Inhibition of the specific binding of human lactotransferrin to human peripheral-blood phytohaemagglutinin-stimulated lymphocytes by fluorescein labelling and location of the binding site

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Labelling of human lactotransferrin with fluorescein 5'-isothiocyanate (FITC) in an equimolar ratio inhibits the binding of the protein to phytohaemagglutinin-activated human peripheral-blood lymphocytes. Therefore it can be assumed that FITC reacts at, or near, the receptor-binding site. Three FITC-labelled peptides have been purified from a tryptic digest of the FITC-labelled lactotransferrin. The determination of their amino acid sequence and their localization on the primary structure of the protein permitted the identification of two FITC-accessible areas in the *N*-terminal lobe and one in the *C*-terminal lobe. In fact, only 10 % of the total FITC was conjugated to one lysine residue (Lys⁵⁷⁹) of the *C*-terminal lobe, whereas most (80 %) of the FITC was conjugated to three close lysine residues [Lys²⁸³ (65 % of total fluorescence), Lys²⁸⁰ and Lys²⁸² (15 % of total fluorescence)] located in β -turn structures, of the *N*-terminal domain I of human lactotransferrin. The results obtained show that the receptor-binding site should be located in the vicinity of the FITC-accessible Lys²⁸³, Lys²⁸⁰ and Lys²⁸², and corroborate our preliminary results reporting the involvement of the *N*-terminal domain I in the binding of human lactotransferrin to mitogen-stimulated lymphocytes [Rochard, Legrand, Mazurier, Montreuil & Spik (1989) FEBS Lett. 255, 201–204]. In any case, FITC labelling is not suitable for studying the binding of lactotransferrin to activated lymphocytes and its use may lead to erroneous interpretations of cell binding experiments.

INTRODUCTION

Previous findings have demonstrated that human lactotransferrin (also named lactoferrin), an iron-binding glycoprotein first isolated from milk [1–3], further characterized in external secretions [4,5] and in neutrophils [6], is involved in the iron delivery to target cells [7,8]. This biological role is mediated by specific receptors which have been found at the surface of enterocytes [9–11] and of human phytohaemagglutinin-stimulated peripheral-blood lymphocytes [12]. As regards the mitogen-stimulated lymphocytes, the receptor has been isolated and characterized as a 110 kDa single-polypeptide-chain glycoprotein [12].

Using fluorescein 5'-isothiocyanate (FITC)-labelled human lactotransferrin we tried to determine the characteristics of human lactotransferrin binding to the activated lymphocytes by flow cytometry and by fluorescence microscopy. As all attempts to demonstrate the binding of the FITC-labelled human lactotransferrin to lymphocytes failed, we postulated that the dye might mask the receptor-binding site of the protein and, therefore, we have examined the effect of the labelling on the interaction of lactotransferrin with the lymphocyte receptor.

The present report confirms this hypothesis and describes the purification and the amino acid sequencing of FITC-labelled peptides which define the localization of the receptor-binding site in the *N*-terminal domain I of lactotransferrin.

EXPERIMENTAL

Materials

FITC isomer I was obtained from Sigma (St. Louis, MO, U.S.A.). Lymphoprep was purchased from Nyegaard and Co. (Oslo, Norway), phytohaemagglutinin from IBF (Villeneuve la Garenne, France), RPMI 1640 and fetal-calf serum from Gibco (Grand Island, NY, U.S.A.), culture dishes from Nunc (Kamstrup, Denmark), ¹²⁵I (carrier-free) from Amersham International (Amersham, Bucks., U.K.), Iodogen from Pierce Chemical Co. (Rockford, IL, U.S.A.) and Sephadex G-25 from Pharmacia (Uppsala, Sweden). H.p.l.c. was performed with a Spectra-Physics model-8700 liquid chromatograph equipped with a Spectra-Physics model-8450 variable-wavelength u.v.–visible detector connected to a model-4270 computing integrator (Spectra-Physics, Autolab Division, San Jose, CA, U.S.A.). H.p.l.c.-grade acetonitrile was from Carlo Erba (Milan, Italy) and h.p.l.c.-grade water was obtained from the Milli-Q Millipore system (Millipore S.A., Bedford, MA, U.S.A.). The 10 μ m Waters μ -Bondapak C₁₈ column and the 10 μ m Chromatem C₁₄ column were from Waters/Millipore Corp. (Milford, MA, U.S.A.) and from Touzart and Matignon (Vitry sur Seine, France) respectively. Peptides were sequenced on an Applied Biosystems 470 A gas-phase sequencer, and amino acid phenylthiohydantoin (PTH) derivatives were identified by h.p.l.c. using 120 A on-line

Abbreviations used: FITC, fluorescein 5'-isothiocyanate; RP-, reverse phase; PBS, phosphate-buffered saline (20 mM-sodium phosphate/150 mM-NaCl, pH 7.4); PTH, phenylthiohydantoin.

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PTH analyser (Applied Biosystems, Foster City, CA, U.S.A.). All chemicals were of analytical grade.

Cell preparation

Human peripheral-blood mononuclear cells were obtained by Lymphoprep separation of heparinized venous blood drawn from healthy volunteers. The macrophage-monocyte depletion was achieved by resuspending 5×10^6 cells/ml in RPMI 1640/25 mM-Hepes, pH 7.3, containing penicillin (100 000 units/l) and streptomycin (50 mg/l). Fractions (0.5 ml each) of the suspension were incubated in 24-well culture dishes for 2 h at 37 °C in CO₂/air (1:19). At the end of incubation, non-adherent cells (lymphocytes) were plated in 72-well culture dishes at 10^6 cells/ml (0.25 ml/well). The culture medium used was RPMI 1640/25 mM-Hepes, pH 7.3, containing antibiotics as described above. Fetal-calf serum was added at a concentration of 10%. The cells were stimulated with phytohaemagglutinin at a final concentration of 20 µg/ml, for which the optimal response was obtained [12]. The stimulated cells were incubated for 72 h at 37 °C in CO₂/air (1:19) before the binding experiments.

Preparation of lactotransferrin

Lactotransferrin was prepared from pooled human milk and purified as described by Spik *et al.* [13]. The homogeneity of the protein was checked by SDS/PAGE [14]. Human serotransferrin was from Behring (Marburg, Germany). Iron saturation of lactotransferrin was carried out by adding to the native protein (in a solution of 0.1 M-sodium citrate/bicarbonate buffer, pH 8.2 [15]) the amount of 0.07 M-FeCl₃ (in the citrate/bicarbonate buffer) necessary to complete iron saturation. The excess of reagent was removed by gel filtration through a Sephadex G-25 column (20 cm × 3 cm). The protein concentration was determined as described by Lowry *et al.* [16], with BSA as standard, and the amount of iron was measured with the sulphobathophenanthroline reagent (Biopack-Fer; Biotrol, Paris, France).

Labelling of human lactotransferrin with FITC

Human lactotransferrin was labelled with FITC (isomer I) by incubating 1 µmol of the protein (80 mg) in 10 ml of 0.1 M-NaHCO₃, pH 9.5, with various concentrations of freshly prepared FITC (0.5–2 µmol in 200 µl of dimethyl sulphoxide). The reaction was performed at room temperature in the dark for 1 h with gentle stirring. The reaction was terminated by passing the solutions through a Sephadex G-25 column (20 cm × 3 cm) in 0.1 M-NaHCO₃, pH 9.5, to eliminate unchanged FITC. The ratio of fluorescein to protein was estimated by measuring the absorbances at 495 nm and 280 nm by the method of Jobbagy & Kiraly [17]. The FITC-labelled lactotransferrin solutions were dialysed, either against 0.1 M-Tris/HCl, pH 8.2, for further tryptic hydrolysis, or against phosphate-buffered saline (PBS) for binding assays.

Radiolabelling of lactotransferrin

Pure lactotransferrin and FITC-labelled lactotransferrin were ¹²⁵I-labelled with Iodogen as previously described [12], and the excess of reagent was removed by gel filtration through a Sephadex G-25 column equilibrated in PBS.

Binding assays

The assays of native and FITC-labelled lactotransferrin binding were performed in silicone-treated polypropylene tubes. Before use, phytohaemagglutinin-stimulated lymphocytes were washed twice in RPMI 1640 without fetal-calf serum, re-

incubated for 2 h, washed again, and finally resuspended in RPMI 1640/25 mM-Hepes, pH 7.3, in order to obtain a cell concentration of about 5×10^5 /ml. ¹²⁵I-labelled proteins were added at a concentration ranging between 0 and 0.15 µM. Non-specific binding was measured in the presence of a 100-fold molar excess of unlabelled lactotransferrin. In order to prevent the non-specific binding of human lactotransferrin to cells or to plastic, all binding experiments were performed in the presence of 1% human serotransferrin, since serotransferrin has no inhibitory effect on the binding of lactotransferrin [18]. After 1 h incubation at 4 °C in the presence of CO₂/air (1:19), 0.2 ml aliquots of the cell suspension were centrifuged (2000 g, 10 min) and washed twice in PBS. At the end of washing, the cell pellet was resuspended in 0.2 ml of PBS, and the radioactivity of the suspension was measured with a Compugamma LKB-Wallac (Turku, Finland) γ-radiation counter.

Competitive binding assays

The inhibition of ¹²⁵I-labelled lactotransferrin binding by FITC-labelled lactotransferrin was assayed in the presence of 5–100-fold molar excess of unlabelled or FITC-labelled lactotransferrins. Binding assays were carried out as described above.

Tryptic hydrolysis and peptide purification

FITC-labelled lactotransferrin was digested by trypsin (enzyme/substrate ratio 1:20, w/w) in 0.1 M-Tris/HCl, pH 8.2, at 37 °C for 12 h after reduction (as described by Cresfield *et al.* [19]) and alkylation with iodoacetamide. The peptides generated from the proteolytic digest were first purified by reverse-phase (RP-) h.p.l.c. on a 10 µm Waters µ-Bondapak C₁₈ column (25 cm long × 0.46 cm internal diameter) with a six-step 0.1% (v/v) trifluoroacetic acid-containing water/acetonitrile gradient from 0 to 65% acetonitrile for 1 h. The fractions containing the FITC-labelled peptides were detected by measuring the absorption at 495 nm after addition of 1 M-NaOH (until the pH reached 8.0), concentrated *in vacuo*, and then rechromatographed twice to purity on a 10 µm Chromatem C₁₄ column (25 cm long × 0.46 cm internal diameter) with 0.1% (v/v) trifluoroacetic acid-containing water/acetonitrile gradients from 25 to 30% acetonitrile for 30 min or from 25 to 40% acetonitrile for 70 min.

RESULTS

Incorporation of FITC into human lactotransferrin

The FITC/human lactotransferrin molar ratio was investigated by incubation of the protein with freshly prepared FITC solutions. It was found that, under the above-described experimental conditions, the binding of FITC to human lactotransferrin was linearly concentration-dependent and that a 1.2-fold molar excess of FITC was necessary to bind one molecule of fluorescein per molecule of protein.

Binding and competitive binding experiments of radio- and FITC-labelled lactotransferrin

The effect of FITC conjugation to ¹²⁵I-labelled human lactotransferrin (ratio 1:1) on the binding to phytohaemagglutinin-stimulated human lymphocytes is shown in Fig. 1. Compared with the ¹²⁵I-labelled native protein, which exhibits a concentration-dependent and saturable binding curve on mitogen-activated human lymphocytes with a *K_d* of 46 nM and 300 000 binding sites, as previously reported [12,20], the binding of the radiolabelled and FITC-labelled protein was not saturable and represented only 15% of that of human lactotransferrin. Higher FITC/human lactotransferrin molar ratios led to the

Fig. 1. Specific binding to phytohaemagglutinin-stimulated human peripheral-blood lymphocytes of ^{125}I -labelled iron-saturated human lactotransferrin (●) and of FITC-conjugated (FITC/protein ratio 1:1, mol/mol) and ^{125}I -labelled iron-saturated human lactotransferrin (○)

Equilibrium binding was measured at 4 °C with 5×10^5 cells per sample.

Fig. 2. Competitive binding to phytohaemagglutinin-stimulated human peripheral-blood lymphocytes of ^{125}I -labelled iron-saturated human lactotransferrin and competitors such as unlabelled lactotransferrin (●) and FITC-conjugated lactotransferrin (fluorescein/protein ratio, 1:1, mol/mol) (○)

same results (results not shown). These results are confirmed by the competitive binding experiments, which show that no more than 5% inhibition was obtained for FITC-labelled lactotransferrin at 100-fold molar excess, instead of 55% for the unlabelled protein (Fig. 2).

Isolation of FITC-labelled peptides

Tryptic digestion of FITC-labelled lactotransferrin followed by RP-h.p.l.c. on a C_{18} column with a 0.1% trifluoroacetic acid-containing water/acetonitrile gradient from 0 to 65% acetonitrile for 1 h led to the recovery of two major fluorescent peptide fractions named I and II and corresponding to $15 \pm 5\%$ and $75 \pm 5\%$ of the total fluorescence respectively (Fig. 3).

Both fractions I and II were rechromatographed by RP-h.p.l.c. on a C_{14} column with 0.1% trifluoroacetic acid-containing water/acetonitrile gradients from 25 to 30% acetonitrile for 30 min and from 25 to 40% acetonitrile for 70 min respectively. Fraction I gave only one fluorescent fraction, called 'I-A' (Fig. 4), whereas fraction II contained two fluorescent fractions called 'II-B' and 'II-C' (Fig. 5).

Further purification under the same chromatographic conditions of these three fractions gave three homogeneous fluorescent peptide-containing fractions I-A, II-B and II-C (results not shown) that represented $14 \pm 5\%$, $11 \pm 5\%$ and $65 \pm 5\%$ of total fluorescence respectively.

Amino acid sequences of the FITC-binding sites of human lactotransferrin

Edman degradation of the FITC-labelled peptide from fraction II-C yielded the single sequence:

with an unusual fluorescent product in the second cycle that is co-eluted with the tryptophan PTH derivative and which we attribute to a FITC-substituted lysine residue or, more likely, to its degradation product, by comparison with the residues-262-272 sequence of unlabelled human lactotransferrin (Fig. 6).

The sequence of the fluorescein-labelled peptide isolated from fraction I-A was determined as:

where the third and fifth residues were FITC-substituted lysine residues, as confirmed by alignment with the human lactotransferrin 278-285 sequence (Fig. 6).

Fig. 3. Preparative RP-h.p.l.c. of the total tryptic digest of FITC-labelled human lactotransferrin

About 10 mg of the tryptic digest was injected on to a 10 μm Waters μ -Bondapak C_{18} column (25 cm length $\times$ 0.46 cm internal diameter) at 0% acetonitrile. Peptides were eluted using the gradient shown in the Figure: solvent A, 0.1% trifluoroacetic acid in water; solvent B, acetonitrile containing 0.1% trifluoroacetic acid; flow rate, 1 ml/min. In this Figure and Figs. 4 and 5, A_{495} is a measure of FITC and A_{214} a measure of protein.

Fig. 4. Preparative RP-h.p.l.c. of the FITC-labelled peptide-containing fraction I

Fraction I was concentrated under vacuum and injected on to a 10 μ m Chromatem C₁₄ column (25 cm length $\times$ 0.46 cm internal diameter) at 25% acetonitrile. Peptides were eluted using the gradient shown in the Figure: solvent A, 0.1% trifluoroacetic acid in water; solvent B, acetonitrile containing 0.1% trifluoroacetic acid; flow rate, 1 ml/min.

Fig. 5. Preparative RP-h.p.l.c. of the FITC-labelled peptide-containing fraction II

Fraction II was concentrated under vacuum and injected on to a 10 μ m Chromatem C₁₄ column (25 cm length $\times$ 0.46 cm internal diameter) at 25% acetonitrile. Peptides were eluted by using the gradient shown in the Figure: solvent A, 0.1% trifluoroacetic acid in water; solvent B, acetonitrile containing 0.1% trifluoroacetic acid; flow rate, 1 ml/min.

Fig. 6. Partial amino acid sequence of human lactotransferrin showing the FITC-conjugating lysine residues

Amino acid residues are numbered according to the sequence of human lactotransferrin reported by Metz-Boutigue *et al.* [21] and modified by Anderson *et al.* [22]. The amino acid sequences designed as I-A, II-B and II-C correspond to the sequences of the FITC-labelled peptides isolated from fractions I-A, II-B and II-C respectively. FITC-conjugated lysine residues are symbolized by asterisks. α -helices ($\sim$) and β -strands ($\sim$) are indicated on sections of the polypeptide chain [22]. The broken line ($\sim$) denotes the α -helix extension in the N-terminal half [22]. Gaps (-) have been placed in the amino acid sequence to allow the superposition of the α -helices and β -strands. Abbreviations: Nt, N-terminal; Ct, C-terminal.

Fraction II-B contained a peptide whose sequence was:

LAQFALLCLDG(–)R

and corresponded to the lactotransferrin 568–580 sequence, with Lys⁵⁷⁹ substituted with fluorescein (Fig. 6). However, a glutamine residue was found instead of the expected aspartic acid residue at position 570 [21,22].

DISCUSSION

As previously reported for proteins such as, for example, phosphorylase kinase α -subunit [23], the labelling by FITC does not occur randomly at the surface of proteins, but hits peculiar lysine residues located in an accessible area of the molecule. The sequencing of FITC-labelled peptides isolated from human lactotransferrin reveals that fluorescein is preferentially conjugated to a particular region of the *N*-terminal lobe and, incidentally, to the *C*-terminal lobe, of lactotransferrin. Indeed, only three FITC-accessible areas where four lysine residues are involved have been located. The less FITC-accessible area (10 % of total fluorescence) is located at the *C*-terminal lobe (Lys⁵⁷⁹), whereas the two major FITC-accessible areas (80 % of total fluorescence) are located at the *N*-terminal lobe of human lactotransferrin, 17–19 amino acid residues away from one another on Lys²⁶³ (65 % of total fluorescence), Lys²⁸⁰ and Lys²⁸² (15 % of total fluorescence).

According to Anderson *et al.* [22] and as shown in Fig. 6, the *C*-terminal FITC-accessible Lys⁵⁷⁹ is located in a strongly basic area (KRK) and in a random-coil structure. The absence of labelling of the *N*-terminal counterpart can be explained by the presence of a threonine residue (Thr²³⁵) instead of Lys⁵⁷⁹. The two *N*-terminal FITC-accessible area are both located in two β -turn structures separate from a large 4.7-turn α -helix (amino acid residues 263–279). This region is included in the *N*-terminal domain I of lactotransferrin, whose amino acid sequence comprises residues 1–90 and 252–320 [22]. The results tie in well with those obtained in our previous paper, showing that the *N*-tryptic fragment (residues 3–283) (numbering given by Metz-Boutigue *et al.* [21] and modified by Anderson *et al.* [22]) obtained by mild tryptic digestion of human lactotransferrin [24,25] binds to mitogen-stimulated lymphocytes [20]. The high accessibility of residues 252–320 to small molecules such as FITC or large molecules such as, presumably, the lactotransferrin lymphocyte receptor, is supported by our previous report concerning the proteolytic cleavage of lactotransferrin into the *N*- and *C*-terminal lobes [24,25]. Indeed, under mild conditions, trypsin preferentially and quantitatively cleaved the lactotransferrin molecule between Lys²⁸⁰ and Asp²⁸¹ [24,25]. Interestingly, as shown in Fig. 6, the alignment of the *N*-terminal and *C*-terminal halves of human lactotransferrin, based on the superposition of their three-dimensional structures according to Anderson *et al.* [22], reveals nearly similar secondary structures, comparing the *N*- and *C*-terminal sequences from residues 262 to 285 and from 604 to 630 respectively, but exhibits primary-structure sequence similarity as low as 35 % when comparing the same sequences. Moreover, only one (namely that in position 263) out of the three FITC-substituted lysine residues of the *N*-terminal lobe exists in the *C*-terminal counterpart (Lys⁶⁰⁵), but in a quite different environment (Fig. 6). This lack of sequence similarity could explain why FITC, trypsin or the lymphocyte receptor are not bound to the *C*-terminal counterpart of human lactotransferrin. Conversely, the lysine residues of human lactotransferrin are conserved in similar positions and in a similar environment in human and rabbit serotransferrins [26–28]. Since FITC-labelled human serotransferrin binds to its cell receptor [29], this observation leads one to suppose that, if

FITC is conjugated to human serotransferrin on the same surface lysine residues as in human lactotransferrin, the site of human serotransferrin binding to its receptor is located elsewhere on the protein. This hypothesis infers specific binding of both proteins to their respective cell receptors.

The fact that 80 % of the detected FITC conjugated to *N*-terminal domain I leads to an 85 % inhibition of the binding of human lactotransferrin to the lymphocyte receptor strongly suggests that the FITC molecules which randomly substitute the three lysine residues at positions 263, 280 and 282 are each responsible for the masking of the receptor-binding site. Therefore the FITC molecules could cover a common area at the surface of the protein which represents all or part of the receptor-binding site.

Fluorescein isothiocyanate is commonly used in labelling proteins such as transferrin [29] for immunohistological assays or for ligand–receptor binding analysis. Our results demonstrate that FITC labelling is not suitable for studying the binding of human lactotransferrin to activated lymphocytes, since its use may lead to erroneous interpretations of cell binding assays.

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