Molecular Interactions between Human Lactotransferrin and the Phytohemagglutinin-Activated Human Lymphocyte Lactotransferrin Receptor Lie in Two Loop-Containing Regions of the N-Terminal Domain I of Human Lactotransferrin†

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ABSTRACT: Fluorescein isothiocyanate derivatization of the human lactotransferrin on Lys-264 inhibits the binding of the protein to human PHA-activated lymphocytes [Legrand, D., Mazurier, J., Maes, P., Rochard, E., Montreuil, J., & Spik, G. (1991) *Biochem. J. 276*, 733–738], indicating that part of the receptor-binding site is located in the N-terminal domain I of lactotransferrin. In the present study, a 6-kDa peptide (residues 4–52) was isolated from the N-terminal lobe of human lactotransferrin which inhibited the binding of the protein to its cell receptor. In addition, lactotransferrin was derivatized using sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate (SASD) and sulfosuccinimidyl 6-((4′-azido-2′-nitrophenyl)amino)hexanoate (sulfo-SANPAH), two heterobifunctional reagents generally used for receptor–ligand cross-linking. The azide group of these two reagents was inactivated by photolysis, and only the succinimidyl ester group was allowed to react with lysine residues of the protein. The binding of the derivatized lactotransferrins to the human lymphocyte receptor was assayed. SASD, which binds to Lys-74, was able to inhibit the binding of lactotransferrin to the cell receptor, in contrast to Lys-281-binding sulfo-SANPAH. Molecular modeling showed the position of SASD, sulfo-SANPAH, and fluorescein molecules at the surface of the protein and suggested that SASD and fluorescein could mask residues 4–6 and two loop-containing regions of human lactotransferrin (residues 28–34 and 38–45). The comparison of the primary and tertiary structures of human lactotransferrin and serotransferrin, which bind to specific cell receptors, shows that the above-mentioned regions, which are likely involved in protein–receptor interactions, possess specific structural features.

Human lactotransferrin (also called lactoferrin) is a bilobed iron-binding glycoprotein present in milk (Montreuil et al., 1960a,b; Johansson, 1960), in external secretions (Biserte et al., 1963; Masson, 1970), and in neutrophils (Masson et al., 1969). The primary structure of human lactotransferrin was first determined by Metz-Boutigue et al. (1984) and then by cDNA cloning (Powell & Ogden, 1990; Rey et al., 1990). The 3D structure of the protein is now well elucidated (Anderson et al., 1987, 1989, 1990; Baker et al., 1987).

In the present paper, the molecular interactions between human lactotransferrin and the lactotransferrin receptor evidenced at the surface of the human phytohemagglutinin-stimulated peripheral blood lymphocytes (Mazurier et al., 1989) were investigated. Preliminary results using large protein fragments obtained by tryptic hydrolysis of human lactotransferrin (Legrand et al., 1984, 1986) demonstrated that the N-terminal domain I (residues 1–91 and 251–319, numbered according to Powell and Ogden (1990) and Rey et al. (1990)) of the protein could interact with the mitogen-stimulated lymphocyte receptor (Rochard et al., 1989). Furthermore, we recently showed that preferential FITC[1] derivatization of lactotransferrin on Lys-264 prevented binding of the protein to the lymphocyte receptor (Legrand et al., 1991). The direct involvement of Lys-264 in receptor binding remained uncertain, and because of the volume occupied in space by the fluorescein molecule, a large area of the N-terminal domain I of lactotransferrin could be masked by the fluorescent marker.

In the present study, further proteolysis of the 30-kDa N-tryptic fragment of the protein (Legrand et al., 1984, 1986) was performed and a 6-kDa peptide (residues 4–52) still able to inhibit the binding of lactotransferrin to the PHA-activated lymphocyte was isolated. This result demonstrates that Lys-

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[1] Abbreviations: FITC, fluorescein isothiocyanate; SASD, sulfosuccinimidyl 2-(*p*-azidosalicylamido)ethyl-1,3′-dithiopropionate; sulfo-SANPAH, sulfosuccinimidyl 6-((4′-azido-2′-nitrophenyl)amino)hexanoate; RP-HPLC, reverse-phase high-performance liquid chromatography; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; PHA, phytohemagglutinin.

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264 is not involved in lactotransferrin–receptor interactions. In order to obtain complementary information about the receptor-binding site, masking reagents, other than FITC, were used. SASD and sulfo-SANPAH, two heterobifunctional reagents generally used for receptor–ligand cross-linking, were chosen for their ability to be easily detected. In fact, SASD is radioiodinable and sulfo-SANPAH can be detected at 488 nm. These reagents were allowed to react with the lysine residues of lactotransferrin through *N*-hydroxysuccinimide esters while the photoreactive arylazide groups were inactivated by photolysis. The effects of lactotransferrin derivatization by SASD and sulfo-SANPAH on the receptor recognition were assayed, and the derivatized lysine residues were identified. Molecular modeling was used for setting FITC, SASD, and sulfo-SANPAH molecules at the surface of the whole protein so that they could interact with the receptor-binding domain. The obtained results, as well as the comparison of the primary and tertiary structures of human lactotransferrin and serotransferrin, allowed the definition of the areas of human lactotransferrin that interact with the lymphocyte receptor.

MATERIALS AND METHODS

Cell Preparation. Human peripheral blood mononuclear cells were obtained by Lymphoprep (Nycomed Pharma AS, Oslo, Norway) separation of heparinized venous blood samples drawn from healthy volunteers. The cells were prepared and stimulated with phytohemagglutinin (Industrie Biologique Française, Villeneuve la Garenne, France) as previously described (Mazurier et al., 1989).

Isolation and Purification of the Iron-Binding Tryptic Fragments from Human Lactotransferrin. Lactotransferrin was prepared from pooled human milk and purified according to Spik et al. (1982). Iron saturation of lactotransferrin was carried out according to Mazurier et al. (1983). The iron-binding N-tryptic and C-tryptic fragments were prepared from human lactotransferrin, as previously described by Legrand et al. (1984) and modified as follows: human lactotransferrin (1 g) was incubated in 100 mL of 0.1 M Tris-HCl, pH 8.2, with 50 mL of immobilized trypsin–Sepharose 4B (1 mg of trypsin/mL of gel) for 24 h at 37 °C under gentle stirring. The gel was removed by centrifugation followed by filtration through 0.45-μm filters, and the protein solution was concentrated to 50 mL using a Centricon-30 ultrafiltration unit (Amicon, Beverly, MA). Finally, 10 μL of diisofluorophosphate (Sigma) were added to the protein solution, and complete inhibition of residual tryptic activity was checked by incubating aliquots of the protein solution with Azocoll beads (Calbiochem, La Jolla, CA) for 1 h at 37 °C. The described procedure prevents residual tryptic activity that could spontaneously degrade the isolated N-terminal tryptic fragment into the N2-glycopeptide (Legrand et al., 1984, 1986). The purification of the N- and C-terminal tryptic fragments and the preparation and purification of the N2-glycopeptide were then performed as previously reported (Legrand et al., 1984, 1986).

Isolation and Purification of the 6-kDa N-Terminal Peptide (Residues 4–52) from Human Lactotransferrin. The fractions containing the N-terminal tryptic fragment from human lactotransferrin (120 mg), obtained after chromatography on a Bio-Gel P-60 column (Legrand et al., 1984), were pooled, dialyzed against water for 6 h, and concentrated under vacuum to a final volume of 50 mL. The pH of the solution was adjusted to 4.0 by adding 2 M Tris solution, and the solution was incubated with 3 mg of *Staphylococcus aureus* V8 protease (EC 3.4.21.19) (Boehringer Mannheim, FRG) (Houmard & Drapeau, 1972) for 20 h at 37 °C with gentle stirring. The hydrolysate was centrifuged at 5000*g* for eliminating the precipitate. An aliquot of the endoproteinase digest was separated on 8–25% gradient slab SDS gels using the Pharmacia Phast-System (Uppsala, Sweden). The peptide-containing solution was vacuum-concentrated to 10 mL, filtered through a 0.22-μm filter, and chromatographed as described in the legend to Figure 1. Fractions were collected, lyophilized, and then analyzed by SDS–PAGE on a 16.5% (w/v) acrylamide gel in 6 M urea according to Schägger and von Jagow (1987). The molecular mass markers used were CNBr fragments of myoglobin from horse heart and from sperm whale (PMW kit from Pharmacia).

Derivatization of Human Lactotransferrin with FITC. Human lactotransferrin used for binding and competitive binding experiments was labeled with FITC from Sigma (isomer I) as previously described (Legrand et al., 1991). The ratio of fluorescein to protein was estimated by measuring the absorbance at 495 nm and 280 nm, according to Jobbagy and Kiraly (1966).

Derivatization of Human Lactotransferrin with Iodinated SASD. All experiments, prior to the photolytic reaction, were performed in a dark room under red lighting. A total of 400 μg of SASD (Pierce Chemical Co.) was first dissolved in 100 μL of dimethyl sulfoxide and then mixed with 400 μL of 0.1 M sodium phosphate, pH 7.4. The SASD solution was then transferred into a glass tube coated with 100 μg of Iodo-Gen and was incubated while stirring with 1 mCi of ^{125}I (Amersham, U.K.), at 4 °C for 5 min. Radiolabeling was stopped by adding 200 μL of KI (40 mg/mL) to the solution for 30 min, and purification of ^{125}I-labeled SASD was performed on a Bio-Gel P-2 column (1.1 cm × 50 cm) in 0.1 M sodium phosphate, pH 7.4. ^{125}I-Labeled SASD fractions were collected, and the concentration of ^{125}I-labeled SASD was determined spectrophotometrically at 271 nm by using a molar extinction coefficient of 2.57×10^{-4} M^{-1} cm^{-1} (Shephard et al., 1988). Human lactotransferrin was dissolved in 0.5 M sodium borate, pH 9.0, at a concentration of 12.5 nM and was incubated for 1 h at room temperature with freshly-prepared ^{125}I-labeled SASD, at concentrations ranging from 12.5 nM to 1.25 μM (1–100-fold molar excess). The solution was then concentrated by ultrafiltration on a Millipore Centricon-30 concentrator to give a volume of 500 μL and passed through a Sephadex PD-10 column in PBS to remove unreacted ^{125}I-labeled SASD. The SASD-derivatized lactotransferrin solution was photolyzed at 4 °C for 10 min using a Minuvis Desaga (Heidelberg, Germany) UV lamp at a distance of 10 cm to inactive the azide group. The ^{125}I-labeled SASD to protein ratio was calculated. The SASD-derivatized human lactotransferrin used for competitive binding experiments was produced as per the same protocol described above, but the iodination step of SASD was performed by using KI instead of ^{125}I.

Derivatization of Human Lactotransferrin with Sulfo-SANPAH. All experiments were performed in a dark room under red lighting. Human lactotransferrin was dissolved in 0.1 M sodium carbonate, pH 9.2, at the concentration of 12.5 nM and was incubated for 2 h at room temperature with sulfo-SANPAH (Pierce Chemical Co.), at concentrations ranging from 12.5 to 250 μM (1–20 molar excess). The solution was then passed through a Sephadex PD-10 column in PBS, in order to remove unreacted sulfo-SANPAH. The molar ratio of SANPAH/protein was determined by measuring the absorption at 488 nm of the derivatized protein before photoactivation (Lomant & Fairbanks, 1976). The

protein concentration was estimated by the BCA protein assay reagent from Pierce Chemical Co. The sulfo-SANPAH-derivatized lactotransferrin solution was photolyzed at 4 °C for 10 min using the Minuvis Desaga UV lamp at a distance of 10 cm for inactivating the azide group and then stored at 4 °C before use.

Radiolabeling of Lactotransferrin Samples. Pure lactotransferrin and fluorescein-, SASD-, or sulfo-SANPAH-derivatized lactotransferrin samples were labeled with ^{125}I using Iodo-Gen, as previously described (Mazurier et al., 1989), and the excess reagent was removed by gel filtration through a Sephadex G-25 column equilibrated with 0.15 M NaCl/10 mM sodium phosphate, pH 7.3.

Binding Assays and Competitive Binding Assays. The binding assays of native and fluorescein-, SASD-, or sulfo-SANPAH-derivatized lactotransferrin samples were performed in siliconized polypropylene tubes. Before use, phytohemagglutinin-stimulated lymphocytes were washed twice with RPMI 1640 without fetal calf serum, incubated for 2 h, washed again, and finally resuspended in RPMI 1640, 25 mM Hepes, pH 7.3, in order to obtain a cell density of about 5×10^5/mL. ^{125}I-Labeled proteins were added at concentrations ranging from 0 to 0.15 μM. Nonspecific binding was measured in the presence of a 100-fold molar excess of unlabeled lactotransferrin. In order to prevent the nonspecific binding of human lactotransferrin to cells or to plastic, all binding experiments were performed in the presence of 1% (v/v) human serotransferrin (Behring, Marburg, FRG), since serotransferrin has no inhibitory effect on the binding of lactotransferrin (Mazurier et al., 1989). After a 1-h incubation at 4 °C in the presence of 5% CO_2/95% air, 0.2 mL of the cell suspension was centrifuged (2000*g*, 10 min) and washed twice with 0.15 M NaCl/10 mM sodium phosphate, pH 7.3. At the end of washing, the cell pellet was resuspended in 0.2 mL of the same buffer, and the radioactivity of the suspension was measured. The inhibition of ^{125}I-labeled lactotransferrin binding by fluorescein-, SASD-, and sulfo-SANPAH-derivatized lactotransferrin samples or peptides isolated from the N-tryptic fragment was assayed in the presence of a 5–100-fold molar excess of unlabeled or derivatized proteins or peptides. Binding assays were carried out as described above.

Proteolysis of SASD- and Sulfo-SANPAH-Derivatized Human Lactotransferrins and Peptide Purification. A total of 1 μmol of radiolabeled–SASD-derivatized lactotransferrin (equimolar ratio SASD/protein) was passed through a Sephadex G25-fine column (2 cm × 20 cm) equilibrated with 0.1 M glycine/0.1 M hydrochloride, pH 2.4. Pepsin from Sigma (enzyme to protein ratio 1/20 w/w) was added to the solution, and incubation was performed for 16 h at 37 °C under magnetic stirring. After the pH was raised to 8.0 with 1 M NaOH, the hydrolysate was concentrated under vacuum and was chromatographed on a Bio-Gel P-60 column (2.5 cm × 150 cm). The fractions containing radiolabeled peptide were concentrated under vacuum and filtered prior to RP-HPLC (Spectra-Physics Model 8700 liquid chromatograph equipped with a Spectra-Physics Model 8450 variable wavelength UV/visible detector connected to a Model 4270 computing integrator). ^{125}I-SASD-labeled peptides were purified by RP-HPLC on a 10-μm Chromatem C14 column (Touzart & Matignon, Vitry sur Seine, France), as described in the legend to Figure 4. The fractions containing the ^{125}I-SASD-labeled peptides were detected by counting with a Compugamma LKB-Wallac (Turku, Finland) and lyophilized. A total of 1 μmol of sulfo-SANPAH-derivatized lactotransferrin (molar ratio of SASD/protein of 1) was passed through a Sephadex G25-fine column (2 cm × 20 cm) equilibrated with 0.1 M Tris-HCl, 8 M urea, 0.2% (w/v) EDTA, pH 8.2. The disulfide bridges were reduced overnight by 2-mercaptoethanol and alkylated with iodoacetamide as described by Cresfield et al. (1963). The reduced and alkylated sulfo-SANPAH-derivatized protein was passed through a Bio-Gel P-30 column in 10% (v/v) acetic acid and dried under vacuum. The protein was dissolved in 10 mL of 0.1 M Tris-HCl, pH 8.2, and was incubated for 16 h at 37 °C while being stirred with 2.5 mg of trypsin (Sigma). The tryptic hydrolysis was stopped by lowering the pH to 5.0 with 1 M HCl. The protein solution was concentrated under vacuum and filtered prior to RP-HPLC. Peptides generated from proteolytic digests were purified by RP-HPLC on a 10-μm Waters μBondapack C14 column (Waters/Millipore Corp.), as described in the legend to Figure 5.

Amino Acid Composition and Peptide Sequence Analysis. Amino acid analysis was performed with a Spectra-Physics Model 8100 liquid chromatograph equipped with a Knauer variable wavelength detector (Bad Homburg, FRG) connected to a Model 4290 Spectra-Physics computing integrator. Peptides were sequenced by Edman degradation with an automatic gas-phase sequencer (Model 470 A; Applied Biosystems, Foster City, CA), and phenylthiohydantoin (PTH) derivatives of amino acids released during each cleavage cycle were identified and quantified by HPLC using an Applied Biosystems 120 A on-line PTH analyzer.

Molecular Modeling Studies. The crystallographic data of diferric human lactotransferrin were kindly provided by Dr. Baker (Massey University, New Zealand). All calculations were carried out on an Evans & Sutherland PS 350 graphic station and a Vax 6320 host computer using the Sybyl 5.3 molecular modeling package (Sybyl, 1988). The structures of FITC, SASD, and sulfo-SANPAH were built from fragments extracted from the Cambridge Crystallographic Databank (Cambridge, U.K.), and their geometry was minimized by using the Sybyl Search program (Mayer et al., 1987). The crystallographic data of rabbit serotransferrin used for comparing the three-dimensional structures of human lactotransferrin and rabbit serotransferrin were kindly provided by Dr. Lindley (London University, Great Britain).

RESULTS

Isolation and Characterization of a Receptor-Binding Peptide from Human Lactotransferrin. The 30-kDa N-terminal tryptic fragment from human lactotransferrin, which was found to specifically bind to the lymphocyte receptor (Rochard et al., 1989), was hydrolyzed by the *S. aureus* V8 protease. Figure 1 shows the RP-HPLC profile of the purification of the 30-kDa N-terminal tryptic fragment digest. The 11 fractions obtained were numbered from I to XI. Analysis of these peptide-containing fractions by 16% SDS–PAGE indicates that fractions I–IV contained unstained material, presumably salts or short peptides or amino acids. Fractions VI, VIII, IX, and X contained mixtures of peptides with molecular masses ranging from 30 kDa (undigested N-tryptic fragment) to 1 kDa with major bands between 14 and 3 kDa while fractions V, VII, and IX contained bands with molecular masses close to 6 kDa (not shown). The inhibition of ^{125}I-labeled human lactotransferrin binding to PHA-activated human lymphocytes was assayed in the presence of a 100-fold molar excess of fractions III–XI, the 30-kDa N-tryptic fragment, the 50-kDa C-tryptic fragment, and the 20-kDa N2-glycopeptide. As fractions VI, VIII, IX, and X contain mixtures of peptides, the corresponding competitor solutions

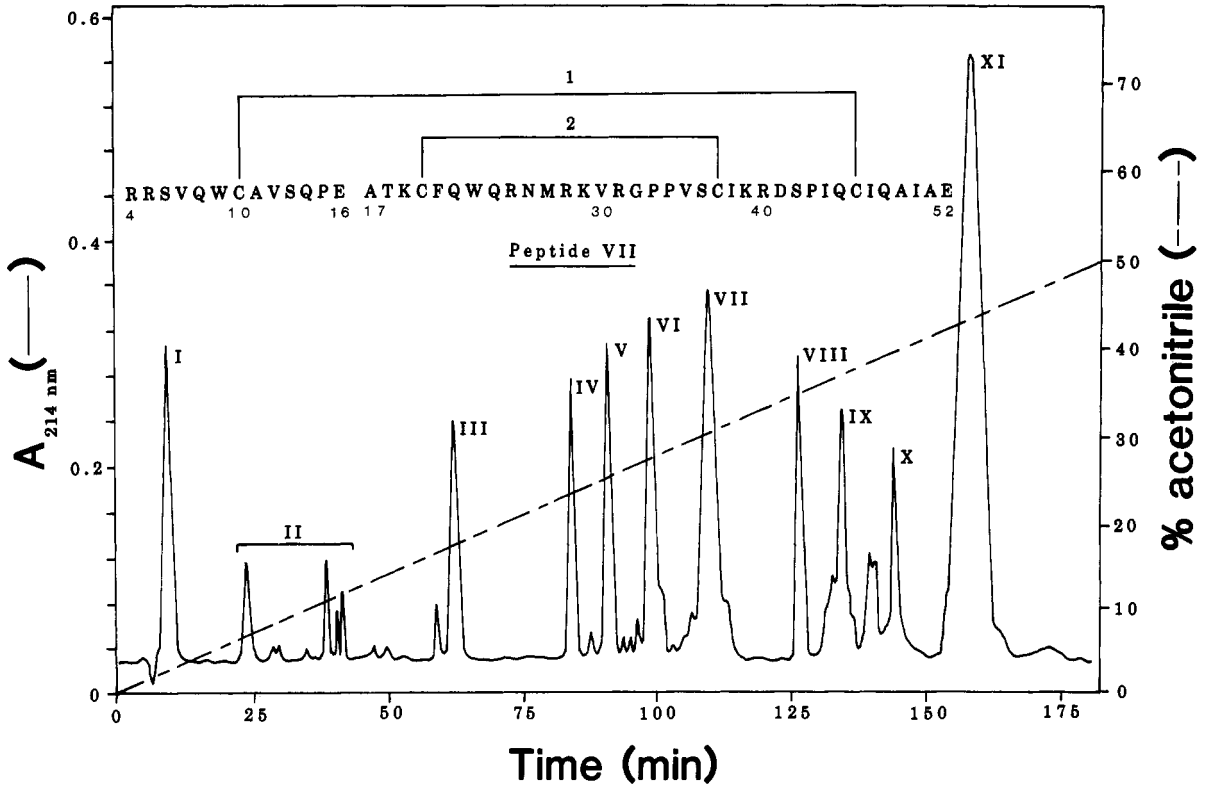

FIGURE 1: RP-HPLC of the *S. aureus* V8 protease digest of the 30-kDa N-tryptic fragment from human lactotransferrin. One-milliliter aliquots were injected onto a reverse-phase Du Pont Instruments Zorbax ODS column (25 cm × 0.94 cm internal diameter). Elution was performed with a 0.1% (v/v) trifluoroacetic acid-containing water/acetonitrile gradient, from 0 to 50% acetonitrile for 180 min (flow rate of 1.5 mL/min). The numbers indicate the different fractions collected. The sequence represents the primary structure of the 6-kDa peptide contained in fraction VII. This sequence is deduced from the N-terminal sequencing of the peptide and from the amino acid composition. The sequence numbering is that of Powell and Ogden (1990) and Rey et al. (1990).

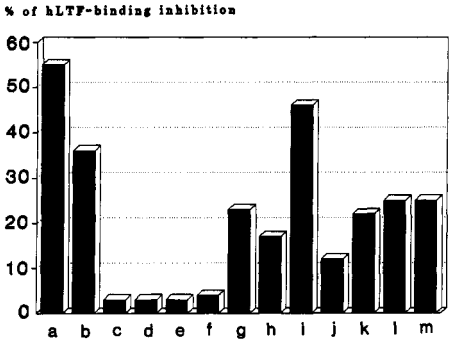

FIGURE 2: Competitive binding assays of radiolabeled human lactotransferrin (hLTF) with a 100-fold molar excess of different polypeptides: (a) human lactotransferrin; (b) 30-kDa N-tryptic fragment, (c) 50-kDa C-tryptic fragment, (d) 20-kDa N2-glycopeptide from human lactotransferrin (Legrand et al., 1984), (e–m) fractions III–XI obtained after RP-HPLC of the *S. aureus* V8 protease digest of the 30-kDa N-tryptic fragment from human lactotransferrin.

were prepared assuming a mean molecular mass of 6 kDa for the peptides. As shown in Figure 2 and as reported elsewhere (Rochard et al., 1989), the binding of radiolabeled lactotransferrin to mitogen-stimulated human lymphocytes was 55% and 33% inhibited by 100-fold molar excesses of unlabeled lactotransferrin and 30-kDa N-tryptic fragment, respectively, whereas no more than 5% inhibition was noted for both 20-kDa N2-glycopeptide and 50-kDa C-tryptic fragment. A strong inhibition of radiolabeled lactotransferrin (45%) was obtained in the presence of fraction VII which contained a single 6-kDa peptide. A lower percentage of inhibition (16–24%) was obtained for fractions V–VII and IX–XI (Figure 2), which can be explained by the presence in these fractions of peptides related to the above-mentioned 6-kDa peptide (not shown). The inhibition rate obtained by fraction VII rose from 45% to 55–60% when a 100-fold molar excess of unlabeled lactotransferrin was added, demonstrating that lactotransferrin and 6-kDa peptide bound to the same specific lymphocyte–binding sites (data not shown).

Edman degradation of the peptide contained in fraction VII yielded the two N-terminal sequences R-R-S-V-Q and A-T-K-C-F in the same molar ratio. Taking into account the two N-terminal sequences, the amino acid composition analysis of fraction VII (not shown) and the potential selective cleavage sites of lactotransferrin by V8 protease, the peptide was located on the human lactotransferrin sequence from residue 4 to 16 and from residue 17 to 52 (Figure 1). The presence of the disulfide bridge 1 (Cys-10 and Cys-46) allows the two peptide chains to be covalently associated. The splitting of the first three amino acid residues (one Gly and two Arg) of human lactotransferrin occurred during tryptic proteolysis of the protein, as previously reported (Legrand et al., 1984, 1986).

Derivatization of Human Lactotransferrin with ^{125}I-Labeled SASD and Sulfo-SANPAH. We have described in a previous report that a 1.2-fold molar excess of FITC is necessary for covalent attachment of one molecule of fluorescein per molecule of lactotransferrin (Legrand et al., 1991). When increasing concentrations of sulfo-SANPAH were incubated with human lactotransferrin, a 1.7-fold molar excess of reagent was necessary for covalent attachment of one molecule of sulfo-SANPAH per molecule of protein. In the case of ^{125}I-labeled SASD derivatization of lactotransferrin, a large excess (25-fold molar excess) was required to get an equimolar ratio of ^{125}I-labeled SASD to protein. This may be related to partial inactivation of SASD during labeling steps.

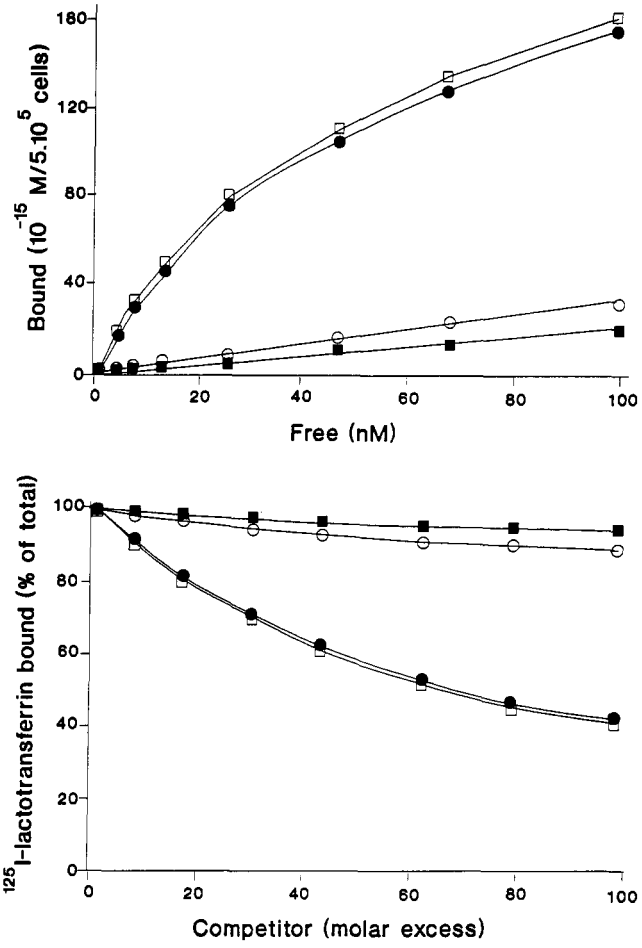

FIGURE 3: Binding assays (A, top) and competitive binding assays (B, bottom) of nonderivatized (□), FITC-derivatized (■), iodinated-SASD-derivatized (○), or sulfo-SANPAH-derivatized (●) diferric human lactotransferrin. The experimental conditions are indicated in the text. All derivatized lactotransferrins used in the experiments possess 1 mol of reagent/mol of protein.

Binding Assays and Competitive Binding Assays of Nonderivatized and Derivatized Lactotransferrins. The effect of iodinated-SASD and sulfo-SANPAH conjugation to ^{125}I-labeled human lactotransferrin (ratio 1/1) on the binding of the protein to phytohemagglutinin-stimulated human lymphocytes is shown in Figure 3A. As previously reported (Mazurier et al., 1989), the radiolabeled nonderivatized lactotransferrin exhibited a concentration-dependent and saturable binding curve to mitogen-activated lymphocytes with a K_d of 43 nM and a number of binding sites of 300 000. A very similar binding curve was obtained with the sulfo-SANPAH-derivatized lactotransferrin, while no more than 5–10% binding was observed for both FITC-, used as control, and iodinated-SASD-derivatized lactotransferrins. The results we obtained are in total agreement with the competitive binding experiments (Figure 3B) which have shown that the binding of radiolabeled human lactotransferrin was 55% inhibited by either nonderivatized lactotransferrin or sulfo-SANPAH-derivatived lactotransferrin whereas no binding inhibition was observed for both FITC- and iodinated-SASD-derivatized lactotransferrins.

Isolation of ^{125}I-SASD-Labeled and Sulfo-SANPAH-Labeled Peptides from Human Lactotransferrin. Since derivatization of human lactotransferrin with reagents such as iodinated SASD and sulfo-SANPAH can modify the behavior of lactotransferrin binding to mitogen-stimulated human lymphocytes, it was of interest to identify the derivatized amino acids by isolating the labeled peptides from lactotransferrin. A similar approach has previously (Legrand et al., 1991) been used for the isolation of peptides derivatized with ^{125}I-labeled SASD and sulfo-SANPAH. Human lactotransferrin was derivatized with ^{125}I-radiolabeled SASD (ratio of SASD to protein 1/1), and the derivatized protein was submitted to proteolytic digestion with pepsin. Pepsin was used because of its ability to efficiently cleave the N-terminal moiety of human lactotransferrin and liberate the intact C-terminal lobe as a 40-kDa C-peptic fragment (Line et al., 1976). The use of other proteases requires previous reduction and alkylation steps which are not suitable with SASD since this bifunctional reagent includes a disulfide bridge. Chromatography of the lactotransferrin peptic hydrolysate was first performed on a Bio-Gel P-60 column in order to separate short peptides from both undigested lactotransferrin and 40-kDa C-terminal peptic fragment (not shown). All radioactive material was found in the peptide-containing fractions, indicating that the radiolabeled SASD was not linked to the C-terminal lobe of human lactotransferrin. RP-HPLC of the radioactive SASD-labeled peptides provided two major radioactive fractions S-I and S-II (Figure 4) containing 20 ± 5% and 60 ± 5% of total radioactivity, respectively. Both fractions S-I and S-II were rechromatographed by RP-HPLC on the same column from 0 to 20% acetonitrile for 105 min and from 0 to 30% acetonitrile for 110 min, respectively (not shown). Chromatography of fraction S-I revealed the presence of a single major radioactive fraction called S-I_1 and corresponding to 15 ± 5% of total radioactivity. Fraction S-II gave two major radioactive fractions called S-II_1 and S-II_2, corresponding to 10 ± 5% and 45 ± 5% of total radioactivity, respectively. Human lactotransferrin derivatized with sulfo-SANPAH in the ratio of 1/1 was reduced, alkylated, and then submitted to tryptic digestion. RP-HPLC of the tryptic digest on a C-14 column led to the recovery of a single major fraction (fraction P-I) containing sulfo-SANPAH-derivatized material, as detected by measuring the absorbance at 488 nm (Figure 5).

Localization of the SASD- and Sulfo-SANPAH-Derivatized Amino Acids on the Human Lactotransferrin Sequence. The sequences of the SASD- and sulfo-SANPAH-derivatized peptides are indicated in Figures 4 and 5, and the localization of modified lysine residues on human lactotransferrin molecule is shown in Figure 6. As peptide S-II_2 contained 45% of total SASD, the main SASD-derivatized amino acid was Lys-74. Lys-264 (peptide S-I_1) and Lys-278 (peptide S-II_1) bound 15% and 10% of total SASD, respectively. The sequence of the unique SANPAH-derivatized peptide was F-G-*-D-K, so indicating that SANPAH was exclusively bound to Lys-281. These results, in conjunction with the results previously obtained with FITC derivatization of human lactotransferrin (Legrand et al., 1991) and summarized in Figure 6, show that, in the N-terminal domain I, up to five lysine residues (residues 74, 264, 278, 281, and 283) are potential targets for either FITC, SASD, or sulfo-SANPAH molecules. Among these five lysine residues, Lys-74 is the preferential binding site for SASD, Lys-264 is for FITC, and Lys-281 is for sulfo-SANPAH.

Molecular Modeling Studies. The above-mentioned experimental results explain the localization of the derivatized lysine residues at close positions on the peptide chain of human lactotransferrin, but they are unable to explain why the sulfo-SANPAH-derivatized lactotransferrin can bind to the cell receptor while FITC- or SASD-derivatized proteins cannot. Moreover, it is not possible to establish connections between the derivatization experiments and the isolation of a receptor-binding peptide containing residues 4–52. In these perspec-

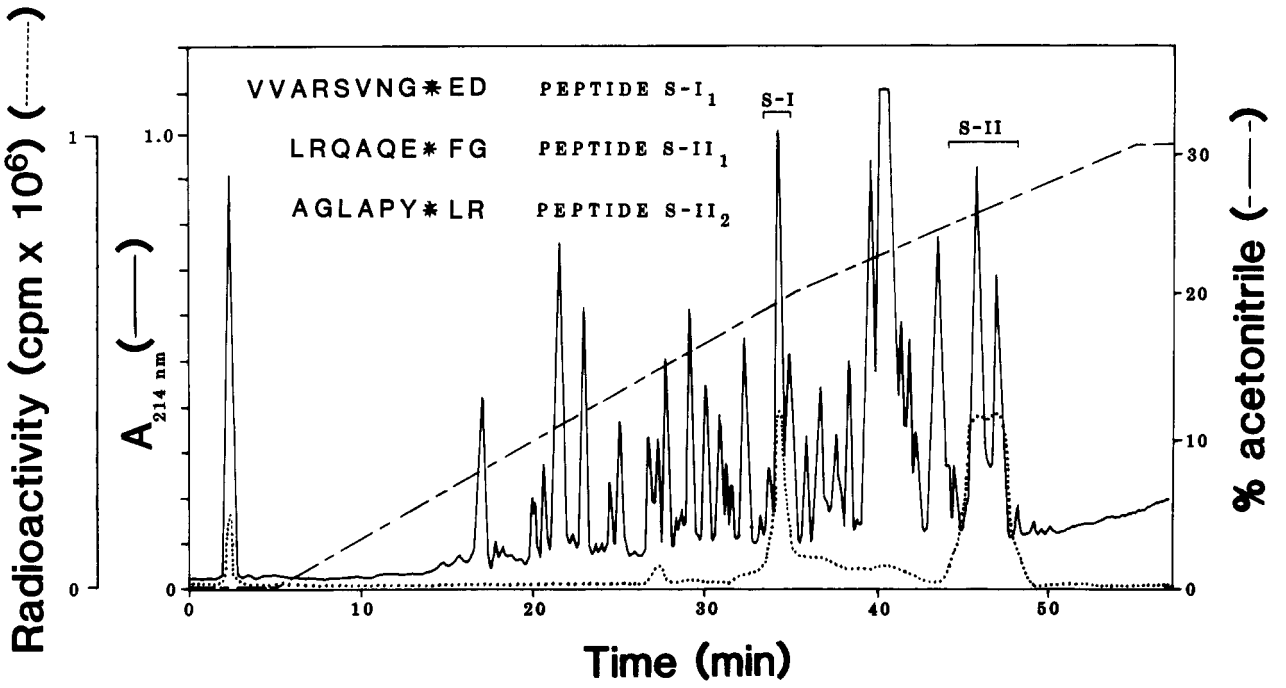

FIGURE 4: RP-HPLC fractionation of the ^{125}I-SASD-derivatized peptides isolated from human lactotransferrin. The pepsic digest of ^{125}I-SASD-derivatized lactotransferrin was concentrated under vacuum, filtered, and injected onto a 10-μm Chromatem C14 column (25 cm × 0.46 cm internal diameter) with a 0.1% (v/v) trifluoroacetic acid-containing water/acetonitrile gradients from 0 to 30% acetonitrile for 55 min. The ^{125}I-SASD-containing fractions S-I and S-II were rechromatographed on the same column from 0 to 20% acetonitrile for 105 min and from 0 to 30% acetonitrile for 110 min to yield radioactive fractions S-I_1 and S-II_1, S-II_2, respectively. The amino acid sequences of fractions S-I_1, S-II_1, and S-II_2 are represented. An asterisk (*) symbolizes the derivatized amino acid residue found as an unusual product after Edman degradation and coelution with phenylthiohydantoin-Trp.

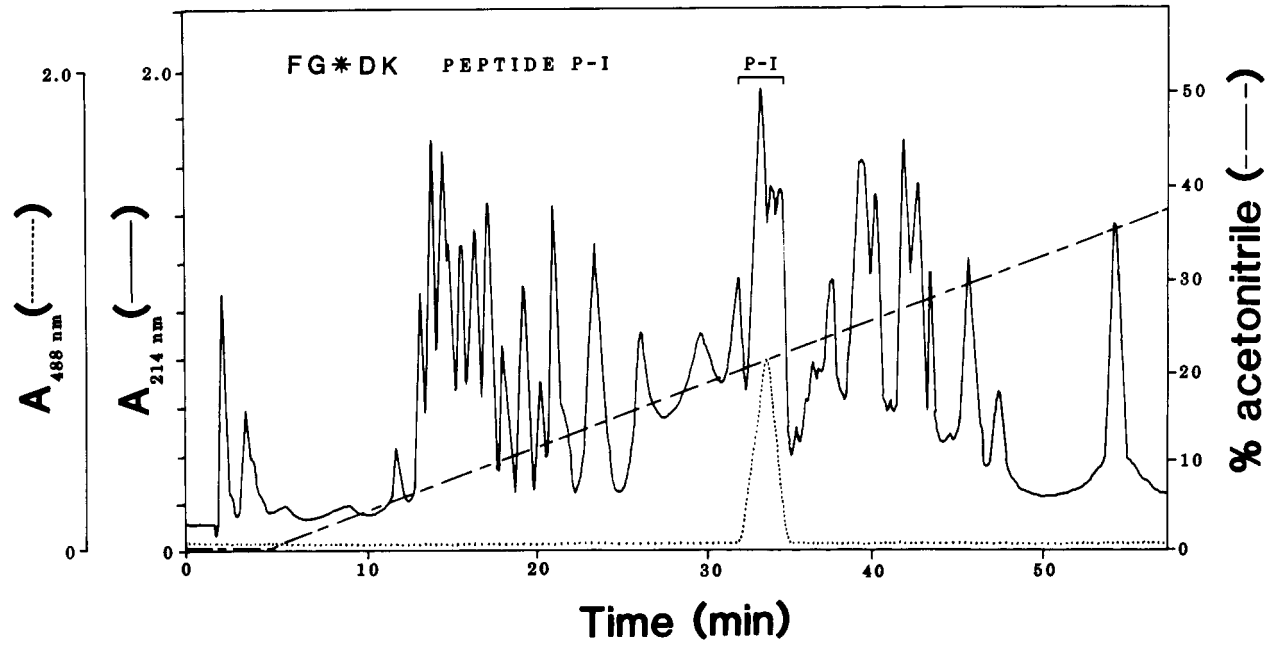

FIGURE 5: RP-HPLC fractionation of the sulfo-SANPAH-derivatized peptides isolated from human lactotransferrin. The trypic digest of the sulfo-SANPAH-derivatized lactotransferrin was concentrated under vacuum, filtered, and injected onto a 10-μm Waters μ-Bondapack C14 column (25 cm × 0.46 cm internal diameter) with a 0.1% (v/v) trifluoroacetic acid-containing water/acetonitrile gradient from 0 to 40% acetonitrile for 60 min. The fraction containing the sulfo-SANPAH-labeled peptide (fraction P-I) was detected at 488 nm. The amino acid sequence of fraction P-I is represented in the chromatogram. The asterisk (*) symbolizes the derivatized amino acid residue found as an unusual product after Edman degradation and coelution with phenylthiohydantoin-Trp.

tives, molecular modeling allows three-dimensional insights of where and how FITC, SASD, and sulfo-SANPAH molecules fit into human lactotransferrin. SASD, FITC, and sulfo-SANPAH, at the locations found (Lys-74, Lys-264, and Lys-281, respectively), were set in order that they could interact with the receptor-binding domain from residues 4–52. Molecular modeling using molecular mechanics has shown that the position of FITC, SASD, and sulfo-SANPAH at the protein surface corresponds to that illustrated in Figure 7A (personal data to be published). As we can see, though FITC and SASD molecules are 190 amino acid residues away from each other on the polypeptide chain (from Lys-74 to Lys-263), they both cover a part of the receptor-binding peptide from residues 4 to 52 (indicated in white in Figure 7A). On the contrary, the sulfo-SANPAH molecule bound on Lys-281, which does not inhibit the binding of lactotransferrin on

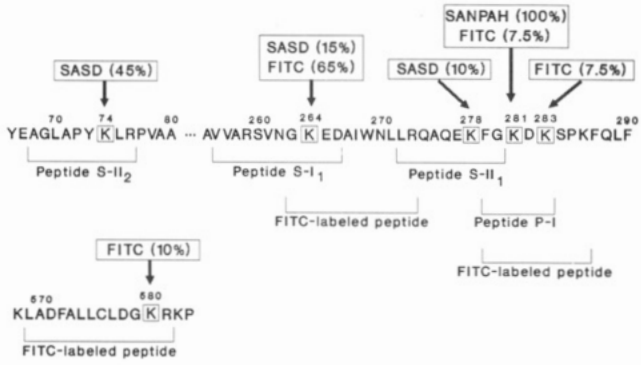

FIGURE 6: Localization of the FITC-, ^{125}I-labeled-SASD-, and sulfo-SANPAH-derivatized lysine residues on human lactotransferrin. Boxed amino acids on the human lactotransferrin peptide chain correspond to the derivatized lysine residues. Brackets delimit the sequences of the derivatized peptides described in Figures 4 and 5. The FITC-derivatized lysine residues have been previously reported by Legrand et al. (1991). The relative binding percentages of FITC, ^{125}I-labeled-SASD, and sulfo-SANPAH for each derivatized lysine residue are indicated in boxes.

the activated lymphocyte receptor, is located at the opposite side of domain I.

As shown in Figure 7B, the peptide chain from residues 4 to 52 possesses a β-(residues 6–11) α-(residues 16–28) β-(residues 34–39) α (residues 46–52) folding. Taking into account the accessibility of the amino acid side chains to solvent, three main regions may interact with a cell receptor: the first one includes residues 4–6 and a loop from residues 28 to 34; the second one comprises the solvent-accessible amino acid residues of a α-helix structure (residues 16–28), and the third one is composed of residues 38–45. FITC could easily mask residues 4–6 and 28–34, but its masking effect for the two other solvent-accessible regions is unlikely. In the same way, only a part of the loop from residues 38 to 45 could be masked by the SASD molecule.

Comparisons of Transferrin Structures. The primary structure of human lactotransferrin from residues 4 to 52 was compared to the homologous sequences of the C-terminal lobe of the protein (residues 343–387) and the N- and C-terminal lobes of human serotransferrin (Figure 8A). As human serotransferrin (Mazurier et al., 1989) and the C-terminal lobe of human lactotransferrin (Rochard et al., 1989) were unable to bind to the lactotransferrin lymphocyte receptor, we postulated that the receptor-binding site should correspond to specific amino acid sequences between residues 4 and 52 of human lactotransferrin. The compared amino acid sequences shown in Figure 8A demonstrate that, except for residues 30, 32, 33, 39, and 42, residues 4-6, 28-34 and 38-45 are specific to the N-terminal moiety of human lactotransferrin. Moreover, the region from residues 29 to 32, which is found to be involved in the receptor-binding site, does not exist in the corresponding areas of the C-terminal lobes of human lactotransferrin and serotransferrin and is three amino acids longer in the N-terminal lobe of human serotransferrin (Figure 8A). Figure 8B shows the superposed 3D structures of residues 4–52 of human lactotransferrin and the corresponding regions of the C-terminal lobe of the protein and the N-terminal lobe of rabbit serotransferrin. The main conformational differences between the four structures concern the solvent-accessible area including residues 4–6 and the loop from residues 28 to 34.

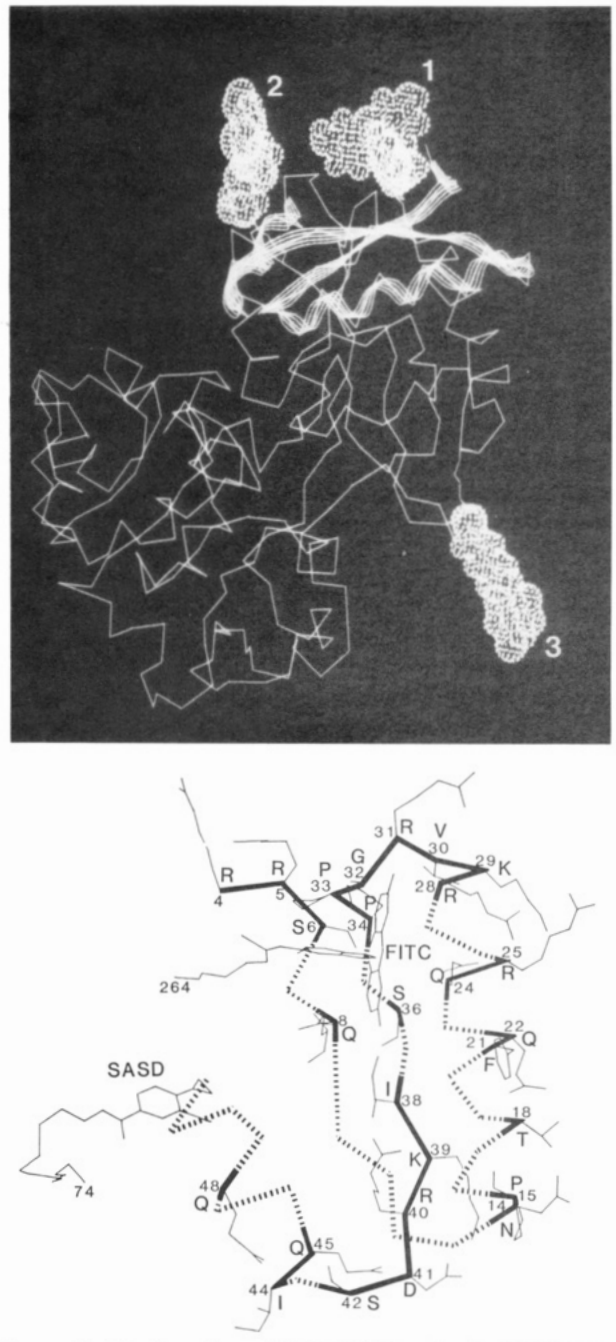

FIGURE 7: Masking effect of FITC, SASD, and sulfo-SANPAH on the human lactotransferrin polypeptide chain. (A, top) Backbone α-carbon tracing of the N-terminal lobe of human lactotransferrin (residues 1–330) showing the folding of (ribbon) the receptor-binding peptide from residue 4 to 52 and the relative positions of (1) FITC, (2) iodinated-SASD, and (3) sulfo-SANPAH molecules, represented with van der Waals surfaces. The docking studies of FITC, iodinated-SASD, and sulfo-SANPAH on the protein are described in the text. (B, bottom) Backbone α-carbon tracing of the receptor-binding peptide from residues 4 to 52 (thick lines) with the side chains of the solvent-accessible amino acid residues (thin lines). The backbone α-carbon tracing corresponding to solvent-inaccessible amino acid residues is indicated with thick dotted lines. The positions of FITC and SASD molecules have been represented. Numbers and one-letter amino acid codes correspond to the solvent-accessible amino acid residues.

DISCUSSION

The aim of the present study was to continue in the characterization of the part of human lactotransferrin which interacts with the human PHA-activated lymphocyte receptor. Previous studies using binding experiments with large lactotransferrin proteolytic fragments suggested that the N-terminal lobe of the protein could interact with the lymphocyte receptor (Rochard et al., 1989). More recently (Legrand et al., 1991), we showed that the covalent binding of FITC on

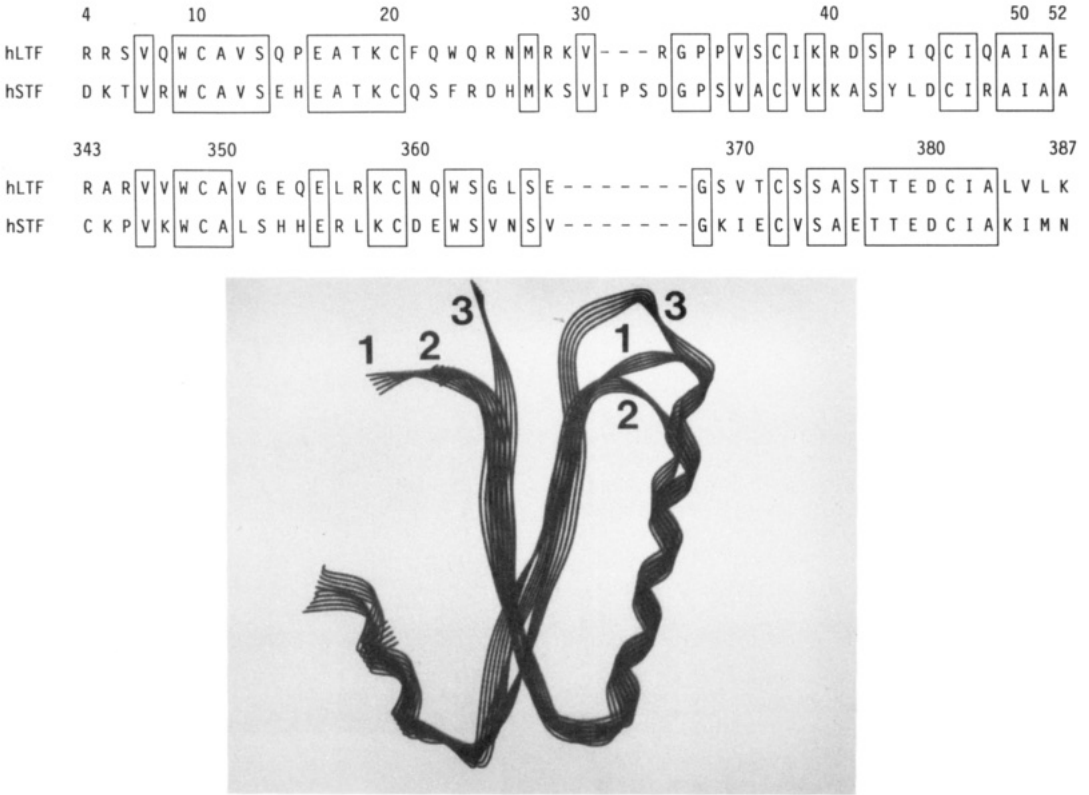

FIGURE 8: Compared structures of residues 4–52 of human lactotransferrin with the homologous sequences of the C-terminal lobe of this protein and the N- and C-terminal lobes of human serotransferrin. (A, top) Alignment of sequences 4–52 and 343–387 of human lactotransferrin (hLTF) with the homologous sequences of the N- and C-terminal moieties of human serotransferrin (hSTF) (Mac Gillivray et al., 1983). (B, bottom) Superposed foldings of sequences (1) 4–52 and (2) 343–387 of human lactotransferrin and (3) the corresponding region of the N-terminal lobe of human serotransferrin (Bailey et al., 1988).

the N-terminal domain I of human lactotransferrin (residues 1–91 and 251–319), essentially on Lys-264, prevented the binding of the protein to its receptor. On the basis of these results, one could conclude that Lys-264 and/or the neighboring of Lys-264 were masked by fluorescein and thereby prevented its binding to the receptor. Thus, it was first postulated that the receptor-interacting area was included in the C-terminal half of the N-terminal domain I, from residues 251 to 319.

The present results obtained by derivatizing human lactotransferrin with SASD and sulfo-SANPAH seem to contradict the above hypothesis. Indeed, we show that SASD mainly conjugated to Lys-74 inhibits the binding of lactotransferrin to the lymphocyte receptor while sulfo-SANPAH conjugated to Lys-281, close to Lys-264, does not. Moreover, the isolation of a 6-kDa peptide able to inhibit the specific binding of human lactotransferrin to the lymphocyte receptor brings direct evidence that the amino acid residues involved in receptor binding are included between residues 4 and 52.

In fact, as shown by molecular modeling in Figure 7, sulfo-SANPAH conjugated to Lys-281 is unable to prevent molecular interactions between residues 4–52 and the lymphocyte receptor. On the contrary, FITC and SASD molecules could mask two among the three main solvent-accessible areas from residues 4 to 52: the area including residues 4–6 and residues 28–34 and the area from residues 38–45. The solvent-accessible amino acid residues of the α-helix from residues 16 to 28 are unlikely to be hidden by either FITC or SASD molecules (Figure 8). Moreover, it is worth noting that FITC could only mask the solvent-accessible area comprising residues 4–6 and 28–34, while SASD could only mask part of the loop 38–45 (Figure 8). Since lactotransferrin undergoes significant receptor-binding inhibition when derivatized by either FITC or SASD, the two above-mentioned regions could be involved in receptor binding.

Anderson et al. (1989) have already focused attention on the basic region from residues 1 to 50 of human lactotransferrin (6 Arg and 3 Lys residues are present in the polypeptide chain), as a possible recognition site for a cellular receptor. Our study definitively demonstrates that the homologous region of human lactotransferrin acts as a receptor-binding site.

Moreover, the involvement of the two loop-containing regions from residues 28 to 34 and from residues 38 to 45 in receptor binding is not quite unexpected. A synthesized tetrapeptide K-R-D-S, which corresponds to residues 39-42 of human lactotransferrin, was indeed recently found to interact with binding sites at the surface of ADP-induced human platelets (Raha et al., 1988; Mazoyer et al., 1990; Drouet et al., 1990). The tetrapeptide K-R-D-S is a structural analog of the sequence R-G-D-S encountered in human fibrinogen and recognized by the glycoprotein GPIIb–IIIa complex. However, the mechanism of action of the tetrapeptide K-R-D-S remains unclear, and some results on thrombosis inhibition, associated with the in vitro effects, suggest that it may act on a receptor which may be different from the glycoprotein GPIIb-IIIa complex (Mazoyer et al., 1990). On the basis of our results, it may be assumed that a protein, related to the human PHA-activated lymphocyte lactotransferrin receptor, is present on platelets. This was confirmed, in our lab, by observing a human lactotransferrin receptor, similar to the lymphocyte receptor, on human platelets (B. Leveugle, J. Mazurier, D. Legrand, and G. Spik, personal communication).

Furthermore, by comparing the primary and spatial structures from residues 4 to 52 of human lactotransferrin with the homologous sequences of the C-terminal lobe of the protein

and the N- and C-terminal lobes of serotransferrin (Figure 8), it clearly appears that the N-terminal end (residues 4–6) and the loop from residues 28 to 34 possess structural features specific to the N-terminal moiety of human lactotransferrin. This observation thus confirms the specificity of this part of lactotransferrin in receptor binding.

In conclusion, the present report gives evidence that the binding site of human lactotransferrin to the PHA-activated lymphocyte receptor is included between residues 4 and 52. Two solvent-accessible areas are likely to be involved in receptor recognition: the first one includes all or part of residues 4–6 and 28–34 and the second one consists of all or part of residues 38–42. Peptide synthesis and site-directed mutagenesis experiments are now underway to characterize the receptor-binding amino acid residues.

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