The heterodimer of the Ca^{2+}-binding proteins MRP8 and MRP14 binds arachidonic acid

Martin Klempt[a,*], Harutyun Melkonyan[a], Wolfgang Nacken[a], Dieter Wiesmann[a], Ulrike Holtkemper[b], Clemens Sorg[a]

[a] *Institute of Experimental Dermatology, University of Münster, von Esmarchstraße 56, 48149 Münster, Germany*
[b] *Institute of Biochemistry, University of Münster, 48149 Münster, Germany*

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Abstract The S100 proteins MRP8 and MRP14 have been shown to be expressed by myeloid cells during inflammatory reactions. Since the majority of S100 proteins exhibit their biological activity when associated as complex it was investigated whether murine MRP8 and MRP14 form heterodimers and whether this complex may bind lipids of the cell membrane. This is of particular importance since their anchoring into the plasma membrane is unclear although upon calcium binding the proteins translocate from the cytoplasma to the cytoskeleton and the plasma membrane. Using recombinant proteins we could show that not the monomers but only the heterodimers specifically bind arachidonic acid. This finding opens new perspectives for the role of MRP8 and MRP14 in acute and chronic inflammatory processes.

Key words: S100 protein; Arachidonic acid; Mouse MRP8/MRP14

1. Introduction

The S100 proteins MRP8 and MRP14 are expressed during myeloid differentiation and are found in high amounts in granulocytes and in monocytes during an early differentiation stage, yet are absent from lymphocytes and mature tissue macrophages [1]. Phagocytes, expressing MRP8 and MRP14, are found in many inflammatory conditions, e.g. rheumatoid arthritis, allograft rejection, inflammatory bowl and lung diseases [2,3]. Expression of MRP8 and MRP14 by infiltrating myelo-monocytic cells has been described to correlate with disease activities in murine models of experimental inflammation, e.g. irritant and allergic contact dermatitis, Leishmaniasis or angiogenesis [4,5].

Elevated serum levels of MRP8 and MRP14 were found in various inflammatory diseases [2]. Therefore, it was proposed to introduce MRP8/14 as a sensitive novel inflammation marker [3]. It has been described earlier that human MRP8 and MRP14 may form homo- and heteropolymers in a calcium-dependent manner [6].

The principal complex is a heterodimer which is found in e.g. monocytes. Upon calcium-binding the complex translocates from the cytoplasma to the cytoskeleton and the plasma membrane [7,8]. How the complex is anchored in the membrane is not clear since the proteins do not contain a transmembrane region [9]. Nevertheless, the proteins can be detected on the surface of the cells. Upon activation of protein kinase C the proteins are also secreted via a novel pathway requiring an intact microtubule network [10].

So far a number of functions has been proposed for MRP8, MRP14 and the heterocomplexes, e.g. antimicrobial, cytostatic and chemotactic activities. For some of these a molecular mechanism has been elaborated [11,12]. However, no ligands or receptors have been found so far for the individual proteins or the complexes.

In view of the lack of a transmembrane domain and the failure to identify a protein ligand or anchor in the membrane, the question was raised whether MRP8 and/or MRP14 might become lipophilic after calcium-binding. Here we could show that heterocomplexes of recombinant murine MRP8 and MRP14 specifically bind arachidonic acid in a strictly calcium-dependent manner, whereas the individual proteins are inactive. This finding suggests an intimate link of MRP8/14 to arachidonic acid and its metabolites which are principally involved in the initiation, propagation and termination of inflammatory processes.

2. Material and methods

2.1. cDNA cloning

Mouse spleen RNA was extracted using the guanidine-isothiocyanate phenol extraction and first strand cDNA was synthesized according to common molecular biology techniques [13]. Using primers MRP8-1 (5′-ATGCCGTCTGAACTGGAG), MRP8-2 (5′-TACTCCTTCTGGCTGTCT), MRP14-1 (5′-ATGGCCAACAAAGCACCT) and MRP14-2(TTACTTCCCACAGCCTTT) double-stranded cDNA of murine MRP8 and MRP14 was synthesized using polymerase chain reaction (95°C 1 min; 50°C 1 min, 72°C 1 min, 35 cycles) and subsequently cloned into pSP72 (Promega, Heidelberg, Germany).

2.2. Expression and purification of recombinant proteins

After subcloning into pQE32 (Qiagen, Hidden, Germany) MRP8 and MRP14 were expressed and purified: bacterial cultures were centrifuged ($3000 \times g$, 20 min) and subsequently resuspended in buffer 1 (8 M urea, 50 mM NaH_2PO_4, 15 mM Imidazol, 10 mM Tris-HCl, 100 mM NaCl; pH 8.0 (all chemicals are obtained from Boehringer Ingelheim Bioproducts, Heidelberg, Germany, unless otherwise stated). After sonification the cell lysate was centrifuged at $20\,000 \times g$ for 30 min. The supernatant was purified using immobilized metal affinity chromatography (Clontech, Palo Alto, CA); elution was performed using buffer 1 at pH 6.0 supplemented with 100 mM Imidazol. The proteins were dialysed in 20 mM NH_4Ac, pH 5.0, and lyophilized. Subsequent analysis by polyacrylamide gel electrophoresis (PAGE) revealed that the recombinant murine MRPs were 95–98% pure (data not shown).

2.3. Complex formation of MRP8 and MRP14

To investigate the possibility of these proteins to form complexes 50 μg of each protein alone or 25 μg of each protein in combination were resuspended in 50 mM $CaCl_2$, 5% β-mercaptoethanol, boiled for 5 min and dialysed for 36 h against 50 mM $CaCl_2$ in 15 mM HEPES, pH 8.0, exchanging the buffer twice. After dialysis the samples were

*Corresponding author. Tel.: (49) 251-8356552; Fax: (49) 251-8356549. E-mail: klempt@uni-muenster.de

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freeze dried and subsequently resuspended in SDS sample buffer (150 mM Tris-HCl, pH 8.5, 0.1% SDS, 10% glycerol, 0,1% bromphenol blue), boiled for 2 min, and analysed by 15% SDS-PAGE. Gels were stained using Coomassie standard procedure.

2.4. Binding to fatty acids

In a first set of experiments recombinant mouse MRP8 and MRP14 proteins were treated according to the procedure described above, with the exception that the dialysis buffer was TBS (50 mM Tris-HCl, 150 mM NaCl) containing 50 mM $CaCl_2$. When dialysis was completed, the samples were adjusted to a concentration of 100 μg/ml and equally divided into two vials. One vial was assayed using the procedure described below and the other sample was supplemented with EDTA at a final concentration of 25 mM. An aliquot of 100 μl containing 10 μg of protein of each sample was incubated with 20 μl of 4.8 μM ^{14}C-labelled arachidonic or oleic acid (Amersham, Braunschweig, Germany) for 30 min at 37°C. Unbound fatty acids were removed from fatty acids associated to MRPs using micro G-25 spin columns (Pharmacia, Freiburg, Germany) according to the manufactors' instruction. To verify Ca^{2+}-dependent binding of the arachidonic acid to the MRPs, the proteins were dialysed against TBS in the absence of Ca^{2+}. To establish different Ca^{2+} concentrations, different amounts of $CaCl_2$ were added to give a final concentration of 25, 50, 100 and 1000 μM. After incubation for 30 min at 37°C radioactive labelled arachidonic acid was added and the experiment was further continued as described above. Coelution of the recombinant mouse MRP8/14 and arachidonic acid was performed following incubation of the protein with labelled arachidonic acid in TBS in the presence of 100 μM Ca^{2+} for 30 min at 37°C. Subsequently, the protein-bound fatty acid was removed from unbound fatty acid by gel filtration (PD-10 columns, Pharmacia). Aliquots of 500 μl were collected and analysed for protein and fatty acid content by UV-VIS spectroscopy and liquid scintillation counting, respectively.

3. Results and discussion

A SDS-PAGE using non-reducing conditions loaded with recombinant mouse MRP8 or recombinant mouse MRP14 reveals two major bands in each lane with a molecular mass of 11 kDa and 22 kDa for MRP8 and 14 kDa and 28 kDa for MRP14, respectively (Fig. 1, lanes 2 and 3). After incubation of both MRPs in Ca^{2+}-rich buffer, an additional band occurs (Fig. 1, lane 1). To verify the assumption that the high molecular mass proteins indeed are homo- and heterodimers, the bands were eluted from the gel, boiled for 5 min with 1% β-mercaptoethanol to reduce the samples and applied to a reducing SDS-PAGE. As expected the homodimers of MRP8 and MRP14 contain only elements of 11 kDa (MRP8) or 14 kDa (MRP14), respectively (Fig. 1, lane A and B). The supposed heterodimer of mouse MRP8 and MRP14 consists of a 11 kDa (MRP8) and a 14 kDa (MRP14) protein (Fig. 1, lane C). Therefore, recombinant mouse MRP8 and MRP14 form homo- and heterodimers in a similar manner as shown for natural human MRPs [6]. Additionally the complexes have been shown to be formed in the presence of calcium.

The recombinant mouse proteins described above were used in different combinations to analyze the binding of MRPs to fatty acids. Only the MRP8/MRP14 complex significantly and specifically binds arachidonic acid. Up to 48% of radioactively labelled arachidonic acids added in substochimetrial amounts remained bound to the heterodimer after separation. In contrast only 9% of the arachidonic acid remained bound to the monomers and homodimers (Fig. 2). It is obvious that the binding of arachidonic acid critically dependents on the formation of the protein complex, which is supposed to be formed in vivo after cell activation [6].

Furthermore, it could be demonstrated that the binding of arachidonic acid is also dependent on the presence of Ca^{2+} reflecting physiological concentrations (Fig. 3A). Additionally, the binding of the MRP8/14 complex to arachidonic acid could be prevented by addition of EDTA to the reaction (Fig. 2), although the presence of EDTA does not destabilize the heterodimer once it has formed (data not shown).

The affinity of MRP complex to arachidonic acid is further demonstrated by a coelution experiment. Bound arachidonic acid coelutes with the heterodimer, whereas free arachidonic acid forms a second, clearly distinguishable peek (Fig. 3B).

The distinct interaction of the MRP8/MRP14 heterodimer with arachidonic acid was in contrast to the faint binding of the complex to oleic acid (13% vs. 48% of the total fatty acid bound to the MRP8/MRP14 complex, Fig. 2). This demonstrates the specific affinity of the MRP heterodimer to arachidonic acid unlike other proteins known to unspecifically associated with a diversity of lipophilic compounds. This conclusion can be drawn as arachidonic acid is a 20 C-atom polyunsaturated molecule ($20{:}4^{\Delta 5,8,11,14}$), whereas oleic acid is a mono-unsaturated 18 C-atom fatty acid ($18{:}1^{\Delta 9}$). Whether the heterodimer exclusively binds arachidonic acid or related derivatives (e.g. prostaglandines) as well, needs to be further investigated. The determination of the physical properties of the binding could add valuable information on whether MRP8/14 act primarily as a transport carrier or has a functional role itself.

The interactions of S-100 proteins with fatty acids have not

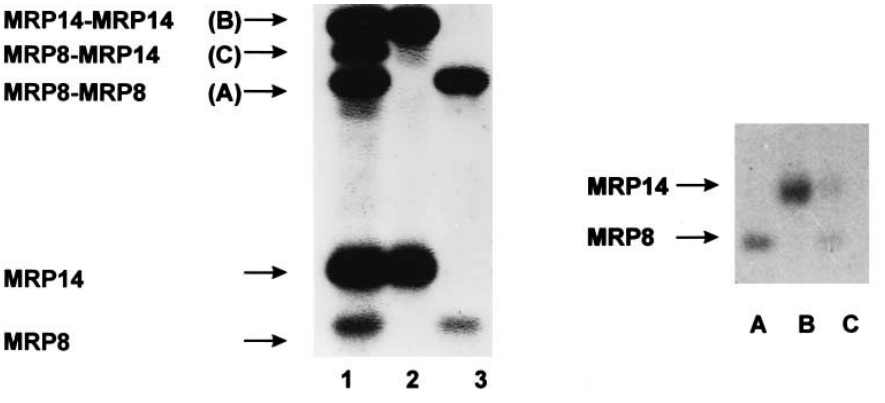

Fig. 1. Recombinant mouse MRP8 and MRP14 on SDS-PAGE (15%) under non-reducing conditions (left): lane 1, MRP8 and MRP14; lane 2, MRP14; lane 3, MRP8. The bands A–C were extracted from the left gel and reloaded on reducing PAGE (right): lanes A–C correspond with the homodimers of MRP8; homodimers of MRP14 and heterodimer of MRP8 and MRP14, respectively.

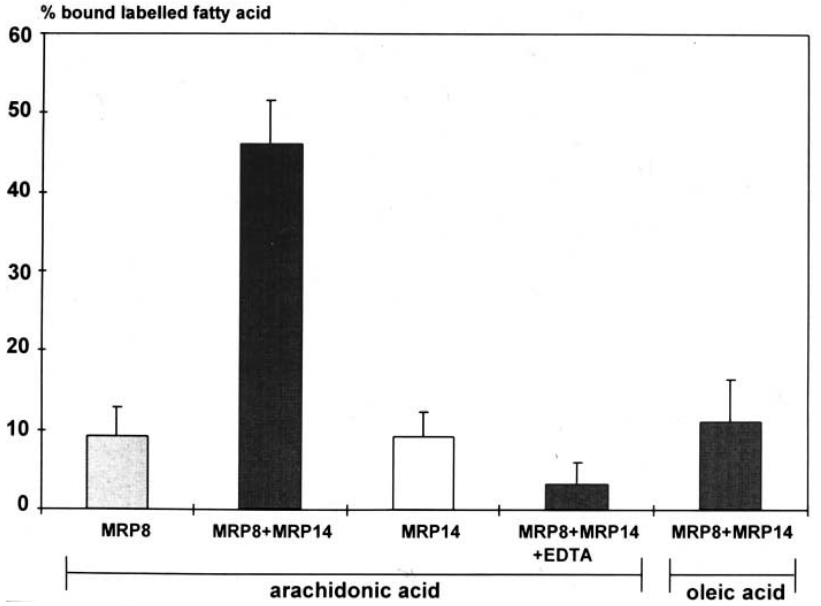

Fig. 2. Binding of recombinant mouse MRP8 and MRP14 to arachidonic and oleic acids. The heterodimer MRP8/MRP14 binds arachidonic acid significantly, whereas the interaction of monomers and homodimers is negligible. Binding of arachidonic acid to the MRP8/MRP14 complex was absent in the presence of EDTA. Control experiments using oleic acid resulted only in a minor binding.

been studied extensively so far. One report suggests the interaction of S-100 proteins with fatty acids in adipocytes [14]. Follow-up studies of this observation have not been published. Recently, another group searching for arachidonic acid binding proteins in human keratinocytes identified MPR8/MRP14 as a potential candidate [15].

The biological significance of our findings remains unclear. It is known that agents that raise calcium levels in cells, e.g. monocytes, induce phospholipase A2 which leads to the cleavage of arachidonic acid from the glycolipid in the membrane. Arachidonic acid is rapidly metabolized via the lipoxygenase and cycloxygenase controlled pathway [16]. Parallel to these events calcium is bound by MRP8/MRP14 complexes which translocate to the cytoskeleton and the plasma membrane. If phosphokinase C is activated, i.e. by arachinoidate [17] this also leads to the secretion via a novel microtubule-dependent pathway of MRP8/14 [10]. An important question is whether MRP8/14 is released as a free complex or with arachidonic acid bound to it. Another question is whether arachidonic acid is the anchor molecule for the protein complex in the membrane. If S-100 proteins in general are bound to fatty acids or subsets of fatty acids, this aggregation would increase the hydrophobicity. Subsequent translocation to the cell membrane could be the initial event which may finally lead to secretion of the S-100 proteins.

The interaction with free arachidonic acid allows to conceive several possibilities. Binding of arachidonic acid could be a way to limit the pro-inflammatory action of arachidonic acid. These actions are very specific, since arachidonic acid but not eicosapentaenoic acid pretreatment of granulocytes results in a release of elastase [18], which decreases endothelial integrity. Furthermore, the export to the extracellular space and the high amounts of MRP8/14 complexes in body fluids of patients with acute and chronic inflammatory diseases [2,3] raises the question whether MRP8/14 is merely a transport protein or the arachidonic acid protein complex has functions

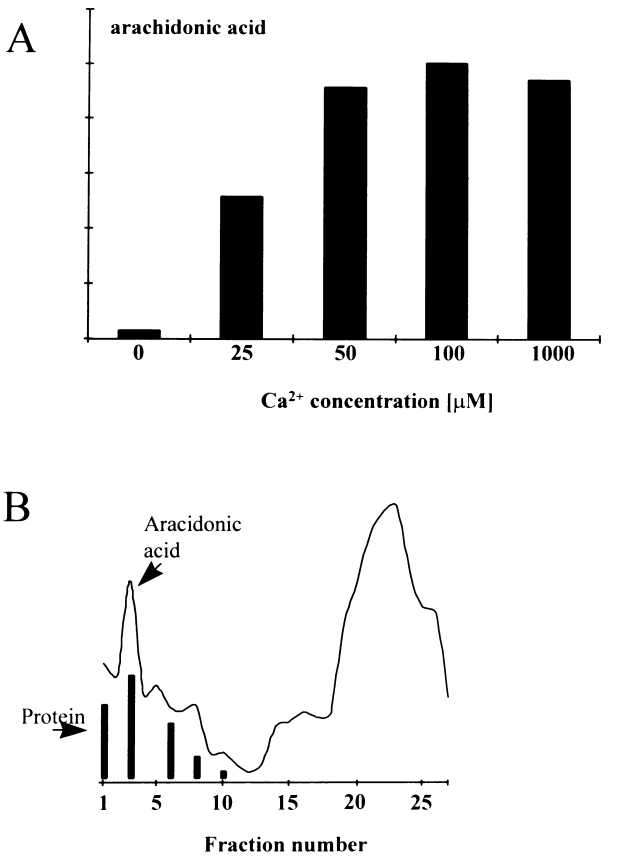

Fig. 3. Binding of recombinant mouse MRP8/MRP14 complex to arachidonic acid is Ca^{2+} dependent (A) even at low concentrations and is saturated at 50 μM Ca^{2+}. Coelution of arachidonic acid together with the MRP8/MRP14 protein complex as demonstrated using 100 mM Ca^{2+} (B) further supports the specificity of this interaction.

of its own. A number of reports on biological functions would have to be revisited in view of the fact that the protein complex isolated from sera of patients with inflammatory diseases bear arachidonic acid or not.

In summary, the specific binding of MRP8/MRP14 complex to arachidonic acid opens a new horizon for the biology of these proteins in combination with fatty acids. It links a well-established system of inflammatory mediators with a group of proteins whose involvement in the inflammatory process has been well documented.

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