A Phage Display Screen and Binding Studies with Acetylated Low Density Lipoprotein Provide Evidence for the Importance of the Scavenger Receptor Cysteine-rich (SRCR) Domain in the Ligand-binding Function of MARCO

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Yunying Chen‡1, Marko Sankala‡1, Juha R. M. Ojala‡, Yi Sun‡, Ari Tuuttila§, David E. Isenman†, Karl Tryggvason‡, and Timo Pikkarainen‡2

From the ‡Division of Matrix Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, S-171 77 Stockholm, Sweden and the §Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada

MARCO is a class A scavenger receptor capable of binding both Gram-negative and -positive bacteria. Using the surface plasmon resonance technique, we show here that a recombinant, soluble form of MARCO, sMARCO, binds the major Gram-negative and -positive bacterial surface components, lipopolysaccharide and lipoteichoic acid. Yet, the interaction of these two polyanions with sMARCO is of much lower affinity than that of polyinosinic acid, a polyionic inhibitor of bacterial binding to MARCO. To further elucidate the ligand-binding functions of MARCO, we performed a phage display screen with sMARCO. The screening resulted in the enrichment of only a handful of phage clones. Contrary to expectations, no polyanionic peptides, but only those with a predominantly hydrophobic nature, were enriched. One peptide, VRWGSFAAWL, was displayed on two-thirds of the phages recovered after four rounds of screening. The VRWGSFAAWL phage-sMARCO interaction had significantly slower dissociation kinetics than that between sMARCO and lipopolysaccharide or lipoteichoic acid. Further work with this phage, and the second most enriched phage, displaying the peptide RLNWAWWLSY, demonstrated that both peptides bind to the SRCR domain of MARCO, and that they probably bind to the same site. Data base searches suggested that the VRWGSFAAWL peptide represents complement component C4, but we could not convincingly confirm this suggestion. A study with chimeric scavenger receptors indicated that even minor sequence changes in the MARCO scavenger receptor cysteine-rich (SRCR) domain can have profound effects on the binding of the prototypic scavenger receptor ligand, acetylated low density lipoprotein. As shown by differential binding of glutathione S-transferase-VRWGSFAAWL, these differences were very likely due to conformational changes. These findings led to experiments that demonstrated a crucial role of the SRCR domain for acetylated low density lipoprotein binding in MARCO. Thus, our results strengthen the notion that the SRCR domain is the major ligand-binding domain in MARCO. Furthermore, they suggest that the domain may contain multiple ligand-binding sites.

MARCO, a close relative of scavenger receptor A (see Ref. 1), is a trimeric type II transmembrane protein with an N-terminal intracellular domain, a transmembrane domain, and an extracellular portion composed of a short spacer domain, a long triple-helical collagenous domain, and a C-terminal scavenger receptor cysteine-rich (SRCR) domain (2). MARCO has a very restricted expression pattern in adult mice living under pathogen-free conditions. It is expressed at significant levels only in the marginal zone macrophages of the spleen, in macrophages of the medullary cord of lymph nodes, and in the peritoneal macrophages (2). In bacterial infections MARCO expression is up-regulated in macrophages of most tissues (3–6). Cells transfected with a plasmid encoding MARCO avidly bind both Gram-negative and -positive bacteria, but not yeast (2, 3). MARCO also has other than microbial ligands, because it was identified as the main receptor for large environmental particles in the lung alveolar macrophages (7). Recent studies indicate that MARCO has endogenous ligands, too. Indeed, the interaction between MARCO and a cell surface determinant on the marginal zone B cells was found to contribute to the retention of the marginal zone B cells within the marginal zone (8). In another recent study, the uroglobulin-related protein 1, UGRP1, was identified as an endogenous ligand of MARCO in the lung (9). Studies with MARCO knock-out mice have demonstrated a role for MARCO in lung defense against pneumococcal pneumonia and inhaled particles (10). MARCO may also have a role in the macrophage adhesion/spreading processes, because ectopic MARCO expression induces the formation of long dendritic processes (11). The SRCR domain is crucial for this activity of MARCO (11). Similarly, this domain is the major bacteria-binding domain in MARCO (3, 12).

We have recently succeeded in establishing a production system for the recombinant soluble MARCO (sMARCO), the extracellular part of this scavenger receptor. The protein was found to have assembled into a collagenous triple-helix, and bind both heat-killed and living Escherichia coli (13). Furthermore, purified lipopolysaccharide (LPS), a surface component of Gram-negative bacteria, was found to interact with the polyhistidine-tagged sMARCO conjugated to nickel-nitrioltriacetic acid beads, but not with beads containing a polyhistidine-tagged control protein. Here, we have extended our studies on sMARCO, and first...
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examined the interaction of LPS, lipoteichoic acid (LTA), a surface component of Gram-positive bacteria and a putative ligand of MARCO, as well as that of the polyribonucleotide poly(I) with the protein using surface plasmon resonance. We show that all these molecules bind MARCO, but poly(I), an inhibitor of bacterial binding to MARCO, has clearly the highest affinity. We then performed a phage display screen with immobilized sMARCO, as well as AcLDL binding studies with transfected cells, and both studies provide further support for the notion that the SRCR domain has a major role in the ligand-binding function of MARCO.

EXPERIMENTAL PROCEDURES

Materials—The rat anti-mouse MARCO monoclonal antibody ED31 was kindly provided by Dr. G. Kraal (Free University, Amsterdam, The Netherlands). Cell culture reagents were obtained from Invitrogen. Polynosinic acid (poly(I)), LPS (from E. coli serotype 0111:B4), and LTA (from Staphylococcus aureus) were from Sigma. The LPS was phenol-extracted and purified by ion-exchange chromatography (L-3024). Construction of the random decapeptide phage library displayed on the minor M13 coat protein P3 has been described (14). Human complement components C4 and C4b were from Advanced Research Technologies (>95% pure by SDS-PAGE). Recombinant human C4d of the B isotype corresponding in length to the physiological degradation fragment of C4b was expressed in bacteria, and purified to the end of the DEAE-Sephalac step as described previously (15). SDS-PAGE analysis indicated that the preparation was ~90% pure. Mouse anti-M13 monoclonal antibody and goat anti-GST polyclonal antibodies were from Amersham Biosciences. Affinity-purified rabbit anti-GST polyclonal antibodies were generated in our laboratory. Rabbit anti-human complement component C4 antibodies were from Sigma, or obtained from Dr. A. Blom (Lund University, Sweden). Secondary antibodies were purchased from Dako or Molecular Probes. Sulfo-NHS-biotin was from Pierce. Acetylated LDL and FITC-labeled 1g/ml GST or BSA were added, and the plate was incubated for 2 h at room temperature. The well was washed with PBS containing Tween 20 to remove unbound phases. Bound phases were eluted with a low pH buffer, neutralized, and used to infect competent K91kan E. coli. Three more rounds of panning were carried out in the same manner, except that less sMARCO was coated onto the micotiter plates for rounds three and four (50 and 500 ng/well), and the phage solution was incubated with the immobilized sMARCO for 1 h only. During these rounds, enrichment was verified by comparing the phage binding onto sMARCO- and BSA-coated surfaces. Randomly selected clones were sequenced as described (18).

Binding of individual phases to surfaces coated with sMARCO, recombinant SRCR domain of MARCO, recV (13), or recombinant nephrin encompassing the first two IgG domains (nNephrin) was tested in the same manner (1 × 10⁶ transducing units of phases added per well). Proteins were coated at the concentration of 10 µg/ml overnight at 4 °C (100 µl/well). Two wells were coated with each protein. Control wells were coated with a GST protein for 45 min. Some of the peptide precipitated in this blocking solution. A fraction of the peptide precipitated in this

Surface Plasmon Resonance Experiments—All experiments were run at 25 °C at a flow rate of 5 µl/min in PBS or 10 mM Hepes, pH 7.4, 150 mM NaCl, using a BIAcore 3000 instrument and NTA sensor chips (BIAcore AB). Buffers were degassed and filtered through 0.2-µm cut-off filters. Soluble MARCO was purified as described previously (13). The NTA sensor chips were used according to the manufacturer’s instructions. Prior to an experiment, purified sMARCO (ligand) in the eluent buffer (PBS) was coupled to the flow cells at densities ranging from 1000 to 3000 RU. Flow cells without immobilized sMARCO were used as reference cells. Analytes were injected over the flow cell surfaces at the following concentrations: LPS and LTA, 25–100 µg/ml; poly(I) and heparin, 5–15 µg/ml. The LPS and LTA solutions were sonicated 3 times for 15 s before the use. The injection time was 7 min. A fresh ligand was applied to the flow cells before each run. Thus, after a run, the chip was washed with 250 mM EDTA, followed with 100 mM NaOH. Thereafter, the NTA surface was first recharged with nickel ions before applying sMARCO. The control flow cell was not loaded with sMARCO. This reloading procedure was chosen, because we observed that a fraction of sMARCO was stripped away from the nickel-nitritolriactric acid surface when LPS and LTA were passed over the chip (see “Results”).

Data Processing—Prior to analysis, the data were zero-adjusted and the reference cell signal was subtracted. Sensorgrams exhibiting instrumental artifacts were excluded from the analysis. The association and dissociation rate constants (Kₐ and Kₜ) were calculated according to the BIAcore evaluation program. The ratio of Kₐ and Kₜ yields the value of the apparent equilibrium dissociation constant (Kₐ). A simple 1:1 Langmuir model was used to fit the data. The molar concentrations of the injected analytes were calculated using the following molecular weight values: E. coli 0111:B4 LPS, 10,000 (16); S. aureus LTA, 7,000 (17); poly(I), 500,000 (manufacturer’s information).

Selection of sMARCO-binding Phages—Selection of phases binding to sMARCO was done essentially as described by Koivunen et al. (14). Briefly, sMARCO (100 µg/ml PBS) was first coated onto a Nunc Maxisorp microtiter well overnight at 4 °C. After blocking 1–2 h with 2% BSA/PBS at room temperature, the phage library solution (1 × 10⁹ transducing units in 2% BSA/PBS) was added, and the plate was incubated for 2 h at room temperature. The well was washed with PBS containing Tween 20 to remove unbound phases. Bound phases were eluted with a low pH buffer, neutralized, and used to infect competent K91kan E. coli. Three more rounds of panning were carried out in the same manner, except that less sMARCO was coated onto the micotiter plates for rounds three and four (50 and 500 ng/well), and the phage solution was incubated with the immobilized sMARCO for 1 h only. During these rounds, enrichment was verified by comparing the phage binding onto sMARCO- and BSA-coated surfaces. Randomly selected clones were sequenced as described (18).

Production of the GST-VRWGSFAAWL Peptide Fusion Protein—A construct encoding a GST protein with the VRWGSFAAWL decapptide extension was generated as follows. A fragment encoding the phage insert was first produced by PCR with the forward and reverse primers containing, respectively, BamHI and EcoRI recognition sites (the forward primer: 5’-AGGTCGAGGATCCTGGGCCGACGGGGC-3’, the reverse primer: 5’-AGTTCTAGAATTCCCCAGGCCCGCCC-3’) using phage DNA as a template. The fragment was gel-purified, digested with BamHI and EcoRI, and cloned into the BamHI-EcoRI-digested pGEX-2TK. The correctness of the construct was verified by DNA sequencing. The GST fusion protein and GST alone were expressed in E. coli BL21 strain. The proteins were produced and purified according to the manufacturer’s instructions (Amersham Biosciences).

In Vitro Phase-binding Assays in the Presence of the GST Proteins or the Synthetic VRWGSFAAWL Peptide—Some of the in vitro phase-binding assays were carried out in the presence of 250 µg/ml GST or GST-VRWGSFAAWL. Also, before adding the test solution to wells coated with the different proteins, the wells were preincubated in the blocking solution containing a GST protein for 45 min. Some of the assays were carried out in the presence of the synthetic VRWGSFAAWL peptide. The peptide was dissolved in Me₂SO to obtain a stock solution of 4 mg/ml, and then diluted to the concentration of 200 µg/ml in the blocking solution. A fraction of the peptide precipitated in this solution, and therefore the solution was cleared by centrifugation before applying into the wells. Control binding assays were carried out in the presence of a similar vehicle concentration.

Binding Assays with Transfected Cells—CHO cells transfected with the various MARCO expression constructs were tested for the binding

5 J. Öjala, unpublished data.
of the VRWGSFAAWL phage, the RLNWAWWLSY phage, and a randomly picked control phage. Cells were prepared for the assay as described previously (12). At the time of assay, the culture medium was removed, and the cells were first incubated for 10 min in ice-cold DMEM containing 10 mM Hepes, pH 7.5, and 2% BSA (incubation medium). This solution was then removed, and the phage-containing solution was added (1 × 10^9 transducing units of phage in the ice-cold incubation medium). After 45 min incubation on ice, the cells were washed five times with ice-cold PBS. The cells were fixed with 4% paraformaldehyde, permeabilized for 5 min with 0.1% Triton X-100/PBS, and incubated in PBS containing 2% BSA (blocking solution) before staining for M13 phage and MARCO. All antibodies were diluted in the blocking solution. To detect the M13 phage, the cells were first incubated overnight at 4 °C with a mouse anti-M13 monoclonal antibody (10 µg/ml), then rinsed several times with PBS, and incubated with FITC-conjugated F(ab')2 fragment of goat anti-mouse IgG (10 µg/ml) for 45 min. After rinsing several times in PBS, the cells were fixed again in the paraformaldehyde solution, and stained for MARCO using polyclonal rabbit anti-mouse MARCO antibodies recognizing the intracellular domain of mouse MARCO (2). TRITC-conjugated swine anti-rabbit IgG was used as a secondary antibody.

The binding of the GST proteins was tested in the same manner. The cells were incubated on ice with 100 µg/ml of a GST protein in DMEM/Hepes with or without 2% BSA for 45 min. After fixation, the permeabilization step was omitted, and the cells were directly stained for GST using either polyclonal goat or rabbit anti-GST antibodies. The antibodies were used, respectively, at the concentration of 25 and 10 µg/ml blocking solution. FITC-conjugated secondary antibodies were used to detect the bound GST proteins. We did not double stain for GST and MARCO in these assays. It was separately verified that the transfections were successful. In some experiments, we tested the binding of biotinylated GST proteins. The proteins (in PBS) were biotinylated by incubating 2 h on ice with sulfo-NHS-biotin (25:1 molar ratio of sulfo-NHS-biotin to protein). Unreacted biotin was then quenched by adding Tris-HCl, pH 7.5, to the final concentration of 20 mM, and the reaction mixtures were extensively dialyzed against PBS at 4 °C. Biotinylation was confirmed by Western blotting with a streptavidin-horseradish peroxidase conjugate.

In some assays, the binding of AcLDL and FITC-labeled heat-killed E. coli was tested. Briefly, the cells were first washed once with DMEM, then incubated in DMEM containing either 2.5 µg/ml AcLDL or different concentrations of FITC-labeled E. coli for 1 h in a humidified atmosphere with 5% CO₂ at 37 °C, after which the cells were washed two times with DMEM, 20 mM Hepes, pH 7.5, and two times with PBS before fixation with 4% paraformaldehyde.

When testing the binding of human complement proteins C4 and C4b, the proteins (50 µg/ml in DMEM/Hepes) were incubated with the cells on ice for 1 h. Thereafter, the cells were processed as described above, and stained with rabbit anti-human complement C4 antibodies. The binding of biotinylated C4d was tested in a similar manner, except that the bound protein was detected with a streptavidin-horseradish peroxidase conjugate. We used the biotinylated form of C4d, because the anti-C4 antibodies did not recognize this fragment. Biotinylation of C4d was performed as described above.

**Antii-VRWGSFAAWL Peptide Antibodies**—Antibodies were raised against the VRWGSFAAWL peptide-keyhole limpet hemocyanin conjugate. A cysteine residue was added to the C terminus of the peptide to facilitate coupling to keyhole limpet hemocyanin. Immunoglobulins were purified from the antiserum using a Gama-bind Sepharose column. Alternatively, anti-peptide antibodies were affinity purified from the antiserum using the GST-VRWGSFAAWL peptide fusion protein coupled to CNBr-activated Sepharose (10 mg of the fusion protein conjugated to 2 ml of the beads), or on a peptide affinity matrix (10 mg of the synthetic peptide coupled via its C-terminal cysteine residue to 2 ml of thiopropyl-Sepharose). 5 ml of antiserum, diluted to 25 ml with 10 mM Tris, pH 7.5, 50 mM NaCl (column buffer), was passed over the columns three to four times. After washing with 20 volumes of the column buffer, and 10 volumes of 10 mM Tris, pH 7.5, 0.4 M NaCl, the antibodies were eluted with 0.2 M glycine, pH 2.5, 150 mM NaCl. Eluted antibodies were immediately neutralized with 1 M Tris, pH 8.3, and dialyzed against PBS.

**RESULTS**

**Characterization of the Binding Properties of sMARCO Using the BIAcore System**—We have previously described initial characterization of the binding properties of recombinant sMARCO (13). Although the main aim of this study was to use sMARCO in a phage display screen to identify potential novel ligands for this scavenger receptor, we first wanted to further characterize sMARCO, and studied its binding properties using the BIAcore system. Another motive for setting up this system was that if obtaining enrichment in the phage display screen, the BIAcore system would provide a method for comparing the binding kinetics of the phage-sMARCO interaction to those between sMARCO and its other ligands.

Polyhistidine-tagged sMARCO was bound onto NTA chips. Binding to the chip was very stable and there was no detectable dissociation (Fig. 1A). This was probably due to the fact that each sMARCO-molecule contains three polyhistidine tails. Interaction between sMARCO and LPS, LTA, poly(I), or heparin was then examined. Of these polyanionic compounds, LPS, LTA, and poly(I) are ligands of scavenger receptor A (19–22). Both LPS and LTA have a tendency to form micelles in an aqueous environment (16, 23, 24). As mentioned in the Introduction, we have found previously that sMARCO interacts with LPS in a test tube assay (13). MARCO-transfected cells have been shown to bind not only Gram-negative, but also Gram-positive bacteria, suggesting that LTA, the major surface component of this latter class of bacteria, might also be a ligand of MARCO (2). The polyribonucleotide poly(I) is clearly a ligand of MARCO, because it can be used to block bacterial binding to MARCO-transfected cells. As shown in Fig. 1B, sMARCO binds LPS in the BIAcore system, too, but the binding is followed by rapid dissociation. Curiously, the curve falls below the baseline that might be due to stripping of sMARCO from the NTA surface. A survey of the literature indicates that LPS can interact with nickel (25). Thus, if a fraction of NTA-chelated nickel is removed when LPS is passed over the flow cell surface, the amount of bound sMARCO also decreases. Fig. 1C shows that sMARCO binds LTA in a similar manner as it binds LPS. Interestingly, no interaction between immobilized sMARCO and LPS or LTA was detected, if sonicated solutions were left to stand for some hours before the BIAcore assay. Because it is known that sonication causes dispersion of large aggregates into smaller and more uniform micelles (26), micelle size appears to be a parameter that affects the interaction of LPS and LTA with sMARCO in this system. Analysis of the binding curves indicated variation in the K_D values ranging from the high nanomolar to low micromolar level for both interactions. This variation is very likely due to the two above described potential sources of variability.

As shown in Fig. 1D, poly(I) was found to interact with sMARCO with very high affinity. Determination of the binding constants indicated that this polyanionic macromolecule bound immobilized sMARCO with the association (K_A) and dissociation rate (K_D) constants of 4.4 (±2.9) × 10^5 M⁻¹ s⁻¹ and 1.6 (±0.8) × 10⁻⁸ s⁻¹, respectively, yielding a K_D value of 1.0 s⁻¹.
4.3 \times 10^{-10}\, M (n = 4). Heparin, instead, did not interact with sMARCO coated onto the chip surface (data not shown). This finding is in line with the observation that heparin does not affect bacterial binding to MARCO.

Isolation of sMARCO-binding Phages—The results described above, together with the known property of scavenger receptors to recognize also a variety other polyanionic ligands (1), suggested that polyanionic peptides might become enriched in a phage display screen with sMARCO. However, this was not the case. In the screen, four rounds of selection were performed to select sMARCO-binding phages from a random decapeptide phage display library. Randomly picked clones were selected for sequencing primarily from round four. Altogether, of the 31 clones sequenced from this round, only five different sequences were obtained (Table 1). This indicates selection of specific phages. The two most enriched sequences were VRWGSFAWL and RLNWAWWLSY. In particular, the former one was highly enriched, because this sequence was recovered from 20 clones. The latter sequence was recovered from five clones. Sequencing of a few clones from the first three rounds revealed that the sequence encoding the peptide VRWGSFAWL was not recovered from round one clones, but it represented 40 and 60% of the phages recovered, respectively, after rounds two and three. The RLNWAWWLSY sequence was not recovered from the phages sequenced after the first two rounds, but it represented 20% of the phages recovered from round three (1 of 5 clones sequenced). As indicated in Table 1, 3 other clones were recovered after round four. In the following experiments, these 3 clones were not studied further, as we focused on the two most enriched ones, particularly on the VRWGSFAWL phage.

To confirm the interaction between sMARCO and the VRWGSFAWL phage with another cell-free approach, we employed the BIAcore analysis. Soluble MARCO coated onto the NTA chips did not bind a control phage (Fig. 2A), whereas it bound the VRWGSFAWL phage well, and the phage dissociated clearly much slower than LPS or LTA (Fig. 2B).

**TABLE 1**

| Sequence          | Clones |
|-------------------|--------|
| VRWGSFAWL         | 20     |
| RLNWAWWLSY        | 5      |
| LRLQWRAWLA        | 2      |
| IPVKWLLRWR        | 3      |
| PVRWRWASWL        | 1      |

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**Phage-binding Site Resides in the SRCR Domain of MARCO—**sMARCO is a large homotrimeric molecule with a molecular mass of 220 kDa. Each subunit chain is composed of three domains, a 75-residue long spacer domain, a long collagenous domain formed by 89 Gly-X-Y triplets, and a 99-residue long SRCR domain, a compact domain with three intrachain disulfide bridges (3, 27, 28).
The injection time was 6 min. The reference cell signal was subtracted from the results (RU: BIAcore resonance units; 1 RU = 1 pg/mm²).

This form is expressed at equal levels to the full-length mouse MARCO over, cell-surface biotinylation experiments have previously shown that the cytoplasmic domain of mouse MARCO for the identification of MARCO-expressing cells cannot be the case, because less phage-binding sites might be accessible on the surface coated with the small recV molecule than on that coated with sMARCO. However, these experiments clearly confirm that the SRCR domain contains the phage-binding site.

Next, by exploiting the purified recombinant SRCR domain of MARCO, recV, we confirmed this result with a cell-free approach. Microtiter wells were coated with BSA, sMARCO, recV, or recombinant nephrin (rNephrin), and phage binding was measured. We used rNephrin as a control, because it is, similarly to sMARCO, a polyhistidine-tagged protein. This experiment demonstrated that the VRWGSFAAWL phage bound avidly to the recV- and sMARCO-coated surfaces (Fig. 4, A and B), but there was practically no binding to the surfaces coated with rNephrin or BSA (Fig. 4, C and D). None of the surfaces bound a control phage (data not shown). In another experiment, we quantitated the VRWGSFAAWL phage binding to the surfaces coated with these three recombinant proteins. When comparing a plating dilution giving only 1 or 2 colonies on the rNephrin plate, there were ~200 colonies on the recV plate and more than 1000 colonies on the sMARCO plate (Fig. 4E).

These results indicate that there might be a difference in the strength of interaction between the VRWGSFAAWL phage and the trimeric sMARCO molecule or the monomeric SRCR domain. However, we cannot conclude with certainty that this is the case, because less phage-binding sites might be accessible on the surface coated with the small recV molecule than on that coated with sMARCO. In any case, these experiments clearly confirm that the SRCR domain contains the phage-binding site.

Next, the VRWGSFAAWL peptide was produced as a GST fusion protein, and the binding of the GST fusion protein and GST alone to the MARCO transfectants was tested. Similarly to the phage-binding assays, this assay was carried out on ice. The experiment confirmed that it is the SRCR domain of MARCO that recognizes the VRWGSFAAWL peptide. Indeed, the full-length mouse MARCO transfectants bound GST-VRWGSFAAWL (Fig. 5A), but not GST alone (Fig. 5C), whereas cells expressing the mouse MARCO form lacking the SRCR domain bound neither GST-VRWGSFAAWL (Fig. 5B) nor GST alone (data not shown). Neither did cells expressing the MARCO truncation extending 17 residues to the SRCR domain bind the fusion protein, although they exhibit avid bacterial binding (data not shown).

The GST proteins were also tested for their ability to block phage binding to sMARCO and recV immobilized onto microtiter plate wells. We found that the GST-VRWGSFAAWL fusion protein reduced bind-

To identify the phage-binding region on MARCO, we first tested transient CHO transfectants expressing either the full-length mouse MARCO or a truncated form lacking the SRCR domain for the binding of the VRWGSFAAWL and RLNWAWWLSY phage clones. The experiment was performed on ice, so only cell binding was measured. Cells expressing the full-length MARCO avidly bound both phages (Fig. 3, A and C), but did not bind a randomly picked control phage (Fig. 3D). Interestingly, cells expressing the truncated MARCO form did not bind any of the phage clones. In panel D, the result from the binding assay with the VRWGSFAAWL phage is shown.

FIGURE 2. Soluble MARCO interacts with the VRWGSFAAWL phage in the BIAcore system. Analysis of the interaction between the VRWGSFAAWL phage and a control phage with sMARCO using the BIAcore biosensor. Immobilized sMARCO does not bind a control phage (A), but binds well the VRWGSFAAWL phage with slow dissociation kinetics (B). The injection time was 6 min. The reference cell signal was subtracted from the results (RU: BIAcore resonance units; 1 RU = 1 pg/mm²).

FIGURE 3. Cells expressing the full-length mouse MARCO, but not those expressing the truncated form lacking the SRCR domain, bind avidly the VRWGSFAAWL and RLNWAWWLSY phage clones. CHO transfectants were tested for their capability to bind the VRWGSFAAWL and RLNWAWWLSY phage clones. The green dots represent individual phages. The transfectants were also stained with the antibodies recognizing the SRCR domain of mouse MARCO for the identification of MARCO-expressing cells (red). Expression of the full-length MARCO gains the cells capability to bind both the VRWGSFAAWL phage (A) and the RLNWAWWLSY phage (C), but not a randomly chosen control phage (D). Cells expressing the MARCO form lacking the SRCR domain bind none of the phage clones. In panel B, the result from the binding assay with the VRWGSFAAWL phage is shown.

Macrophage Scavenger Receptor MARCO

A

B

C

D

E

F

G

H

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K

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N

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P

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R

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T

U

V

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X

Y

Z

[Image 49x554 to 563x733]

[Image 212x26 to 239x38]

[Image 256x158]
ing of the VRWGSFAAWL phage onto the sMARCO- or recV-coated surfaces to background levels, i.e. to that seen on the rNephrin-coated surface (data not shown). As expected, a similar concentration of GST alone did not have any effect. Furthermore, the GST-VRWGSFAAWL fusion protein blocked the binding of the RLNWAWWLSY phage onto these two recombinant MARCO-coated surfaces to background levels (data not shown). Again, GST alone was without any effect. Similarly, the synthetic VRWGSFAAWL peptide, which was diluted from a stock prepared in Me2SO, blocked the binding of both phages onto immobilized sMARCO or recV (data not shown). A similar vehicle concentration was without any effect.

Finally, we wanted to study whether the VRWGSFAAWL peptide is recognized only by mouse MARCO, or also by its counterpart from another species. To this end, we performed a binding assay with the full-length human MARCO transfectants. The amino acid sequence identity between the SRCR domains of mouse and human MARCO is 74% (3). Notably, these cells bound GST-VRWGSFAAWL equally well as the mouse MARCO transfectants (data not shown), demonstrating that the interaction is not species-specific. As expected, the transfectants failed to bind GST alone.

Data Base Searches with the Phage Sequences Suggest That Complement Component C4 May Be a Ligand of MARCO, but Experimental Evidence Does Not Confirm the Suggestion—We analyzed the VRWGSFAAWL and RLNWAWWLSY phage sequences using the BLAST program (NCBI) to find out whether they show similarity to known or hypothetical proteins deposited in the data bank. The sequences have, with their many tryptophan residues, a hydrophobic nature, and therefore gave many hits to transmembrane sequences of various proteins. Additionally, when restricting the search to mammalian proteins, one of the best hits obtained with the VRWGSFAAWL peptide was a match with a sequence in complement component C4. Indeed, if counting also a conservative change, there was a 7-residue continuous match with the GSYAAWL segment encompassing residues 1064–1070 in human complement C4. Interestingly, all the other enriched peptides were also found to contain the WL motif. Because the complement system is an important effector system of innate immunity, and MARCO plays a role in this same system of immunity, several efforts were made to find out whether C4 is a true ligand of MARCO. We could detect weak binding of C4b, the activated form of C4, and of C4d, a physiological degradation fragment of C4b, to the full-length MARCO transfectants (data not shown), but the binding was not inhibitable by a 30-fold molar amount of GST-VRWGSFAAWL. Neither did the fusion protein inhibit the
interaction between sMARCO and biotinylated C4d in an enzyme-linked immunosorbent assay-type assay. The antipeptide antibodies also failed to recognize C4 or C4b in the enzyme-linked immunosorbent assay or Western blots, although they well recognized the GST fusion protein, but not GST alone, in similar assays.

Reciprocal Binding of GST-VRWGSFAAWL and AcLDL to Transfectants Expressing Chimeric Scavenger Receptors with Different Lengths of the MARCO SRCR Domain Segment—Aiming to map the peptide-binding site in the SRCR domain of mouse MARCO, we generated a novel set of constructs encoding chimeric scavenger receptors. Here, the entire SRCR domain of a novel scavenger receptor A-like molecule Tesr (29), or large portions of the domain, was replaced by a corresponding segment of mouse MARCO. Cells expressing chimeric receptors containing MARCO segments 423–481 (form “IW”); from the beginning of the MARCO SRCR domain to tryphophan 481) and 423–507 (form “NC”; from the beginning of the MARCO SRCR domain to cysteine 507) were found to bind the GST fusion protein well (Fig. 6A), whereas there was no binding to cells expressing an intact form of Tesr (data not shown). It was in fact the case that cells expressing these two chimeric receptors bound GST-VRWGSFAAWL better than those expressing the chimera with an intact MARCO SRCR domain (form “MARCO SRCR”; residues 423–518). Identical results were obtained when detecting the bound fusion protein with anti-GST antibodies from two different sources. Staining of living cells on ice with the monoclonal antibody ED31 indicated that the MARCO SRCR form was not expressed at lower levels on the cell surface than IW or NC. Indeed, the MARCO SRCR and IW forms were found to be expressed at equal levels, and NC at slightly lower levels (data not shown). In striking contrast to the results in the binding assays with the GST fusion protein, cells expressing the MARCO SRCR form bound AcLDL very avidly, whereas there was only marginal binding to cells expressing IW and NC (Fig. 6B). No significant differences were found in bacterial binding between the transfectants (data not shown). Our interpretation of these results is that differential binding of the fusion protein to the chimeric receptors indicates conformational changes, and that AcLDL binding, but not bacterial binding, appears to be sensitive to these changes.

The SRCR Domain Is Crucial for AcLDL Binding in MARCO—Although the above described results clearly indicated AcLDL binding activity for the MARCO SRCR domain, it still remained at least a formal possibility that the AcLDL-binding site of the chimeric receptors resides outside the SRCR domain, and the domain sterically hinders AcLDL binding in the IW and NC forms. To confirm that the MARCO SRCR domain indeed exhibits AcLDL binding, we assayed AcLDL binding to transfectants expressing different variants of mouse MARCO. Cells transfected with an irrelevant control plasmid did not bind AcLDL (Fig. 7A), whereas those expressing the full-length MARCO bound avidly (data not shown). Similarly, transfectants expressing a form containing only the first 8 Gly-X-Y triplets of the 89-triplet long collagenous domain (12), but with an intact SRCR domain, exhibited avid binding (Fig. 7B). However, no binding to transfectants expressing the truncated MARCO form lacking the SRCR domain was detected (Fig. 7C). Collectively, these results not only confirm that the MARCO SRCR domain exhibits AcLDL binding, but they also demonstrate that this domain is crucial in MARCO for the binding of this prototypic SR ligand.

DISCUSSION

The results presented here strengthen the notion that the SRCR domain has a major role in the ligand-binding function of MARCO. The main aim of our study was to employ the phage display approach, an unbiased method, and isolate short MARCO-binding peptides that could probably be used to predict physiological ligand(s) of MARCO with the help of data base searches, or, at the very least, the peptides could shed light on the ligand-binding characteristics of MARCO. These expectations were based on the knowledge that the phage-display system often targets functionally active sites on proteins (for recent papers, see e.g. Refs. 18 and 30–35). Taken into account the results from our BlAcore studies and the knowledge of the features of the many scavenger receptor ligands, we expected that, if obtaining any enrichment at all, polyamionic peptides might get enriched in the screen. The screening of a random decapptide phage library with the recombinant, soluble MARCO indeed resulted in the enrichment of a handful of peptides. Surprisingly, no polyamionic peptides were enriched. The isolated peptides were not found to have extensive sequence identity, but they have strikingly similar features, i.e. a basic residue near or at the N
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terminus followed by a stretch of mostly hydrophobic residues. Notably, each peptide enriched after round four contains a hydrophobic motif WL. One phage clone, VRWGSFAAWL, was highly enriched after four rounds of selection, and accounted for more than 60% of the phages recovered at this final step of screening. The phage carrying this peptide was rapidly enriched in the screening process, because it accounted for 40% of the phages already after round two. The BIA-core analysis indicated that the VRWGSFAAWL phage-sMARCO interaction had slower dissociation kinetics than that between sMARCO and LPS or LTA.

When viewed by rotary-shadowing microscopy, sMARCO appears as a ~80 nm long collagenous rod connecting two small globules that correspond to the spacer and SRCR domains (13). We found that the binding site for the VRWGSFAAWL peptide, as well as for the second most enriched peptide, RLNWAWWLSY, displayed on about 15% of the phages recovered after round four, does not reside within the spacer domain or the long collagenous domain, but in the SRCR domain. In addition, the results from the blocking experiments indicate that the peptides probably bind to the same site in the SRCR domain. We have previously found that this domain is the predominant bacteria-binding domain in MARCO (3, 12). It is also crucial for the capability of MARCO to induce formation of long dendritic processes in transfected cells (11). Moreover, as shown in this study, the domain is crucial for AcLDL binding. All these activities are blocked by the polyanion poly(I), indicating a polyanionic nature for the interacting ligands. The enriched peptides are not polyanionic, and therefore it was not very surprising that the GST-VRWGSFAAWL fusion protein did not block any of these activities of MARCO. On the other hand, one should mention here that the synthetic VRWGSFAAWL peptide had a marked inhibitory effect on AcLDL binding. When diluted from a stock prepared in Me₂SO, a fraction of the peptide tended to precipitate, but the solution was clarified before adding on cells. If the blockage is not because of the binding of soluble peptide aggregates, the results may indicate that the peptide and AcLDL bind to the same or overlapping sites on the SRCR domain. In this regard, it is worth mentioning that the crystal structure of the recombinant SRCR domain, recV, indicates the presence of a cluster of arginine residues,7 which may be important for the binding of AcLDL. We have also been trying to crystallize the VRWGSFAAWL peptide-recV complex, but we have so far had difficulties in finding conditions where the recV crystals have a high peptide occupancy. The low peptide occupancy may be because of the low solubility of the peptide in aqueous solutions. In any case, we can observe in the complex extra electron density between the two arginines. This supports the view that the binding sites of the peptide and AcLDL are at least partially overlapping. Still, whatever is the case, the domain appears to have multiple binding interfaces, because the mode of interaction of these two ligands with MARCO has to be quite different. Related to the presence of the arginine cluster, it is of interest to note that the long side chain of arginine can be involved both in polar and nonpolar interactions. Thus, whereas the guanido group is involved in ionic interactions, the rest of the side chain may be involved in hydrophobic interactions (36). Considering the structures of the VRWGSFAAWL and RLNWAWWLSY peptides, it is worth mentioning that the C-terminal GSFAAWL and AWWLSY segments of the peptides are predicted to fold into an a-helical conformation. This would place some of the hydrophobic residues on the same face of the helix, likely providing part of the surface for interaction with MARCO. The other important determinant for binding may be the arginine residue preceding the a-helix in both peptides.

As suggested above, it is possible that the phage display screen may have uncovered a novel binding activity on the SRCR domain of MARCO. Data base searches suggested the intriguing possibility that the VRWGSFAAWL sequence represents complement component C4. Examination of the C4d structure (15) (Protein Data Bank accession code 1HZF) indicated a close proximity of our phage sequence match and the highly reactive internal thioester bond that becomes exposed upon C4 activation. Cleavage of the bond very likely induces local conformation changes, which initially provided a reasonable explanation for our findings that we could detect interaction only between MARCO and C4b/C4d. However, we could not convincingly demonstrate that C4b and C4d are real ligands of MARCO. Moreover, a close examination of the C4d structure does not support this possibility, because, of the 7-residue match, GSFAAWL, only the segment WL is fully exposed. Consequently, it is not clear at the moment which molecule the peptide(s) represents. In this regard, one has to keep in mind that it is not uncommon that an isolated peptide is only a structural mimetic of the ligand and does not bear high enough sequence similarity to it to give a hit in the BLAST search. This problem might possibly be overcome with the help of anti-peptide antibodies (37). We generated antibodies against the VRWGSFAAWL peptide, but they have not so far been useful tools for the ligand identification.

Significantly, the cell binding studies with GST-VRWGSFAAWL led to the experiments that not only demonstrated a crucial role for the SRCR domain in the interaction of MARCO with AcLDL, but those performed with the chimeric forms of the scavenger receptor Tesr with its SRCR domain replaced either entirely or in large part by the corresponding MARCO sequences also illustrated how minor changes in the primary structure of the SRCR domain can have profound effects on binding of this form of LDL. The constructs encoding the chimeric receptors were originally intended only for the mapping of the GST-VRWGSFAAWL binding site on the MARCO SRCR domain. Indeed, we found that the chimera with an intact MARCO SRCR domain bound the fusion protein, but also that those changed back to the Tesr sequence 11 or 37 residues before the end of the domain did not exhibit decreased binding, but rather increased binding. On the contrary, these two latter chimeras had a dramatically reduced AcLDL binding capability compared with the chimera with an intact MARCO SRCR domain. Although we cannot exclude the possibility that the two chimeras whose SRCR domain is not composed only of MARCO sequences lack some residues directly involved in AcLDL binding, this is probably not the case at least with the chimera missing only the last 11 residues of the MARCO SRCR domain. This notion is based on the fact that neither this segment of MARCO (VHNEDAGVECS) nor the corresponding one of Tesr (GHAEDAGVTCCTVP) contains any positively charged residues, which are considered to be crucial for the interaction with AcLDL, a polyanionic macromolecule. The differences in AcLDL binding cannot be because of any major structural alterations either, because both segments contain a cysteine residue, which participates in the formation of an intradomain disulfide bond (3, 27, 28). This bond fixes the position of the very C-terminal end. Instead, examination of the crystal structure of the MARCO SRCR domain suggests that the single amino acid changes may alter the conformation of arginine residues in a six-stranded B-sheet in which the C-terminal segment AGVVECS participates (28), thereby affecting AcLDL binding, and that of the GST fusion protein, too.

7 J. Ojala, manuscript in preparation.
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REFERENCES

1. Peiser, L., Mukhopadhyay, S., and Gordon, S. (2002) *Curr. Opin. Immunol.* 14, 123–128
2. Elomaa, O., Kangas, M., Sahlgren, C., Tuukkanen, J., Sormunen, R., Liakka, A., Theesfeld, I., Kraal, G., and Tryggvason, K. (1999) *Cell* 80, 603–609
3. Elomaa, O., Sankala, M., Pikkarainen, T., Bergmann, U., Tauteila, A., Raatikainen-Aho, A., Sariola, H., and Tryggvason, K. (1998) *J. Biol. Chem.* 273, 4530–4538
4. van der Laan, L. J., Kangas, M., Dopp, E. A., Brogh-Holub, E., Elomaa, O., Tryggvason, K., and Kraal, G. (1997) *Immunol. Lett.* 57, 203–208
5. van der Laan, L. J., Dopp, E. A., Haworth, R., Pikkarainen, T., Kangas, M., Elomaa, O., Dijkstra, C. D., Gordon, S., Tryggvason, K., and Kraal, G. (1999) *J. Immunol.* 162, 939–947
6. Ito, S., Naito, M., Kohayashi, Y., Takatsuka, H., Iwada, S., Usuda, H., Umezua, H., Hasegawa, G., Arakawa, M., Shultz, L. D., Elomaa, O., and Tryggvason, K. (1999) *Arch. Histol. Cytol.* 62, 83–95
7. Palecanda, A., Paulauskis, J., Al-Mutairi, E., Imrich, A., Qin, G., Suzuki, H., Kodama, T., Tryggvason, K., Koziel, H., and Kobzik, L. (1999) *J. Exp. Med.* 189, 1497–1506
8. Karlsson, M. C., Guinamard, R., Bolland, S., Sankala, M., Steinman, R. M., and Ravetch, J. V. (2003) *J. Exp. Med.* 198, 333–340
9. Bin, L. H., Nielsen, L. D., Liu, X., Mason, R. J., and Shu, H. B. (2003) *J. Immunol.* 171, 924–930
10. Arredouani, M., Yang, Z., Ning, Y., Qin, G., Soininen, R., Tryggvason, K., and Kozbik, L. (2004) *J. Exp. Med.* 200, 267–272
11. Pikkarainen, T., Brannstrom, A., and Tryggvason, K. (1999) *J. Biol. Chem.* 274, 10975–10982
12. Brannstrom, A., Sankala, M., Tryggvason, K., and Pikkarainen, T. (2002) *Biochem. Biophys. Res. Commun.* 290, 1462–1469
13. Sankala, M., Brannstrom, A., Schulteuss, I., Bergmann, U., Morguanova, E., Engel, J., Tryggvason, K., and Pikkarainen, T. (2002) *J. Biol. Chem.* 277, 33378–33385
14. Koivunen, E., Restel, B. H., Rajotte, D., Lahdenranta, J., Hagedorn, M., Arap, W., and Pasqualini, R. (1999) *Methods Mol. Biol.* 129, 3–17
15. van den Elsen, J. M., Martin, A., Wong, V., Clemenza, L., Rose, D. R., and Isenman, D. E. (2002) *J. Mol. Biol.* 322, 1103–1115
16. Aurell, C. A., and Wistrom, A. O. (1998) *Biochem. Biophys. Res. Commun.* 253, 119–123
17. Greenberg, J. W., Fischer, W., and Joiner, K. A. (1999) *Infect. Immun.* 67, 3318–3325
18. Koivunen, E., Ranta, T. M., Annala, A., Taube, S., Uppala, A., Jokinen, M., van Willigen, G., Ihanus, E., and Gahmberg, C. G. (2001) *J. Cell Biol.* 153, 905–916
19. Hampton, R. Y., Golenbock, D. T., Pennman, M., Krieger, M., and Raetz, C. R. (1991) *Nature* 352, 342–344
20. Ashkenas, J., Penman, M., Vasile, E., Acton, S., Freeman, M., and Krieger, M. (1993) *J. Biol. Chem.* 268, 3546–3554
21. Dunne, D. W., Resnick, D., Greenberg, J., Krieger, M., and Joiner, K. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 1863–1867
22. Santos, N. C., Silva, A. C., Castanho, M. A., Martins-Silva, J., and Saldanha, C. (2003) *ChemBiochem.* 4, 96–100
23. Labischinski, H., Naumann, D., and Fischer, W. (1999) *Eur. J. Biochem.* 262, 1269–1274
24. Wellingshausen, N., Schromm, A. B., Seydel, U., Brandenburg, K., Luhm, J., Kirchner, H., and Rink, L. (1996) *J. Immunol.* 157, 3139–3145
25. Risco, C., Carrascosa, J. L., and Bosch, M. A. (1991) *J. Histochem. Cytochem.* 39, 667–675
26. Resnick, D., Chatterton, J. E., Schwartz, K., Slattery, H., and Krieger, M. (1996) *J. Biol. Chem.* 271, 26924–26930
27. Hohenester, E., Sasaki, T., and Timpl, R. (1999) *Nat. Struct. Biol.* 6, 228–232
28. Sarraj, M. A., McClive, P. J., Wollmore, H. P., Loveland, K. L., and Sinclair, A. H. (2005) *Dev. Dyn.* 234, 1026–1033
29. Koolpe, M., Dail, M., and Pasquale, E. B. (2002) *J. Biol. Chem.* 277, 46974–46979
30. Murai, K. K., Nguyen, L. N., Koolpe, M., McLennan, R., Krull, C. E., and Pasquale, E. B. (2003) *Mol. Cell. Neurosci.* 24, 1000–1011
31. Hettian, L., Ping, A., Shwame, S., Xiaoying, L., Jih, W., Lin, M., Meisberg, L., Junshan, Y., and Chengchao, S. (2002) *J. Biol. Chem.* 277, 43137–43142
32. El-Mousawi, M., Tchistiakova, L., Yurchenko, L., Pietrzynski, G., Moreno, M., Stanimirovic, D., Ahmad, D., and Alakhov, V. (2003) *J. Biol. Chem.* 278, 46681–46691
33. Pillutla, R. C., Hsiao, K. C., Beasley, J. R., Brandt, J., Ostergaard, S., Hansen, P. H., Spetzler, J. C., Danielsen, G. M., Andersen, A. S., Brissette, R. E., Lennick, M., Fletcher, P. W., Blume, A. J., Schaffer, L., and Goldstein, N. I. (2002) *J. Biol. Chem.* 277, 22590–22594
34. Stefanidakis, M., Bjorklund, M., Ihanus, E., Gahmberg, C. G., and Koivunen, E. (2003) *J. Biol. Chem.* 278, 54674–54684
35. Andrew, C. D., Penel, S., Jones, G. R., and Doig, A. J. (2001) *Proteins* 45, 449–455
36. Cardo-Vila, M., Arap, W., and Pasqualini, R. (2003) *Mol. Cell.* 11, 1151–1162