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Lactotransferrin receptor of mouse small-intestinal brush border

Binding characteristics of membrane-bound and Triton X-100-solubilized forms

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A specific lactotransferrin receptor was identified in the mouse small-intestinal brush-border membrane and the binding features were investigated in homologous and heterologous systems. The receptor was found to be specific for lactotransferrins isolated from milk of various species, but the affinity was higher toward the homologous ligand ($K_a = 3.5 \times 10^6$ M^{-1} compared with 2.6×10^6 M^{-1} for both human and bovine lactotransferrins). However, the number of binding sites (n) was the same for the three lactotransferrins, namely $0.53 \times 10^{12}/\mu$g of membrane protein. The binding of mouse lactotransferrin to its receptor was found to be pH-dependent, with an optimal binding at pH 5.5, and seemed unlikely to be carbohydrate-mediated. The receptor was demonstrated to be devoid of any affinity for human and mouse serotransferrins or for a 'serotransferrin-like' protein isolated from mouse milk. The receptor was solubilized with 1% Triton X-100 with good yield. The solubilized receptor was found to retain lactotransferrin-binding activity and sensitivity to pH.

INTRODUCTION

Since the discovery of lactotransferrin (or lactoferrin) in human milk, Montreuil *et al.* (1960*a*,*b*) proposed a role of this glycoprotein in the iron-donating function of milk in newborns. This view was reinforced by the characterization of lactotransferrin in duodenal fluids as well as at the surface of intestinal mucosa (Masson *et al.*, 1966) and by the marked ability of human and bovine lactotransferrins to resist, in large part, proteolytic attack during the transit through the digestive gut in newborn humans (Spik *et al.*, 1982*a*; Davidson & Lönnerdal, 1985*a*). The demonstration by Cox *et al.* (1979) and by Yoshino *et al.* (1983) that human and bovine lactotransferrins were capable of donating iron to intestinal mucosa, and the characterization by Mazurier *et al.* (1985) of a lactotransferrin receptor in the rabbit jejunal brush-border vesicles, provide more arguments for the possible role of lactotransferrin in intestinal iron absorption. Since it was previously reported that the binding of human lactotransferrin to human alveolar macrophages can be inhibited by fucoidin (Campbell, 1982) or by fucosylated bovine serum albumin (Goavec *et al.*, 1985), we thus investigated the role of carbohydrate in the binding of lactotransferrin to mouse intestinal brush-border-membrane vesicles. The presence in some mammalian milks, in addition to lactotransferrin, of a 'serotransferrin-like' protein led us to compare the role of both proteins in intestinal iron absorption. As mouse milk contains 3 mg of a 'serotransferrin-like protein'/ml as against 1 mg of lactotransferrin/ml (Leclercq *et al.*, 1987), the recognition by mouse intestinal brush-border membrane of both iron-binding proteins and of serotransferrin isolated from mouse serum was analysed. Moreover, the solubilization of the lactotransferrin receptor was performed by using Triton X-100 as a detergent and the binding features of the solubilized receptor were compared with those of the membrane-bound receptor.

MATERIALS AND METHODS

Proteins

Iron-free human serotransferrin was obtained from Behring (Marburg, Germany) and hen's-egg lysozyme (grade I) from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Human and bovine lactotransferrins were prepared as described elsewhere (Chéron *et al.*, 1977; Spik *et al.*, 1982*b*). Mouse serotransferrin was isolated from mouse plasma as described by Sawatzki *et al.* (1981). Mouse 'serotransferrin-like' protein and lactotransferrin were isolated from mouse milk by the method of Sawatzki & Kubanek (1983) and Leclercq *et al.* (1987). The three mouse transferrins were homogeneous as assessed by SDS/polyacrylamide-gel electrophoresis, gel chromatography and immunoelectrophoresis. The iron-free and iron-saturated forms of transferrins were obtained as detailed previously (Mazurier & Spik, 1980). The α-L-fucosyl-(bovine serum albumin)(α-L-Fuc-BSA; 17 residues of fucose per molecule of bovine serum albumin) was kindly given by Dr. M. Monsigny (University of Orléans, Orléans, France) and prepared as described by Monsigny *et al.* (1984).

Other reagents

PMSF, aprotinin, pepstatin A, DTT, *o*-phenanthroline, L-leucine *p*-nitroanilide, disodium *p*-nitrophenyl

Abbreviations used: PBS, phosphate-buffered saline (0.02 M-sodium phosphate/0.15 M-NaCl, pH 7.4); α-L-Fuc-BSA, α-L-fucosyl-(bovine serum albumin); PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol.

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phosphate and Triton X-100 were purchased from Sigma. Iodogen was obtained from Pierce Chemical Co. (Rotterdam, The Netherlands) and $Na^{125}I$ (carrier-free) (625 MBq of $^{125}I/\mu g$ of iodine) was purchased from Amersham International; the nitrocellulose membranes BA85 from Schleicher and Schüll (Dassel, Germany).

Protein iodination

Proteins were ^{125}I-labelled at 4 °C for 10 min by using Iodogen as a catalyst in PBS according to the manufacturer's instructions. The amount of added iodine (including non-radioactive iodine) was so calculated as to give an average of one iodine atom per molecule of protein. The specific radioactivity was about 100000 c.p.m./μg of protein. The unbound iodine was removed by gel filtration on a Sephadex G-25 PD-10 column equilibrated with the incubation buffer. Iodinated proteins were used within 2 weeks to avoid radiation damage.

Preparation of brush-border-membrane vesicles

Swiss mice aged 4–5 weeks were used for each experiment. The animals were fed on a standard laboratory diet *ad libitum* and were starved overnight before being killed by cervical dislocation. The proximal third of the small intestine was removed and rinsed immediately with ice-cold 0.15 M-NaCl. Membrane vesicles were prepared as described by Kessler *et al.* (1978). The final vesicle suspension was used immediately or frozen and stored at −70 °C. The purification procedure was monitored by measuring aminopeptidase (EC 3.4.11.2) by the method of Benakiba & Maroux (1980) and $(Na^+ + K^+)$-dependent ATPase (EC 3.6.1.3) as described by Murer *et al.* (1976). Protein content was determined by the method of Peterson (1977), with BSA as standard. The vesicles were routinely examined by electron microscopy using a JEOL 120 CX electron microscope.

Solubilization of membrane vesicles

Membrane vesicles corresponding to a protein concentration of 3 mg/ml were incubated for 30 min at 4 °C in a solution of 10 mM-NaOH/Mes (pH 5.5)/150 mM-NaCl, containing 0.1–5% (w/v) Triton X-100 as required, and 1 mM each of the following proteinase inhibitors: PMSF, aprotinin, pepstatin A, DTT and *o*-phenanthroline. After centrifugation at 120000 *g* for 60 min at 4 °C, the supernatant (referred to hereafter as 'Triton X-100 extract') was used for protein-content determination and for binding assays.

Binding assay of membrane vesicles

The binding assays were conducted in a standard incubation solution containing, in a 140 μl final volume, 10 mM-NaOH/Mes, pH 5.5, 50 mM-NaCl, 50 mM-D-mannitol, 1 mM-D-glucose and, when necessary, other compounds (see the Figures). ^{125}I-labelled lactotransferrin was normally at about 0.1 μM, except for the saturation assay, in which the concentration was varied from 0.01 to 2 μM. Reactions were initiated with addition of membrane vesicles (30 μg of membrane proteins). Incubations were performed at 4 °C for 10 min in all cases, except for kinetic assays, and terminated by centrifugation at 100000 *g* in a Beckman airfuge. The pellets were washed twice by repeated suspension–sedimentation and then transferred to haemolysis tubes for radioactivity counting in a LKB 1282 CompuGamma counter. Non-specific binding was estimated by performing parallel incubations to which a 100-fold excess of unlabelled analogue was added.

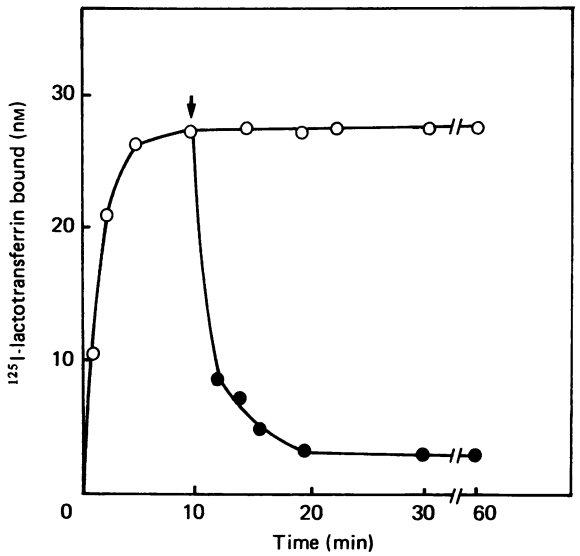

Fig. 1. Time course of binding (○) and depletion by unlabelled mouse lactotransferrin (●) of ^{125}I-labelled mouse lactotransferrin to mouse intestinal brush-border-membrane vesicles

^{125}I-labelled mouse lactotransferrin (0.01 μM) was incubated with membrane vesicles as described in the Materials and methods section. At time shown by the arrow, unlabelled mouse lactotransferrin was added in a minimum volume to give a final concentration of 0.5 μM. Assays were carried out by stopping the incubation after various intervals of time. Each value represents the average for duplicate determinations from two independent experiments.

Binding assay of Triton X-100 extracts

Portions (5 μl) of Triton X-100 extracts were spotted on to nitrocellulose strips and then incubated under gentle agitation with ^{125}I-labelled mouse lactotransferrin in 5 ml of an incubation buffer containing 0.1% Triton X-100, 10 mM-NaOH/Mes, pH 5.5, 150 mM-NaCl, for 1 h at 4 °C. After twice washing with the incubation buffer, the nitrocellulose strips were dried, cut into squares, and the retained radioactivity determined.

Competition binding assay

The competition between different transferrins, bovine serum albumin, α-L-Fuc-BSA, hen's-egg lysozyme and ^{125}I-labelled mouse lactotransferrin for receptor binding was assayed by introducing into the incubation medium increasing concentrations of each of the reagents, ranging from 0.1 to 2 μM or more. Assays were carried out as described above.

Effect of bivalent ions and chelating agents on lactotransferrin binding

The effect of several bivalent ions was assayed on Triton X-100 extracts. The incubation was carried out as described above with or without various concentrations of $CaCl_2$ (0–50 mM), $MgCl_2$, $MnCl_2$ (10 mM), EGTA or EDTA (10 mM).

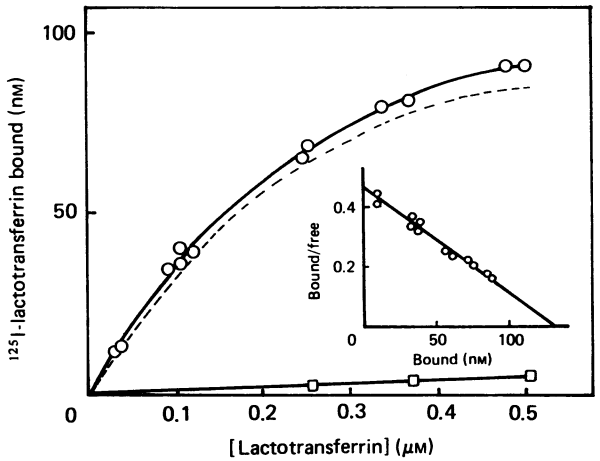

Fig. 2. Binding of ^{125}I-labelled mouse lactotransferrin to mouse intestinal brush-border-membrane vesicles as a function of lactotransferrin concentration

Assays were carried out by incubating 30 μg of membrane vesicle protein with an increasing amount of radiolabelled mouse lactotransferrin as outlined in the Materials and methods section. ○, Total binding; □, non-specific binding; ----, specific binding. The inset shows a Scatchard (1949)-plot analysis of the specific binding data. Results are typical for three separate experiments.

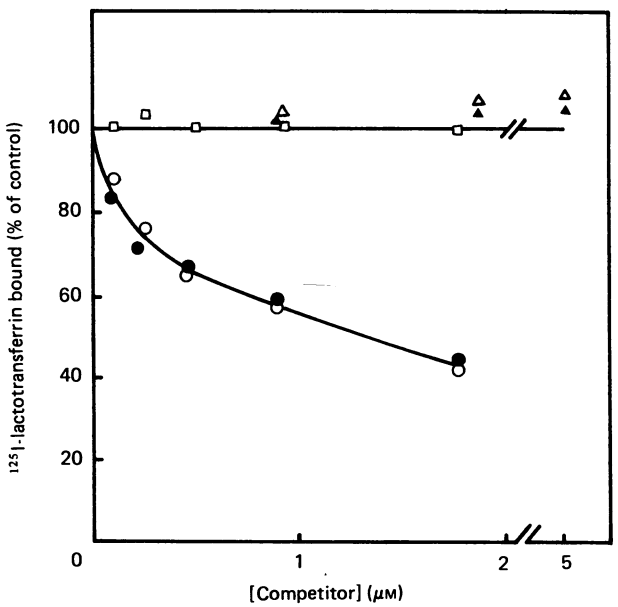

Fig. 3. Competitive binding to membrane vesicles of ^{125}I-labelled mouse lactotransferrin with human (○) and bovine (●) lactotransferrins, hen's-egg lysozyme (□), BSA (△) and α-L-Fuc-BSA (▲)

Assays were carried out by incubating 0.1 μM-^{125}I-labelled mouse lactotransferrin with membrane vesicles in the presence of increasing concentrations of competitors as described in the Materials and methods section. Each point represents the average of duplicate measurements from two separate experiments.

RESULTS

Characterization of mouse intestinal brush-border-membrane vesicles

The mouse intestinal brush-border-membrane vesicles finally obtained were essentially sealed and free of any other contaminant organelles as checked by electron microscopy. The specific activity of the brush-border marker leucine aminopeptidase in the final membrane was increased about 15-fold over the homogenate of intestinal scrapings, at a yield of about 45%. The activity of basolateral marker $(Na^+ + K^+)$-ATPase was not detectable.

Mouse lactotransferrin binding

As shown in Fig. 1, binding of ^{125}I-labelled iron-saturated mouse lactotransferrin to mouse intestinal brush-border membrane vesicles was rapid, an equilibrium being reached in less than 10 min and a plateau maintained over a period of 60 min. In subsequent experiments, incubations of membrane vesicles were performed for 10 min. The binding was found to be reversible, since the addition of 50 M excess of unlabelled mouse lactotransferrin to the equilibrium system displaced the bound ^{125}I-labelled mouse lactotransferrin. As shown by the results in Fig. 2, the binding of mouse lactotransferrin to brush-border-membrane vesicles was saturable at concentrations ranging from 0.01 to 0.5 μM, whereas the non-specific binding was low, representing less than 5% of the total binding. Scatchard (1949)-plot analysis of the specific binding data revealed an apparent unique category of binding sites with K_a(affinity constant) $= 3.5 \times 10^6$ M^{-1} and n (number of binding sites) $= 0.53 \times 10^{12}/\mu$g of membrane protein. In the same experiment, iron-free mouse lactotransferrin showed a similar affinity ($K_a = 3.6 \times 10^6$ M^{-1}, with an identical number of binding sites).

Binding specificity

In order to illustrate the specificity of lactotransferrin binding, competitive assays were carried out with human and bovine lactotransferrins as inhibitors. The results obtained are shown in Fig. 3. Human and bovine lactotransferrins inhibited the binding of ^{125}I-labelled mouse lactotransferrin and exhibited the same inhibition profile. These results show that the three kinds of lactotransferrin bound to the same receptor sites. Further experiments with ^{125}I-labelled human and bovine lactotransferrins revealed that the two proteins possess a similar affinity for brush-border-membrane receptor ($K_a = 2.6 \times 10^6$ M^{-1}; c.f. mouse lactotransferrin: $K_a = 3.5 \times 10^6$ M^{-1}). The number of binding sites remained identical. In the same experiment, hen's-egg lysozyme was also chosen as inhibitor, based on the fact that the hen's-egg lysozyme is a cationic protein just like lactotransferrins and would inhibit receptor binding if the lactotransferrin binding was electrostatic in nature. In fact, hen's-egg lysozyme did not compete with mouse lactotransferrin for receptor binding (Fig. 3), indicating that the lactotransferrin binding to mouse intestinal brush-border membrane vesicles cannot be explained by a non-specific ionic binding. When up to 50 M excess of α-L-Fuc-BSA was used as competitor, the binding of lactotransferrin to the membrane was not inhibited (Fig. 3), suggesting that lactotransferrin does not bind to membrane via a fucose receptor.

Absence of serotransferrin receptor

Binding of ^{125}I-labelled mouse milk 'serotransferrin-like' protein as well as of mouse serotransferrin to the

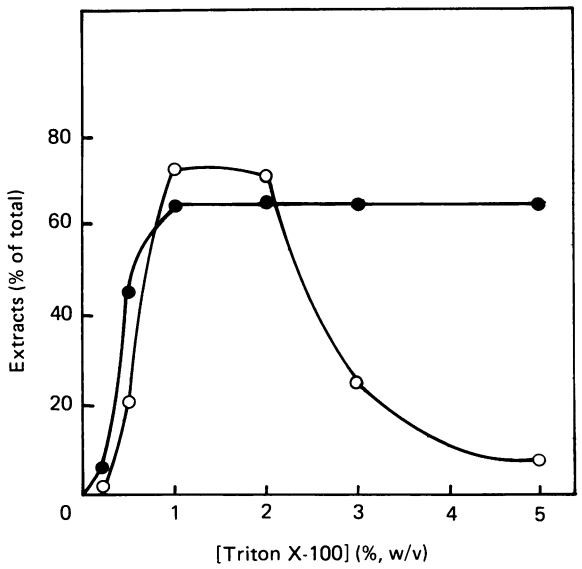

Fig. 4. Extraction with Triton X-100 of brush-border-membrane proteins (●) and mouse lactotransferrin-binding activity (○)

Assays were performed by incubating membrane vesicles (3 mg/ml) with increasing concentrations of Triton X-100 as described in the Materials and methods section. Protein and lactotransferrin-binding activity were assayed on the supernatant after 60 min centrifugation at 120000 *g*. Results are the average of duplicate determinations from two separate experiments.

mouse intestinal brush-border-membrane vesicles was assayed at various pH values (5–8). No specific binding could be found at ligand concentrations ranging from 1 nM to 2 μM. These results indicate that the mouse intestinal brush-border-membrane vesicles lack a specific receptor for the 'serotransferrin-like' protein isolated from mouse milk and for the mouse serotransferrin.

Extraction of membrane proteins

Successful receptor solubilization underlies receptor purification. In our experiments, about 65% of the membrane proteins were recovered in the supernatant after 60 min centrifugation at 120000 *g* with 1% Triton X-100, at a protein concentration of about 3 mg/ml (Fig. 4). By using this concentration of detergent, about 70% of the lactotransferrin binding activity was extracted. Higher concentrations of Triton X-100 did not improve the extraction of proteins; moreover, at concentrations higher than 2%, the binding activity of the receptor was inhibited.

Effect of Triton X-100 concentration on the binding assay

The binding of ^{125}I-labelled mouse lactotransferrin to the extracts of membrane vesicles was assayed in the presence of various concentrations of Triton X-100. As an optimal binding was found with a detergent concentration of 0.1% (w/v) (Fig. 5), this concentration was used throughout this experiment.

Effect of pH on lactotransferrin binding

The pH-dependency of lactotransferrin binding was investigated for the brush-border membrane vesicles and the Triton X-100 extracts. In both preparations the optimal binding was observed at about pH 5.5–6.0 (Fig. 6). In the case of membrane vesicles, the apo- and iron-saturated mouse lactotransferrins exhibited a rather similar profile, whereas with Triton X-100 extracts, interestingly, a shifted profile was observed. As a consequence, the binding of iron-saturated lactotransferrin was more diminished at pH values higher than 6.

Effect of bivalent cations on lactotransferrin binding

Since the membrane vesicles contained large amounts of bound Ca^{2+}, which are extremely difficult to remove with a chelator (Kessler *et al.*, 1978), only Triton X-100 extracts were used in our experiment for the study of the effect of bivalent cations. As depicted in Fig. 7(*a*), Ca^{2+} enhanced the mouse lactotransferrin binding with a linear correlation at concentrations lower than 8 mM; at higher concentrations a plateau was reached. Mg^{2+} and Mn^{2+} could also increase lactotransferrin binding, but at a concentration of 10 mM, their effect was only 40 and 30% respectively of the effect of Ca^{2+}. Without addition of bivalent cations, the binding amounted to about half of the maximal binding. Binding assays with or without addition of 10 mM-Ca^{2+} revealed, by the Scatchard (1949)-plot analysis, that Ca^{2+} increased the number of binding sites by 58% (1.12×10^{10} cf. 0.71×10^{10}/μg of membrane protein), while only slightly altering the apparent affinity constants (1.45×10^{8} M^{-1} cf. 1.19×10^{8} M^{-1}). These values are about 30-fold higher than that in the case of membrane-bound receptor. Furthermore the binding was not totally abolished by the addition of 10 mM-EGTA or -EDTA, although EDTA

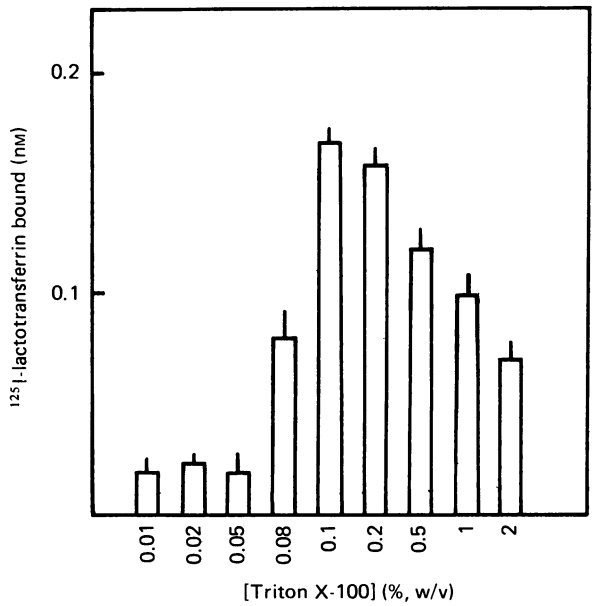

Fig. 5. Effect of Triton X-100 concentration on lactotransferrin-binding activity

Assays were carried out as described in the Materials and methods section with various concentrations of Triton X-100 in the incubation buffer. Results are the averages and ranges for duplicate determinations from two separate experiments.

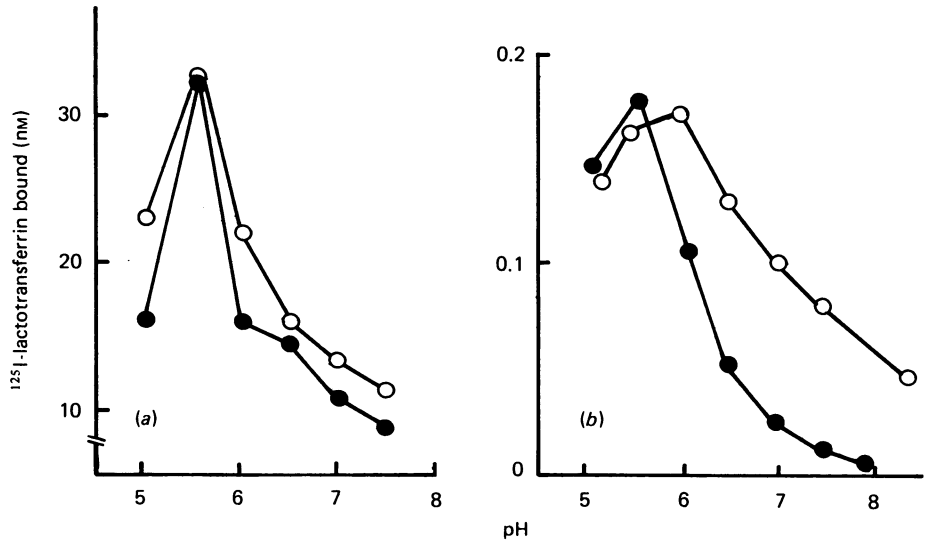

Fig. 6. ^{125}I-labelled apo-(○) and iron-saturated (●) mouse lactotransferrin binding to (*a*) membrane-bound and (*b*) Triton X-100-solubilized receptor as a function of pH

Assays were carried out as indicated in the Materials and methods section at various pH values. Each point represents the average of duplicate determinations from two separate experiments.

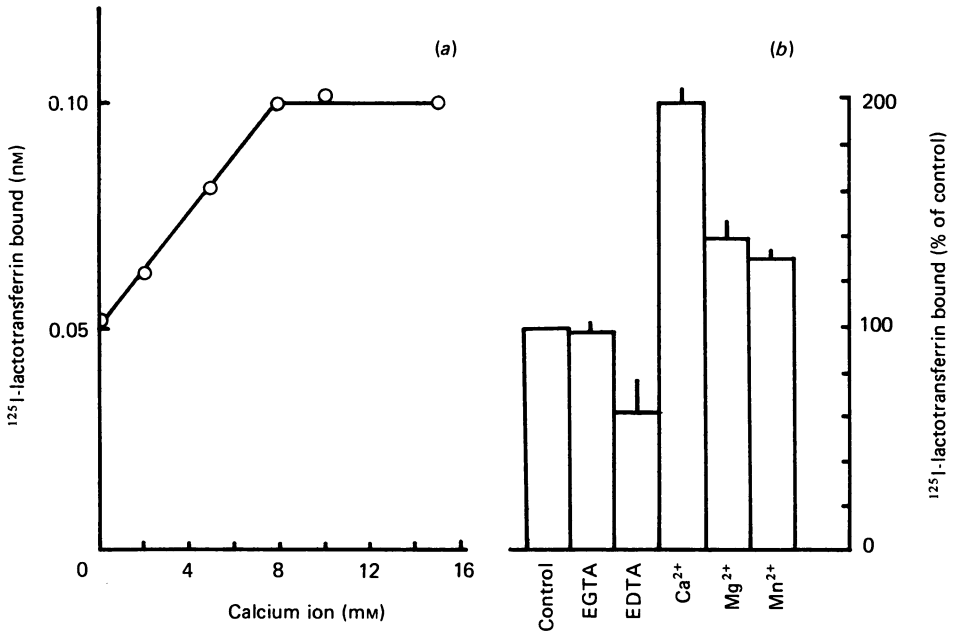

Fig. 7. Binding of ^{125}I-labelled mouse lactotransferrin Triton X-100-solubilized receptor as a function of increasing concentrations of Ca^{2+} (*a*) and at a fixed concentration (10 mM) of Ca^{2+}, Mg^{2+} and Mn^{2+} ions, EGTA and EDTA

Assays were carried out as described in the Materials and methods section. Results are the averages and ranges for duplicate determinations from three separate experiments.

decreased the binding to a greater extent (Fig. 7B). One possible explanation is that two types of binding sites were concomitantly present, with one bivalent-cation-dependent and the other bivalent-cation-independent. An alternative explanation is that there is only one class of binding site, which is bivalent-cation dependent, although their affinity for the bivalent cation is different, being higher for Ca^{2+} than for Mg^{2+} or Mn^{2+}. The bivalent cations required for binding are, however, not completely chelated in a complex detergent-containing system and, consequently, the binding is only partly diminished. Since lactotransferrin polymerizes in the presence of Ca^{2+} ions (Bennett *et al.*, 1981) and the effect of polymerization on receptor binding was unknown, we performed gel-filtration experiments (results not shown), which indicated that no polymerisation of mouse lactotransferrin occurred under our experimental conditions.

DISCUSSION

We have demonstrated that the lactotransferrins bind to mouse small-intestinal brush-border-membrane vesicles with an apparent affinity constant compatible with the values published for the brush-border-membrane receptor of other species (Mazurier *et al.*, 1985; Davidson & Lönnerdal, 1985*b*). The finding that the homologous lactotransferrin possesses higher affinity constant for receptor may be relevant to the data obtained by Cox *et al.* (1979) indicating that the homologous lactotransferrin shows a relatively higher effectiveness in delivering the bound iron to human intestinal biopsies. The failure of cationic proteins, such as hen's-egg lysozyme, to inhibit lactotransferrin binding suggests that the interaction between the lactotransferrin and the mouse intestinal brush-border membrane is unlikely to be electrostatic in nature, contrary to the suggestion of Debanne *et al.* (1985) in the case of the human lactotransferrin–rat liver membrane interaction. Human and bovine lactotransferrins exhibited the same binding features and the same competition profile against mouse lactotransferrin for receptor binding. This behaviour seems to indicate that the receptor has no strict species specificity. In our previous experiments (Goavec *et al.*, 1985) the binding of human lactotransferrin to human alveolar macrophages was inhibited by α-L-Fuc-BSA and by α-D-mannosyl (Man)-BSA, suggesting that the interaction of the lactotransferrin may be mediated by Man/Fuc receptor possessing similar properties to the Man/Fuc rat alveolar macrophage receptor (Shepherd *et al.*, 1981). If such a fucose receptor were present in mouse enterocytes, human lactotransferrin containing Fuc(α1–6)- and Fuc-(α1–3)-linked (Spik *et al.*, 1982*b*), mouse 'serotransferrin-like' protein and mouse lactotransferrin containing Fuc(α1–6)-linked (Leclercq *et al.*, 1987) and bovine lactotransferrin containing oligomannosidic glycans (van Halbeek *et al.*, 1981) and fucosylated *N*-acetyllactosaminic glycans (Spik *et al.*, 1985) would interact with this receptor. In fact, the 'serotransferrin-like' protein did not bind to membrane vesicles, and the α-L-Fuc-BSA was not able to inhibit the binding of mouse lactotransferrin, indicating that, in mouse enterocytes, the lactotransferrin binding site was not the Man/Fuc receptor. These results suggest that the polypeptide chain rather than the glycan moiety in the molecule of lactotransferrins is implicated in the ligand–receptor interaction. The lactotransferrin binding was found to be dependent on pH, with an optimum at pH 5.5. For the moment, we question the physiological relevance of these data, since the pH at the cell surface remains unknown, although in rats the cell-surface microclimate pH of the proximal jejunum was measured by pH-microelectrode and found to be 5.5 (Lucas *et al.*, 1975).

Triton X-100 at 1% was used to solubilize brush-border membranes with good yield in protein and lactotransferrin-binding activity. Lower concentrations in detergent resulted in poor recovery of lactotransferrin-binding activity in the supernatant. This may suggest that the receptor is an integral, rather than an extrinsic, membrane protein. The Triton X-100-solubilized receptor retained lactotransferrin-binding activity and, more importantly, its sensitivity to pH. Obviously properties of the solubilized receptor cannot totally reflect those of its membrane-bound counterpart, since the binding affinity constant was increased by about 30-fold.

In agreement with early study in rabbit (Mazurier *et al.*, 1985), we were not able to demonstrate any specific binding activity in mouse intestinal brush-border membrane for 'serotransferrin-like' protein isolated from mouse milk and for human and mouse serotransferrins. Recent ultrastructural studies using electron microscopy and an immunoperoxidase assay also failed to identify serotransferrin receptor on the surface of enterocyte microvilli (Marx *et al.*, 1985; Parmley *et al.*, 1985; Banerjee *et al.*, 1986). It has been reported that the serotransferrin failed to give its iron to human intestinal tissue *in vitro* (Cox *et al.*, 1979) and that the iron absorption *in vitro* in rat jejunal and ileal segments was independent of its mucosal transferrin content (Schümann *et al.*, 1986). If a specific receptor for serotransferrin in the intestinal brush-border membrane is absent, it would be necessary to invoke a mechanism for iron uptake from the lumen involving another membrane component, which could participate in the internalization of iron into the mucosa cells. The lactotransferrin receptor could be one of several possible candidates. The results that we have obtained, in association with those previously obtained by Cox *et al.* (1979) and Mazurier *et al.* (1985) favour a role for lactotransferrins in intestinal iron absorption (Montreuil *et al.*, 1985). However, the exact role of lactotransferrin receptor in the mechanism of iron absorption awaits further study, and the unusually high concentration of 'serotransferrin-like' protein in mouse milk remains to be explained.

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