigen.** Human erythrocytes (2 x 10⁶ cells/ml) and K562 cells (4 x 10⁶ cells/ml) were incubated for 1 h at 4°C with $^{125}$-labeled MGSAs (0.2 nM) in the absence or presence of 1 µM concentrations of unlabeled MGSAs, IL-8, RANTES, MCP-1, PF4, MIP-1α, and MIP-1β. The binding reactions were stopped as described under "Experimental Procedures." The data are expressed as counts/min of $^{125}$-labeled MGSA bound (per 10⁶ cells) for K562 cells and (per 10⁶ cells) for human erythrocytes.

**Fig. 3. Scatchard analysis of $^{125}$-labeled MGSAs binding to K562 cells stably expressing the Duffy antigen.** K562 cells (4 x 10⁶ cells/ml) stably expressing the Duffy antigen were incubated for 1 h at 4°C with $^{125}$-labeled MGSA (0.2 nM) in the presence of increasing concentrations of unlabeled MGSA (upper left panel), IL-8 (upper right panel), RANTES (lower left panel), and MCP-1 (lower right panel). The binding reactions were stopped as described under "Experimental Procedures."

**Fig. 4. Inhibition of $^{125}$-labeled MGSAs binding to the Duffy antigen by the monoclonal antibody Fy6.** K562 cells (4 x 10⁶ cells/ml) stably expressing the Duffy antigen were preincubated with increasing concentrations of Fy6 antibody (6) or an irrelevant antibody (3) for 2 h at 4°C. The cells were then incubated for a further hour with 0.2 nM $^{125}$-labeled MGSAs. The binding reactions were stopped as described under "Experimental Procedures." Non specific binding, determined by the addition of 1 µM unlabeled MGSA, was subtracted from total binding to yield specific binding.
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FIG. 5. Covalent cross-linking of $^{125}$I-MGSA to K562 cells expressing the Duffy antigen. Membranes prepared from K562 cells stably expressing the Duffy antigen were incubated with 5 nM $^{125}$I-MGSA in the absence (lane 1) and in the presence (lane 2) of 1 μM unlabeled MGSA. Membranes prepared from mock transfected K562 cells were incubated with 5 nM $^{125}$I-MGSA (lane 3). After 1 h at 4 °C the membranes were washed free of unbound label. The bound $^{125}$I-IL-8 was cross-linked by incubation with EDC for 1 h at 4 °C and then washed with PBS before analysis by SDS-PAGE. 150 μg of protein were applied to the gel. After electrophoresis the gels were dried down and subjected to autoradiography.

is the same molecule as the human erythrocyte chemokine receptor. First, both proteins bind radiolabeled MGSA, and this binding is displaced by the same spectrum of unlabeled chemokines. Second, the binding is not displaced by unlabeled MIP-1α and MIP-1β. Third, a monoclonal antibody to the Duffy antigen dose responsively inhibits $^{125}$I-MGSA binding to both proteins. Finally, both proteins are cross-linked with $^{125}$I-MGSA, and their molecular mass by cross-linking is identical.

REFERENCES