DARC

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SUMMARY

The Duffy blood group antigen is a portal of entry for the malarial parasite *Plasmodium vivax*. Recent work has shown that this protein, also known as DARC, is a promiscuous chemokine receptor. Although there is still no evidence that DARC can signal, its expression in the CNS on neurons (Purkinje cells) and on endothelial cells lining postcapillary venules (the site of leukocyte trafficking) hint at an as yet unknown physiological role. This article reviews the literature, both past and recent, and discusses the biology of this enigmatic protein.

BACKGROUND

Discovery

The Duffy blood group antigen was first identified serologically on human erythrocytes as the target of alloantibodies that can cause *posttransfusion hemolytic reactions* (Cutbush *et al*., 1950; Ikin *et al*., 1951). Characterization of the Duffy antigen revealed that it was degraded by the proteases chymotrypsin and pronase, has a tendency to aggregate when boiled in SDS, and was recognized as a 46 kDa glycoprotein by a monoclonal antibody designated Fy6 (Hadley *et al*., 1984; Nichols *et al*., 1987). There are two major antigens, Fy^a and Fy^b, that were defined by a human erythrocyte agglutination assay. The Duffy antigens are encoded by two codominant alleles FyA and FyB and four different phenotypes exist: Fy(a+b+), Fy(a+b−), Fy(a−b+), and Fy(a−b−). The Fy(a−b−) phenotype, also known as the Duffy-negative phenotype, is found mainly in West Africans and in around two-thirds of Americans of African descent (Sanger *et al*., 1955). This phenotype results in the absence of the Duffy antigen in the erythrocytes of these individuals. Interestingly, these same individuals are resistant to infection by the *malaria* parasite *Plasmodium vivax*, and two separate reports established that the Duffy antigen was a cellular attachment factor for this species of malaria parasite (Miller *et al*., 1975, 1976).

Although these studies identified the Duffy antigen as a portal of entry for the malarial parasite *P. vivax*, the biology of this protein took a totally unexpected turn based on the findings of two interconnected studies. In 1991 Darbonne *et al*. described an erythrocyte protein that was able to bind the chemokine IL-8 with high affinity. During the course of characterizing this chemokine-binding protein, Horuk *et al*. (1993) showed that it bound a range of CXC and CC chemokines, including IL-8, MGSA, RANTES, and MCP-1 and, interestingly, noted that erythrocytes obtained from most African Americans did not bind these chemokines. This observation, together with the fact that the monoclonal antibody Fy6 blocked chemokine binding and that the chemokine MGSA inhibited *P. vivax* invasion of erythrocytes, suggested that the Duffy antigen could also be a chemokine receptor (Horuk *et al*., 1993). These findings were confirmed when the Duffy antigen was cloned (Chaudhuri *et al*., 1993) and the expressed protein, which had a seven transmembrane domain structure similar to that of other chemokine receptors, was shown to bind a range of CXC and CC chemokines (Chaudhuri *et al*., 1994; Neote *et al*., 1994).

The Duffy blood group antigen has a wider range of expression than was at first realized and as we shall see later it is expressed on a number of nonerythroid tissues such as endothelial cells of postcapillary venules and Purkinje cells in the cerebellum (Hadley *et al*., 1994; Horuk *et al*., 1997).

Alternative names

Duffy blood group antigen, Duffy glycoprotein, Duffy antigen receptor for chemokines (DARC).

Structure

DARC is a putative seven transmembrane domain receptor.

Main activities and pathophysiological roles

The involvement of DARC in pathophysiology is exemplified by its role as a transmission factor for *malaria*. The first clues for this were provided by Miller *et al*. (1975) who reported that Duffy-negative human erythrocytes were resistant to invasion by *P. knowlesi*, a simian malaria related to *P. vivax* that also requires DARC to invade human erythrocytes. These studies, together with the observation that almost 100% of individuals in West Africa who are unable to be infected with *P. vivax* malaria are also Duffy-negative, suggested a link between this innate resistance and the Fy(a−b−) phenotype. A number of studies demonstrated a correlation between the Duffy-negative phenotype and resistance to *P. vivax* infection (confirming that DARC was the vehicle of entry for the invasion of human erythrocytes by this malarial parasite). As suggested by Darbonne *et al*. (1991), the physiological role of DARC might be to act as an intravascular sink, which could bind and inactivate circulating chemokines. This would presumably generate a chemokine gradient with higher concentrations of active chemokines found in the subendothelial matrix, possibly bound to sulfated glycans. Clearance of IL-8 from the plasma by binding to erythrocytes has been demonstrated in humans treated with IL-1 (Tilg *et al*., 1993). Although these data suggest a role for the erythrocyte DARC as a chemokine-binding protein, there is no formal evidence to suggest that this is its true biological role. In fact, if the postulated role of DARC in erythrocytes is biologically relevant, then the absence of this protein in Duffy-negative individuals should have clinical consequences. In this context it is interesting that Africans and African Americans have a lower peripheral neutrophil count, although this does not appear to be associated with any pathophysiology (Broun *et al*., 1996). Whether or not this neutropenia is causally related to the Duffy-negative phenotype remains to be seen.

The localization of DARC to endothelial cells that line postcapillary venules is interesting since these structures are a dynamic interface that comprise the site for leukocyte transmigration from the vascular space into the tissue space during inflammation. This process, which is part of the inflammation cascade, is characterized by cytokine-mediated endothelial cell and leukocyte activation, selectin-mediated leukocyte rolling, integrin-mediated leukocyte adherence and ultimately migration of the leukocyte out of the vascular space into the surrounding tissues along chemokine gradients (Springer, 1991, 1994; Lasky, 1992). Endothelial cells, activated by cytokines *in vivo*, produce IL-8 (Hébert *et al*., 1991), which may set up a chemotactic gradient favoring transendothelial diapedesis of leukocytes. The localization of DARC to endothelial cells of postcapillary venules, together with its ability to bind proinflammatory chemokines, suggests that it may play a major role in this inflammatory cascade.

GENE

Accession numbers

Human cDNA: U01839

Sequence

The gene sequence of the human protein has recently been reported (Chaudhuri *et al*., 1993). The successful cloning of DARC was based on sequence information obtained from protease-digested fragments of Fy6 immunoaffinity-purified erythrocyte proteins. The human DARC sequence was found to have an open reading frame of 1014 bases, encoding a protein of 338 amino acids on a single intronless gene (**Figure 1**).

Northern blot analysis revealed a 1.27 kb transcript in kidney, spleen, and fetal liver, while the brain had an additional 8.5 kb transcript (Chaudhuri *et al*., 1993). Although the two different DARC mRNA isoforms in the brain are differentially regulated and differ in their 5′ untranslated sequence, they encode the same polypeptide (Le Van Kim *et al*., 1997). The basis for the Duffy-negative phenotype was recently shown to be due to a single T to C substitution at nucleotide −46 in the gene sequence. This mutation impairs the promoter activity in erythroid cells by disrupting a binding site for the GATA1 erythroid transcription factor (Tournamille *et al*., 1995). In addition, a novel mutation, present in the FY*B coding sequence (271C to T), is associated with some

Figure 1 Nucleotide sequence of human DARC.

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   1 GGCTTCCCCA GGACTGTTCC TGCTCCGGCT CTTCAGGCTC CCTGCTTTGT CCTTTTCCAC
  61 TGTCCGCACT GCATCTGACT CCTGCAGAGA CCTTGTTCTC CCACCCGACC TTCCTCTCTG
 121 TCCTCCCCTC CCACCTGCCC CTCAGTTCCC AGGAGACTCT TCCGGTGTAA CTCTGATGGC
 181 CTCCTCTGGG TATGTCCTCC AGGCGGAGCT CTCCCCCTCA ACTGAGAACT CAAGTCAGCT
 241 GGACTTCGAA GATGTATGGA ATTCTTCCTA TGGTGTGAAT GATTCCTTCC CAGATGGAGA
 301 CTATQATGCC AACCTGGAAG CAGCTGCCCC CTGCCACTCC TGTAACCTGC TGGATGACTC
 361 TGCACTGCCC TTCTTCATCC TCACCAGTGT CCTGGGTATC CTAGCTAGCA GCACTGTCCT
 421 CTTCATGCTT TTCAGACCTC TCTTCCGCTG GCAGCTCTGC CCTGGCTGGC CTGTCCTGGC
 481 ACAGCTGGCT GTGGGCAGTG CCCTCTTCAG CATTGTGGTG CCCGTCTTGG CCCCAGGGCT
 541 AGGTAGCACT CGCAGCTCTG CCCTGTGTAG CCTGGGCTAC TGTGTCTGGT ATGGCTCAGC
 601 CTTTGCCCAG GCTTTGCTGC TAGGGTGCCA TGCCTCCCTG GGCCACAGAC TGGGTGCAGG
 661 CCAGGTCCCA GGCCTCACCC TGGGGCTCAC TGTGGGAATT TGGGGAGTGG CTGCCCTACT
 721 GACACTGCCT GTCACCCTGG CCAGTGGTGC TTCTGGTGGA CTCTGCACCC TGATATACAG
 781 CACGGAGCTG AAGGCTTTGC AGGCCACACA CACTGTAGCC TGTCTTGCCA TCTTTGTCTT
 841 GTTGCCATTG GGTTTGTTTG GAGCCAAGGG GCTGAAGAAG GCATTGGGTA TGGGGCCAGG
 901 CCCCTGGATG AATATCCTGT GGGCCTGGTT TATTTTCTGG TGGCCTCATG GGGTGGTTCT
 961 AGGACTGGAT TTCCTGGTGA GGTCCAAGCT GTTGCTGTTG TCAACATGTC TGGCCCAGCA
1021 GGCTCTGGAC CTGCTGCTGA ACCTGGCAGA AGCCCTGGCA ATTTTGCACT GTGTGGCTAC
1081 GCCCCTGCTC CTCGCCCTAT TCTGCCACCA GGCCACCCGC ACCCTCTTGC CCTCTCTGCC
1141 CCTCCCTGAA GGATGGTCTT CTCATCTGGA CACCCTTGGA AGCAAATCCT AGTTCTCTTC
1201 CCACCTGTCA ACCTGAATTA AAGTCTACAC TGCCTTTGTG
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Duffy-negative phenotypes among non-Ashkenazi Jews and among Brazilian blacks (Parasol *et al*., 1998).

Chromosome location and linkages

The chromosomal location of the Duffy blood group antigen has been mapped to human chromosome 1q22-q23 where it is flanked by the genes for spectrin and Na/K ATPase (Marsh, 1977).

PROTEIN

Accession numbers

Mouse: AF016584, AF016697

Sequence

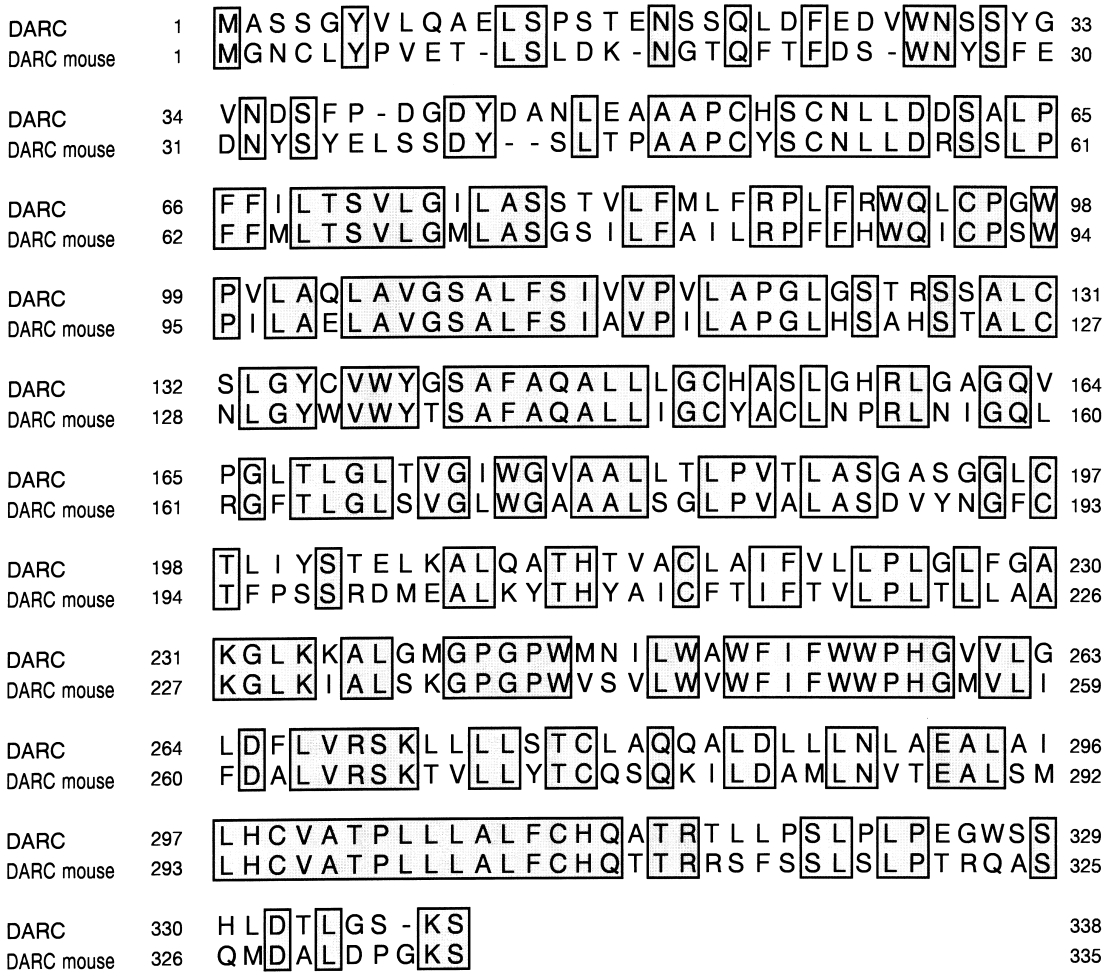
See **Figure 2**.

Description of protein

The open reading frame of the 1014 bp cDNA clone of DARC predicts a hydrophobic protein of 338 amino acid residues with a theoretical molecular mass of around 36 kDa (**Figure 3**). The protein has two *N*-linked glycosylation sites on the N-terminus. Based on hydropathy analysis of the cDNA clone it was originally suggested that the protein contains nine membrane-spanning domains. However, subsequent analysis using alternative computerized hydropathy plots revealed that DARC has seven hydrophobic membrane-spanning segments, more in line with that of the other cloned chemokine receptors. While DARC is biochemically distinct from the other cloned chemokine receptors, it does appear to share their common heptahelical topology, including the conservation of a number of Trp and Pro residues in helices IV, V, VI, and VII, which are highly conserved throughout the entire family of G protein-linked receptors and are postulated to play major roles in receptor binding and function (Wess *et al*., 1993). The cloned DARC protein also has four conserved Cys residues (at the N-terminus, and the first, second, and third extracellular loops) that are thought to be paired to form disulfides which help to stabilize the protein.

Comparison of the primary sequence of DARC with those of the other chemokine receptors does, however, reveal some unique differences. For example, many residues in the predicted cytoplasmic loops, including the Asp-Arg-Tyr (DRY) motif at the end of the third transmembrane-spanning helix, and the C-terminal tail, which have been shown to be important in interacting with and coupling to G proteins, are not conserved in DARC. These changes in primary structure, together with the failure of the receptor to respond to ligands in any biological assay or to stimulate GTPase activity and the absence of any effect on ligand binding by treatment with *pertussis* toxin (Horuk, unpublished), suggest that DARC may not be coupled to G-proteins. In addition, when DARC is expressed in an insect cell line known to be devoid of G_i proteins (Quehenberger

Figure 2 Alignment of the primary structures of the cloned human and mouse DARC. Conserved residues are in orange. (Full colour figure may be viewed online.)

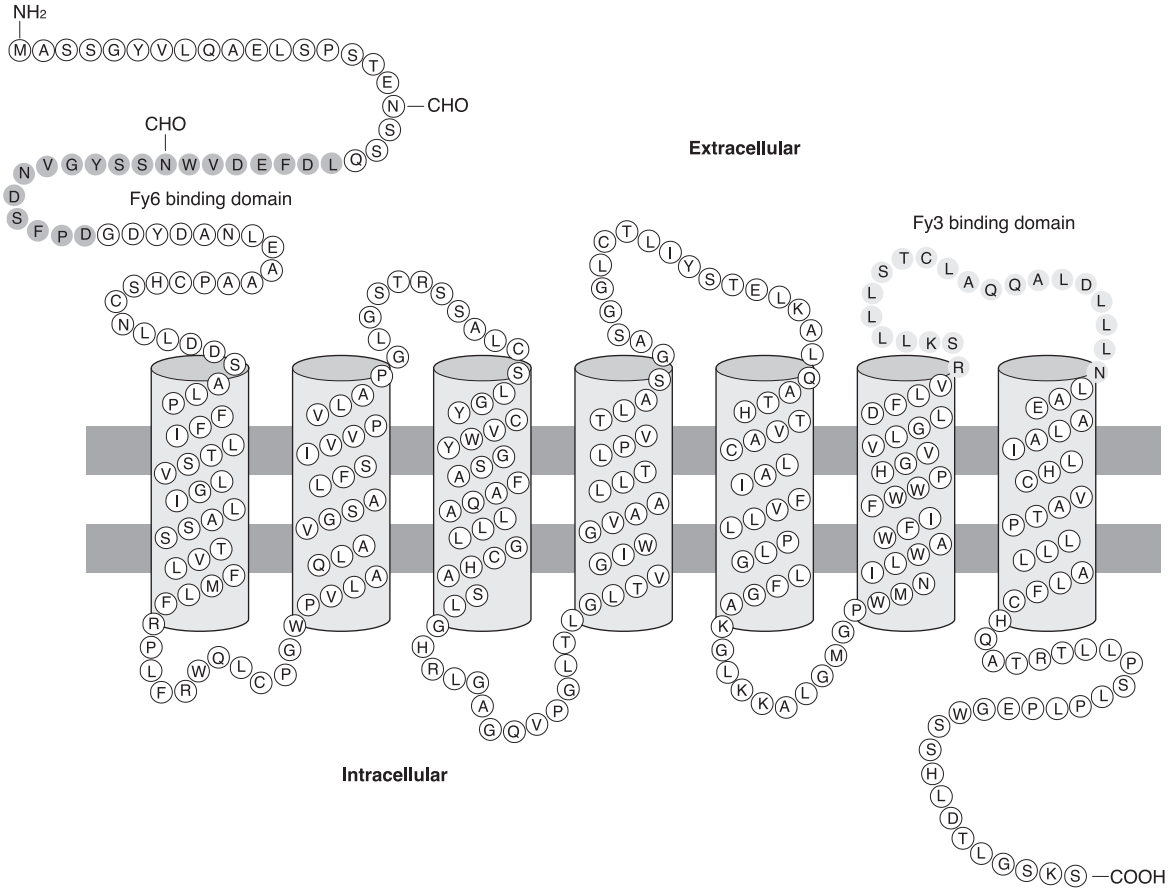


et al., 1992), it demonstrated high-affinity ligand binding, in contrast to CXCR2, which failed to bind IL-8, presumably because the absence of interaction with a G_i subunit caused it to assume a conformation that lacked a functional binding pocket (Horuk and Peiper, unpublished).

Thus DARC appears to belong to a growing family of seven transmembrane receptors that are not coupled to G proteins. These include subtypes of receptors for dopamine, somatostatin, vasoactive intestinal peptide, and angiotensin, all of which appear to bind ligand independently of G protein coupling, although they have a DRY motif (Sokoloff *et al.*, 1990; Rens-Domiano *et al.*, 1992; Gressens *et al.*, 1993; Mukoyama *et al.*, 1993). Other members of this group include two *Drosophila* proteins, the BOSS protein, which has a large N-terminal extracellular domain, is not G protein-coupled, but is, nevertheless, biologically active and appears to be a ligand for a tyrosine kinase receptor called sevenless (Krämer *et al.*, 1991).

In addition, recent work suggests that some G protein-coupled receptors can also signal through alternative independent pathways (Milne *et al.*, 1995). For example, when the G protein-coupled cAMP receptor from the slime mold *Dictyostelium* is expressed in a cell line deficient in G protein β subunits, it is still able to activate some cellular responses such as Ca^{2+} ion influx and receptor phosphorylation (Milne *et al.*, 1995). Based on these, and other findings, Schnitzler *et al.* (1995) have postulated that this receptor signals through two distinct pathways, one being the classical G protein-linked pathway, and the other a G protein-independent pathway which appears to involve the activation of the transcription factor G-box binding factor.

Figure 3 Proposed membrane topography of DARC. Membrane-spanning α helices are defined based on hydropathy analysis. CHO, potential *N*-linked glycosylation sites. Residues involved in binding to the monoclonal antibody Fy6 are shown in green; residues involved in binding to the monoclonal antibody Fy3 are shown in orange. (Full colour figure may be viewed online.)



Relevant homologies and species differences

DARC has very little primary amino acid sequence homology with the other cloned chemokine receptors; it is most closely homologous with CXCR2 with which it shares around 24% homology. Analysis of the nucleotide sequences of DARC from individuals that are of the Fy(a+b−), Fy(a−b+), and Fy(a+b+) phenotype reveals that the Fya and Fyb alleles differ by a single base substitution in the second position of codon 44 that encodes a glycine residue in Fya and an aspartic acid residue in Fyb (Chaudhuri *et al*., 1995). This polymorphism does not appear to have any physiological consequences.

In addition to the human nucleotide sequence, the mouse DARC sequence has also recently been described (Luo *et al*., 1997). Unlike the human sequence which is intronless, the mouse sequence is encoded on two exons: exon 1 of 55 nucleotides, which encodes seven amino acid residues; and exon 2 of 1038 nucleotides, which encodes 327 residues. The single intron consists of 462 nucleotides. The open reading frame shows 60% homology with the human DARC protein. However, mouse erythrocytes are serologically Duffy-negative and mouse erythrocyte membrane proteins do not crossreact with two Duffy-specific rabbit polyclonal antibodies.

DARC has been cloned from a number of primates, including chimpanzee, aotus, squirrel, and rhesus monkeys (Chaudhuri *et al*., 1995; Horuk *et al*., 1996). As expected, homologies between the primate and human proteins are high, ranging from 93 to 99%. In addition, DARC has been partially cloned from cow, pig, and rabbit and the primary structure is conserved approximately 70–75% (Horuk *et al*., 1996; Hadley and Peiper, 1997). The direct demonstration of the existence of a gene encoding a polypeptide highly homologous to DARC in cow, pig, rabbit, and

mouse and the preservation of chemokine-binding function in rodent and avian erythrocytes (Horuk *et al*., 1996) implies that this molecule might play an important biological role in these, and in other species.

Affinity for ligand(s)

Human erythrocytes are able to bind radiolabeled CXC and CC chemokines, including IL-8, MGSA, NAP-2, RANTES, and MCP-1 with high affinity (Horuk *et al*., 1993; Neote *et al*., 1993). However, human MIP-1α and MIP-1β were unable to compete effectively for binding. Scatchard analysis revealed that the affinity of binding ranged from 5 to 10 nM with around 5000 binding sites. The K_d for any combination of labeled chemokine displaced by any other was strikingly similar. These studies revealed that, in contrast to other chemokine receptors, DARC was able to bind both CXC and CC chemokines.

Receptor-binding studies with radiolabeled MGSA and IL-8 in mouse erythrocytes revealed specific binding of the radiolabeled chemokines that were displaceable by the predicted repertoire of unlabeled chemokines characteristic of DARC. However, some clear differences in chemokine binding by murine DARC compared to human DARC were observed. For example, mouse erythrocytes bound human IL-8 poorly compared to human MGSA, whereas both chemokines bound equally well to human erythrocytes. In addition, the CC chemokine MIP-1α readily displaced both radiolabeled MGSA and IL-8 from mouse erythrocytes; however, the K_d for this displacement is around 120 nM compared to a K_d of 5–10 nM for the binding of MGSA, RANTES, and MCP-1 to this receptor. In contrast, MIP-1α has almost no effect on chemokine binding in human erythrocytes.

In summary, chemokines can be classified into five distinct groups with respect to their ability to bind to DARC. Two distinct groups that bind to DARC, both human and mouse, with high affinity are the CXC chemokines that have the ELR motif (IL-8, MGSA, NAP-2, and ENA-78), and the basic CC chemokines (RANTES, MCP-1, and MCP-3). In contrast, the non-ELR CXC chemokines, characterized by PF4 and IP-10, bind to human and mouse DARC with low affinity. The acidic CC chemokines, characterized by the MIP-1 proteins, do not bind to human DARC and bind to mouse DARC with low affinity. Finally, the only member of the C chemokines so far characterized, lymphotactin, does not bind at all to DARC based on displacement studies with radiolabeled MGSA, and with an affinity that is in the low micromolar range based on direct binding studies with radiolabeled lymphotactin.

Chemokine-binding experiments with human, monkey, and chicken erythrocytes demonstrated that all four species bound chemokines with high affinity. The K_d values for MGSA binding were 6 nM human, 6 nM monkey, 13 nM mouse, and 9 nM chicken erythrocytes (Horuk *et al*., 1996). Thus, the chemokine-binding site in DARC appears to be highly conserved from human to bird, suggesting a non-redundant role for this protein.

Previous studies with other chemokine receptors including CXCR1, CXCR2, and CCR5 have revealed that the N-terminal domain of these receptors is at least partly responsible for their ligand-binding specificity (LaRosa *et al*., 1992; Gayle *et al*., 1993; Doms and Peipert, 1997). In a recent communication, Lu *et al*. (1995) show that this is also the case for the DARC. The ligand-binding specificity of DARC was investigated with a receptor chimera composed of the N-terminal extracellular domain of DARC (amino acid residues 1–66) and the seven transmembrane spanning regions and cytoplasmic tail of CXCR2 (amino acid residues 50–355). Receptor-binding studies clearly demonstrated that the DARC/CXCR2 chimera could bind MGSA, IL-8, and RANTES with high affinity. Interestingly, the chimera, in contrast to DARC, did not bind MCP-1: the reasons for this are at present unclear. However, an alanine scan mutant of MGSA, E_6A, displayed high-affinity binding to both DARC and the DARC/CXCR2 chimera but did not bind to CXCR2. These findings suggest that the N-terminal region of the DARC is at least partly responsible for conferring high-affinity chemokine binding to the protein. It may also contain the binding epitopes for the binding of the *P. vivax* malaria parasite ligand, since COS cells transfected with the parasite ligand can bind to the DARC/CXCR2 chimera.

Analysis of erythrocytes treated with sulfhydryl group-modifying reagents have demonstrated that the chemokine receptor function of DARC requires the integrity of disulfide bond(s) but not that of free sulfhydryl group(s) (Tournamille *et al*., 1997). Accordingly, mutation of cysteines 51 and 276 abolished chemokine binding to DARC transfectants. These results suggest that the chemokine-binding pocket of DARC, which includes residues in the N-terminus and the third extracellular loop, are brought into close vicinity by a disulfide bridge.

Recent studies have identified a single-base polymorphism, C286T, of DARC in some individuals: this results in a single amino acid substitution Arg89Cys that affects both Fy6 and chemokine

binding to DARC transfectants (Tournamille *et al*., 1998). This mutation results in a very low expression of DARC on erythroid cells. Examination of DARC sequences shows that this residue, which is in the first extracellular loop of the protein, is conserved or replaced by a homologous charged His residue in other species. The extracellular loops of chemokine receptors and seven transmembrane G protein-coupled receptors in general contain a number of positively charged Lys and Arg residues. It has been suggested that these positively charged amino acids can interact with negative charges on the polar headgroups of phospholipids in the cell membrane and that this may help to maintain receptor topology. Thus, mutation of these residues could lead to a disruption of the overall architecture of the receptor and lead to a decrease in binding affinity for the ligand.

Further insight into the chemokine binding site of DARC has been provided by alanine scanning mutagenesis studies with MGSA (Hesselgesser *et al*., 1995). Previous work with IL-8 showed that the sequence $E_4L_5R_6$ was essential for receptor binding and neutrophil activation of CXCR1 and CXCR2 (Hébert *et al*., 1991). All three residues – arginine in particular – are highly sensitive to modification. In contrast to these findings, recent work with alanine scan mutants of MGSA suggests that the ELR motif is less important for binding to DARC than to CXCR2 (Hesselgesser *et al*., 1995). These studies demonstrated that the binding affinities of the MGSA mutants E_6A, and L_7A for DARC were only about 2-fold and 10-fold less, respectively, than that of wild-type MGSA, suggesting that these residues are not important in determining binding to DARC (Hesselgesser *et al*., 1995). The binding affinity of the MGSA mutant R_8A, however, was approximately 240-fold lower than wild-type MGSA, indicating that a positive charge may be required in this region of the protein for binding. Interestingly, some members of the CC chemokines (which all lack this positive charge as well as the E and L residues) like RANTES and MCP-1, bind to DARC whereas others like MIP-1α and MIP-1β do not bind (Horuk, 1994). Based on analysis of the solution structure of RANTES (Skelton *et al*., 1995), and the amino acid sequence of the CC chemokines, we can speculate that the positively charged lysine residue next 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.

The *P. vivax* and *P. knowlesi* Duffy-binding ligands have been cloned and are large proteins of molecular mass 140 kDa and 135 kDa, respectively (Adams *et al*., 1992). Based on their sequence homologies, these ligands have been divided into six regions, including two cysteine-rich areas (Adams *et al*., 1992). Recently, one of these two cysteine-rich stretches of the parasite ligand (region II) has been identified as the binding domain that binds to the DARC. The region II protein ($\sim$330 amino acids) was expressed in COS7 cells and found to be capable of binding to Duffy-positive but not to Duffy-negative erythrocytes. Furthermore, preincubation with MGSA and IL-8 blocked the binding of the region II protein to Duffy-positive erythrocytes. These studies suggest that the parasite ligand and the chemokines could bind to similar epitopes on the DARC. Examination of the primary sequences of these disparate proteins reveals no regions of homology and we will have to await a detailed comparison of their tertiary structures, which is still lacking for the parasite Duffy binding protein, to obtain more information regarding their binding to DARC.

Cell types and tissues expressing the receptor

A variety of human erythroleukemic cell lines, including Kg1, K562, and HEL cells, were tested for the expression of DARC by screening for [^{125}I]IL-8 binding (Horuk *et al*., 1994). Of the cell lines screened, only the HEL cells showed specific [^{125}I]IL-8 binding. These cells, which were originally derived from a patient with *Hodgkin's disease*, carry the phenotypic markers of erythroid cells that include the ability to synthesize globin (Martin and Papayannopoulou, 1982). Further analysis of HEL cells determined that they appeared to express a protein with the characteristic hallmarks of DARC, i.e. receptor binding of a wide array of chemokines, inhibition of this binding by the Fy6 antibody, crossreactivity by western blotting with Fy6 of a protein of a similar molecular mass, and hybridization of mRNA from HEL cells with a cDNA probe to DARC.

Transcripts encoding isoforms of DARC have been detected in polyadenylated RNA from a variety of human tissues, including kidney, spleen, lung, and brain (Chaudhuri *et al*., 1993; Neote *et al*., 1994). Based on a number of experimental observations, including chemokine binding and immunohistochemical staining, Hadley *et al*. (1994) showed that DARC protein was expressed on endothelial cells lining the small blood vessels of the human kidney. Evidence that the DARC polypeptide is also expressed in

endothelial cells lining postcapillary venules of spleen was provided in a related report (Peiper *et al*., 1995). In the same study the authors demonstrated the specific immunohistochemical staining of soft tissue from a Duffy-negative patient with the monoclonal antibody Fy6. The DARC-specific staining was localized to specialized endothelial cells that line the postcapillary venules of the tissue, but, as expected, erythrocytes within the lumen failed to show binding of the monoclonal antibody. Confirmation of the immunohistochemical identification of this protein was provided by northern blotting, ligand binding, chemical crosslinking, and immunoblotting experiments which were all consistent with the characteristic features of DARC.

These findings indicate that the expression of the DARC is retained on endothelial cells even in the presence of strong negative selection from morbidity and mortality from *P. vivax* which resulted in the loss of expression of this protein by erythrocytes. This raises the possibility that DARC plays a critical role in the biology of endothelial cells. Although there is no evidence that DARC can transduce a biological signal in endothelial cells, it has been shown that the receptor can internalize in response to ligand binding (Horuk *et al*., 1994). In this context it is interesting that a recent study has suggested that DARC might participate in transcytosis and surface presentation of IL-8 by venular endothelial cells (Middleton *et al*., 1997).

Northern blotting experiments demonstrating that mRNA encoding DARC is expressed in human brain (Chaudhuri *et al*., 1993) prompted us to examine archival sections of human brain for DARC expression (Horuk *et al*., 1997). Immunohistochemistry of brain sections revealed that neuronal processes were expressing DARC in the cerebellum and adjacent regions of the brainstem. These immunohistochemical observations were confirmed by ligand-binding studies with isolated membranes from human cerebellum which demonstrated radiolabeled chemokine binding with a pattern of displacement identical to that observed for DARC in erythrocytes. Scatchard analysis of radiolabeled chemokine binding revealed a single class of binding sites in the cerebellum with a K_d of 4 nM. This binding affinity is very similar to that previously reported for erythrocyte DARC (Horuk *et al*., 1993; Neote *et al*., 1993). Given that chemokines such as IL-8, RANTES, and MCP-1 are expressed by resident cells in the CNS, primarily astrocytes, and that DARC is expressed by neurons in the CNS, it is tempting to speculate that DARC may play a important role in the modulation of neuronal activity by astrocytes.

BIOLOGICAL CONSEQUENCES OF ACTIVATING OR INHIBITING RECEPTOR AND PATHOPHYSIOLOGY

Phenotypes of receptor knockouts and receptor overexpression mice

Genetic mutations of receptors both natural and induced (by targeted gene disruption) can help to unravel their biological roles. Although no DARC receptor knockouts have been developed in mice so far, nature has been generous in this regard by providing us with a naturally occurring example of gene inactivation for DARC. Humans homozygous for inherited inactivating mutations of the DARC gene in erythrocytes have been identified, and appear to be phenotypically normal and healthy (Mallinson *et al*., 1995). Indeed, as we have seen, this gene inactivation appears to be beneficial to the host, rendering the individual resistant to *malaria* induced by *P. vivax* which utilizes DARC to attach to and enter erythrocytes. Interestingly, these Duffy-negative individuals are not truly deficient and do express DARC on nonerythroid cells. Although there do not appear to be any noticeable pathophysiologic consequences resulting from a Duffy-negative phenotype, it is of course possible that there are compensatory mechanisms that take over the postulated biological role of DARC as a chemokine sink on erythroid cells. We will have to await the description of a true DARC knockout in mice to determine the physiologic role of DARC on nonerythroid cells.

THERAPEUTIC UTILITY

Effect of treatment with soluble receptor domain

A monoclonal antibody to the Duffy blood group antigen known as Fy6 has been described by Nichols *et al*. (1987). In humans, the epitope to Fy6 is present on the erythrocytes of all persons except those of the Fy(a−b−) type. The Fy6 epitope on DARC was shown to be on the N-terminus and is centered around residues Phe22 and Glu23; mutation of either of these residues destroys both Fy6 and chemokine binding (Tournamille *et al*., 1987). Consistent with the idea that Fy6 and chemokines share the same binding sites for DARC, Fy6 is able to inhibit chemokine binding (Horuk *et al*., 1993). No direct

in vivo studies to inhibit *P. vivax* infection of human erythrocytes have been described. However, given the commonality of chemokine/Fy6/parasite-binding sites for DARC, it might be expected that Fy6 could prove to be effective *in vivo* in inhibiting *P. vivax* and may thus be therapeutically useful.

Effects of inhibitors (antibodies) to receptors

In a recent study the ability of selected MGSA mutants to inhibit the invasion of human Duffy-positive erythrocytes by *P. knowlesi* was assessed (Hesselgesser *et al.*, 1995). The mutants inhibited parasite invasion at ligand concentrations that were consistent with their receptor-binding affinities for DARC. For example, the mutant E_6A was almost as effective as MGSA with an EC_{50} of inhibition of invasion of 8.6 nM, compared to 7 nM for wild-type MGSA. Mutant R_8A which does not bind to the DARC did not inhibit parasite invasion at concentrations up to 1 μM. The mutant E_6A binds to DARC with high affinity and efficiently blocks parasite invasion but does not bind CXCR2 and does not activate neutrophils. Analogs of MGSA, like E_6A, may be useful as receptor-blocking drugs that inhibit erythrocyte invasion by *P. vivax* but do not affect 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 are a new and novel approach in the fight against *P. vivax*-induced malaria.

Recent studies have identified the peptide within the Duffy blood group antigen of human erythrocytes to which the *P. vivax* and *P. knowlesi* ligands bind (Chitnis *et al.*, 1996). Peptides from the N-terminal extracellular region of the Duffy antigen were tested for their ability to block the binding of erythrocytes to transfected COS cells expressing on their surface region II of the Duffy-binding ligands. The binding site on the human Duffy antigen used by both the *P. vivax* and *P. knowlesi* ligands maps to a 35 amino acid region. A 35 amino acid peptide from the human Duffy antigen blocked the binding of *P. vivax* to human erythrocytes with a K_i of 2.9 μM. These studies suggest that it might be possible to design small peptides that are effective therapeutic agents to inhibit *P. vivax*-induced *malaria*.

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