

The N-terminal domain I of human lactotransferrin binds specifically to phytohemagglutinin-stimulated peripheral blood human lymphocyte receptors

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Human lactotransferrin receptors have been recently characterized on mitogen-stimulated human lymphocytes [(1989) Eur. J. Biochem. 179, 481–487]. In order to define the lactotransferrin recognition site by these receptors, the binding to lymphocytes of several tryptic fragments, isolated from human lactotransferrin by mild tryptic hydrolysis [(1984) Biochim. Biophys. Acta 787, 90–96], has been investigated. The 30 kDa N-tryptic fragment (residues 4–281) and the re-associated N,C-tryptic complex bind to lactotransferrin lymphocyte receptor with a dissociation constant of 44 nM and 39 nM, respectively, similar to the value obtained for the native lactotransferrin ($K_d = 46$ nM). However, neither the N-terminal domain II (residues 91–257) nor the 50 kDa C-tryptic fragment (residues 282–703) are recognized. These results suggest that the binding site of human lactotransferrin by the lymphocyte receptor is located in the N-terminal lobe and more precisely in the N-terminal domain I (residues 4–90 and/or 258–281).

Lactotransferrin; Lactoferrin; Phytohemagglutinin stimulation; Receptor; Binding site; (Human lymphocyte)

1. INTRODUCTION

Lactotransferrin, also called lactoferrin, serotransferrin and ovotransferrin are bilobed iron-binding glycoproteins of the transferrin class whose primary structure [1–3] and three-dimensional conformation, as revealed by partial proteolytic hydrolysis studies [4–6] and X-ray diffraction data [7,8], are very similar. However, these glycoproteins differ in the number, the structure and the location of their glycans [9–12]. Otherwise, these transferrins bind to different cells such as reticulocytes (serotransferrin) [13], enterocytes (lactotransferrin) [14,15] and chick embryo red blood cells (ovotransferrin) [16], thus suggesting that specific transferrin receptors are present at the surface of these cells and recognize a glycan, a peculiar region of the polypeptide chain, or both.

Up to now, very few data are available about the transferrin recognition site on these cells. The recent characterization of a specific lactotransferrin receptor, at the surface of phytohemagglutinin-stimulated human peripheral blood lymphocytes [17], prompted us to define the region of the human lactotransferrin molecule which interacts with this kind of receptors. The analysis was performed by using different fragments previously isolated from human lactotransferrin by mild tryptic hydrolysis [6]. Moreover, in order to point out the importance of the lactotransferrin molecule conformation in the interaction with its receptor, the binding and competition experiments were performed in the presence of the re-associated N,C-tryptic complex whose conformation is slightly modified compared to lactotransferrin [18].

2. MATERIALS AND METHODS

2.1. Proteins

Lactotransferrin purified from human lactoserum [11] was iron-saturated [19] and submitted to a mild tryptic hydrolysis [6]

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in order to isolate the 30 kDa monoferric N-tryptic fragment (residues 4–281) and the 50 kDa monoferric C-tryptic fragment (residues 282–703). Both 30 kDa N- and 50 kDa C-tryptic fragments were re-associated into a non-covalent N,C-tryptic complex [18]. In addition, an iron-binding 20 kDa glycopeptide (residues 91–257) called N2-glycopeptide, which corresponds to the N-terminal domain II of human lactotransferrin described by Anderson et al. [7], was prepared from tryptic hydrolysate of the 30 kDa N-tryptic fragment [6].

2.2. Cells

Human peripheral blood lymphocytes were isolated from heparinized whole blood and stimulated with phytohemagglutinin (IBF, Villeneuve la Garenne, France) as previously described [17].

2.3. Binding assays

Proteins were labelled with Na^{125}I (Amersham International, England) by using Iodo-Gen (Pierce Chemical Co., Rotterdam, The Netherlands) as a catalyst [17]. The cell binding of labelled human lactotransferrin and of its different fragments described above was performed in siliconized polypropylene tubes (Minisorb, Nunc, Denmark). Before use, cells were washed twice in RPMI 1640 without fetal calf serum and resuspended in RPMI 1640, 25 mM Hepes, pH 7.3, in order to obtain a cell concentration of about 5×10^5 cells/ml. ^{125}I -labelled lactotransferrin was added to the cell suspension at a concentration ranging from 0 to 100 nM and, after 2 h at 4°C, the cells were run ($2000 \times g$, 10 min) and washed three times with phosphate-buffered saline. All assays were performed in the presence of 0.2% human serotransferrin, not recognized by the lactotransferrin receptors [17], in order to limit the important adsorption of lactotransferrin onto the walls of the incubation vials. Non-specific binding was estimated by adding a 100-molar excess of unlabelled lactotransferrin. The dissociation constants (K_d) and the number of binding sites (n) were determined by Scatchard plot analysis [20].

2.4. Competitive binding assays

The inhibition of ^{125}I -labelled human lactotransferrin binding by the N- and C-tryptic fragments, the N2-glycopeptide, and by the re-associated N,C-tryptic complex was assayed in the presence of 5–100-fold molar excess of each unlabelled polypeptide. Binding assays were carried out as described above.

3. RESULTS

3.1. Binding assays

As shown in fig.1, the binding of the N-tryptic fragment and of the re-constituted N,C-tryptic complex was concentration-dependent from 0 to 20 nM and saturable at about 100 nM, with respective dissociation constants of $K_d = 44 \pm 4$ and 39 ± 6 nM, similar to that of human lactotransferrin which was used as a reference ($K_d = 46 \pm 3$ nM). However, the number of binding sites of both N-tryptic fragment ($n = 140\,000$) and re-

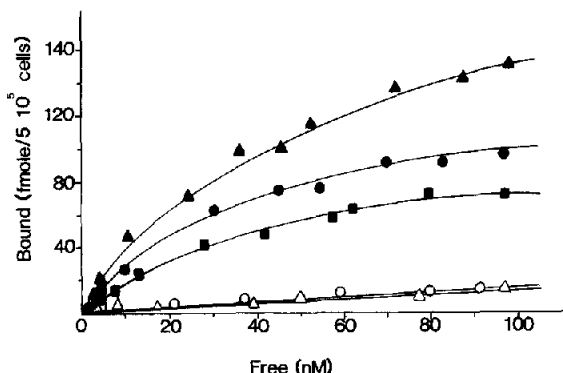


Fig.1. Specific binding of ^{125}I -labelled iron saturated human lactotransferrin (\blacktriangle — \blacktriangle), re-associated N,C-tryptic complex (\bullet — \bullet), N-tryptic fragment (\blacksquare — \blacksquare), C-tryptic fragment (\triangle — \triangle) and N2-glycopeptide (\circ — \circ) to phytohemagglutinin-stimulated human lymphocytes. Equilibrium bindings were measured at 4°C using 0.5×10^6 cells per sample. Results were calculated from three separate experiments.

associated N,C-tryptic complex ($n = 170\,000$) was 2-fold lower than the native protein ($n = 300\,000$). On the contrary, the binding of both C-tryptic fragment and N2-glycopeptide to mitogen-stimulated lymphocytes only represented 10% of human lactotransferrin binding and was not saturable, suggesting a non-specific binding.

3.2. Competitive binding assays

Competition experiments were performed between ^{125}I -labelled lactotransferrin and unlabelled peptides and as shown in fig.2, the radiolabelled lactotransferrin binding to mitogen-stimulated human lymphocytes was 55% inhibited by 100-fold molar excess of unlabelled human lactotransferrin. In the same experimental conditions, the re-associated N,C-tryptic complex and the N-tryptic fragment blocked the labelled lactotransferrin binding with 45% and 32% inhibition, respectively.

Furthermore, neither the C-tryptic fragment nor the N2-glycopeptide significantly inhibited the lactotransferrin binding on mitogen-stimulated lymphocytes since no more than 5% inhibition was obtained for both tryptic fragments (fig.2). These results are in good agreement with the binding experiments described above.

4. DISCUSSION

The preliminary results we have obtained in-

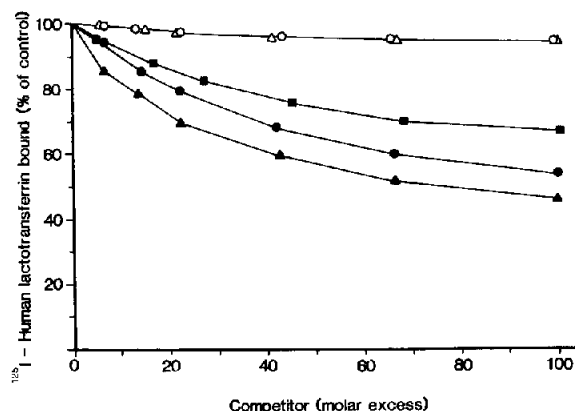


Fig.2. Competitive bindings to phytohemagglutinin-stimulated human lymphocytes between ^{125}I -labelled human lactotransferrin and competitors such as: human lactotransferrin (▲—▲), re-associated N,C-tryptic complex (●—●), N-tryptic fragment (■—■), C-tryptic fragment (△—△) and N2-glycopeptide (○—○). Competitive bindings were measured at 4°C using 1×10^6 cells per sample. Each point represents the average of duplicate measurements from two separate experiments.

dicates, that the N-tryptic fragment and the re-associated N,C-tryptic complex are recognized by the receptors with the same affinity as that of lactotransferrin. Nevertheless, the lower number of binding sites of these tryptic fragments could be explained by secondary-structure modifications, mainly located in the N-tryptic fragment. As a matter of fact, the isolation of the N- and C-tryptic fragments leads to a decrease of about 30% of the α -helical content and during the non-covalent re-association of both N- and C-tryptic fragments into the so-called N,N-tryptic complex, the conformation of the native lactotransferrin is restored only in part [18]. These observations point out the importance of the protein conformation to lymphocyte receptor binding. Moreover, the lactotransferrin recognition site seems to be located precisely in the N-terminal domain I (residues 4–90 and 258–281) described by Anderson et al. [7] because the N2-glycopeptide (residues 91–257), corresponding to the N-terminal domain II, was not bound to the lymphocyte receptor. Up to now, the N-terminal domain I has not been isolated because of its degradation during tryptic hydrolysis. The glycan does not seem to be involved in the binding to lymphocyte receptors since neither the N2-glycopeptide nor the C-tryptic fragment, each containing one glycan, were recognized. In addition, the

binding experiments with labelled fragments and the competitive assays with unlabelled fragments demonstrate that protein iodination does not modify the lactotransferrin binding to lymphocytes. These results differ from those obtained by Mason and Brown [21] who have shown that isolated N- and C-terminal lobes of hen ovotransferrin cannot bind to chick embryo red blood cells. However, when both the lobes, re-associated or not, are present binding occurs [22]. Moreover, iodination experiments have underlined the implication of a tyrosinyl residue at the C-terminal lobe of hen ovotransferrin in receptor recognition [21]. Thus, it appears that the interactions of human lactotransferrin and hen ovotransferrin with their specific receptor are different.

Further investigations are being engaged in order to locate more precisely the human lactotransferrin peptide sequence which interacts with phytohemagglutinin-stimulated human lymphocyte receptors.

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