Lactoferrin Upregulates the Expression of CD4 Antigen Through the Stimulation of the Mitogen-Activated Protein Kinase in the Human Lymphoblastic T Jurkat Cell Line

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Abstract   The main biological properties of lactoferrin are thought to concern inflammation and immunomodulation processes, including maturation of immature B and T cells. Lactoferrin accelerates T-cell maturation by inducing the expression of the CD4 surface marker. In this report, using the Jurkat T-cell line, we have shown that lactoferrin upregulates the expression of CD4 antigen through the activation of a transduction pathway. Using an anti-phosphotyrosine antibody, lactoferrin was demonstrated to induce a cascade of phosphorylation of numerous proteins on their tyrosine residues. This tyrosine-phosphorylation was transient, reaching maxima between 5 and 10 min. We also identified the mitogen-activated protein kinase (MAP kinase) which presented an enhanced catalytic activity, reaching a maximum at 10 min of incubation with lactoferrin. Moreover, the use of inhibitors such as genistein and PD98059, tyrosine kinases and MAP kinase kinase (or MEK) inhibitors respectively, allowed us to correlate the activation of MAP kinase with the upregulation of CD4 expression. Finally, using Lck-defective Jurkat cells, our results showed that the p56[lck] (Lck) kinase is necessary for MAP kinase activity and CD4 expression. This paper demonstrates that lactoferrin activates transduction pathway(s) in lymphoblastic T-cells, and that Lck and the Erk2 isoform of MAP kinase are implicated in the upregulation of CD4, induced by lactoferrin in these cells. J. Cell. Biochem. 79:583–593, 2000.   © 2000 Wiley-Liss, Inc.

Key words: lactoferrin; Jurkat cells; maturation; CD4; MAP kinase; p56[lck]

Lactoferrin, also called lactotransferrin [Montreuil et al., 1960], an iron-binding glycoprotein produced by epithelial cells and neutrophilic leukocytes precursors, is found in most biological fluids [Masson et al., 1969]. Lactoferrin function involves more than simple chelation of iron. Lactoferrin has been shown to be involved in inflammatory, antitumoral, and differentiation processes. In fact, this protein regulates the production of proinflammatory cytokines [Crouch et al., 1992; Sorimachi et al., 1997], and modulates the adhesive properties of leukocytes to the endothelial cells at inflammatory sites [Boxer et al., 1982; Kurose et al., 1994]. In vivo lactoferrin also reduces solid tumour growth and inhibits experimental metastasis [Bezault et al., 1994]. This seems to be due to the activation of the NK cells' cytotoxicity [Nishiya and Horwitz, 1982], and to the inhibition of the proliferation of tumoral cells [Damiens et al., 1998]. Indeed lactoferrin inhibits in vitro growth arrest of breast epithelial cancer cells at the G1 to S transition of the cell cycle by modulating the expression and activity of key regulatory proteins [Damiens et al., 1999]. Finally, lactoferrin accelerates the differentiation process of immature B- and T-lym-

phocytes: CD4$^-$ CD8$^-$ murine thymocytes acquired the CD4 antigen characteristic for the helper cell phenotype [Zimecki et al., 1991]. Similarly, in the presence of lactoferrin, murine splenic B cells show phenotypic changes in the ratio of surface IgM and IgD, which is characteristic of the maturation process [Zimecki et al., 1995]. Biological activities induced by lactoferrin depend on its target cells, and on the presence of specific binding proteins at their surface. Lactoferrin is a multifunctional protein which is able to specifically recognize the low density lipoprotein-receptor-related protein (LRP) [Ziere et al., 1992], the lymphocyte receptor [Mazurier et al., 1989], the asialoglycoproteins receptor [Bennatt et al., 1997], and proteoglycans [Mann et al., 1994].

Up to date, little is known concerning the intracellular molecular events triggered by lactoferrin in target cells. It was suggested that lactoferrin specifically interacts with DNA consensus sequences and acts as a transcription factor [He and Furmanski, 1995]. However it was recently demonstrated that the binding of lactoferrin to LRP in hepatocytes stimulates a G protein-dependent signaling pathway [Goretzki and Mueller, 1998].

Until now, the involvement of transduction pathways in the mechanism by which lactoferrin affects immature T-cells has not been investigated. To gain further insights into the maturation of T-cells through the expression of CD4, we used the human lymphoblastic T Jurkat cell line. These cells, which express the lymphocyte receptor of lactoferrin [Bi et al., 1994; Legrand et al., 1997] and a low density of CD4 antigen at the cell surface, constitute a good model for our study. In the present paper we investigated the effects of lactoferrin on the expression of CD4 at the cell surface and on the tyrosine phosphorylation of cytosolic proteins. The catalytic activity of MAP kinase (also called extracellular signal-regulated kinase, or Erk) was also studied because of its key role in signal transduction from many receptors in response to their ligands. The MAP kinase activation was then correlated with the CD4 up-regulation, using genistein, a tyrosine kinase inhibitor, and PD98059, a specific MEK inhibitor. Finally the implication of the p56^{lck} kinase in the CD4 expression induced by lactoferrin was investigated using a Lck-defective Jurkat cell line.

MATERIALS AND METHODS
Materials and Antibodies

The Jurkat E6 lymphoblastic T-cell line and the J.CaM1.6 cell line, a mutant derivative of Jurkat which is defective in Lck kinase expression, were obtained from the ECACC. RPMI 1640 medium was from Gibco-BRL (Eragny, France) and bovine serum albumine (BSA) was from Boehringer Mannheim (Mannheim, Germany). Enhanced chemiluminescence system (ECL) was from Amersham (UK), DEAE Sepharose CL-6B was purchased from Pharmacia Biotech (Uppsala, Sweden), and PD98059 was from Calbiochem (Meudon, France). Myelin basic protein (MBP), protein kinase inhibitor (PKI), genistein, forskolin, polyclonal anti-MAP kinase, and FITC-conjugated anti-mouse antibodies were from Sigma Chemical Company (St. Louis, MO). Monoclonal anti-phosphotyrosine antibody (clone PY20, IgG2b) was from Transduction Laboratories (Lexington, KY), monoclonal anti-P-Erk antibody (clone E10) was from New England Biolabs (Beverly, UK), whereas monoclonal anti-CD3 (clone X35, IgG2a), anti-CD4 (clone 13B8.2, IgG1κ), and anti-CD71 (clone YDJ.1.2.2., IgG1) antibodies were from Immunotech (Marseille, France). Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were purchased from Sanofi-Pasteur (Steenvoorde, France) and Biosys (Compiègne, France), respectively.

Lactoferrin was prepared from pooled human lactoserum as described by Spik et al. [1982]. Purity of the protein was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Moreover, a saline shock was performed to remove lipopolysaccharides (LPS) bound to lactoferrin. Briefly lactoferrin was passed on a SP-sepharose fast flow column equilibrated in 0.1 M NaCl, followed by injection of 0.5 M NaCl onto the column. Human lactoferrin was then eluted with 2 M NaCl and desalted on PD10G25 column [Baveye et al., 2000]. LPS contamination of lactoferrin was less than 50 pg of endotoxin per mg of protein, as estimated by the Limulus amoebocyte lysate assay (QCL 1000, BioWhittaker).

Cell Culture

Jurkat cells (E6 and CaM1.6) were routinely grown at 37°C in a humidified atmosphere under 5% CO_2 in RPMI 1640 medium pH 7.4,

containing 5 µg/ml gentamycin, 2 mM L-glutamine, 20 mM Hepes, and supplemented with 10% heat-inactivated fetal calf serum. Cells were kept at logarithmic growth phase and used at a cell density of 6×10^5/ml.

Evaluation of Upregulation of CD4 Antigen and Cell Cycle Analysis

Cells were diluted at a density of 2×10^5 per ml and grown for 48 h in the presence of lactoferrin at concentrations ranging from 0 µg to 100 µg per ml. Expression of CD4 and CD71 antigens were measured at the cell surface and cell cycle analysis were performed every 24 h. Briefly, cells (6×10^5) were incubated for 1 h in 100 µl of phosphate buffered saline (PBS) with mouse monoclonal IgG against CD71 or CD4 antigens (diluted 1:25). Cells were then washed twice by centrifugation and incubated for 30 min with the secondary antibody of FITC-conjugated anti-mouse IgG (diluted 1:40). An isotype control (IgG1) was used to evaluate the non-specific binding of the antibody and the fluorescence intensity was measured using a FACScan flow cytometer (Becton and Dickinson, Sunnyvale, CA). The variation of CD4 density was calculated as follow: (mean of CD4− mean of isotype) $\times$ 100/mean of isotype.

For cell cycle analysis, cells (1×10^6) were fixed with cold 70% ethanol for 3 h on ice. Fixed cells were then stained at 37°C with propidium iodide (28 µg/ml) in the presence of RNase (28 µg/ml) for 45 min. After two washings in PBS, cells were analysed on FACScan using the cellFit software program (Becton and Dickinson).

Binding of Fluorescent-Labeled Lactoferrin to the Cells

The fluorescein labeling of lactoferrin was carried out through its glycan moiety using 5-(((2-(carbohydrazino) methyl) thio) acetyl) aminofluorescein (HyF) as previously described [Leveugle et al., 1993]. Cells (3×10^5) were incubated in 100 µl of RPMI medium, containing 0.04% of sodium azide, with various concentrations of [HyF]-labeled lactoferrin ranging from 0 to 40 µg/ml during 45 min at 4°C, and washed with the incubation buffer. Non-specific binding was measured in the presence of a 100 molar excess of unlabeled lactoferrin. The cells were analysed by flow cytometry, and the mean fluorescence variations were calculated from means of total (MT) and non-specific (MNS) fluorescent intensities as follow: (MT-MNS) $\times$ 100/MNS.

Cellular Lysate

Jurkat T cells were incubated without or with lactoferrin (at time varying from 0 to 45 min and at concentrations ranging from 0 to 100 µg/ml) in RPMI 1640 medium pH 7.4 containing 0.1% BSA, washed in PBS pH 7.4, and then lysed in ice cold lysing-buffer (155 mM NaCl, 10 mM Tris-HCl pH 7.6, 1% Triton X-100, 1% deoxycholate, 1 mM EGTA, 0.2 mM Na_3VO_4, 100 mM NaF, 1 mM PMSF, 50 µg/ml leupeptin, 200 µM aprotinin) for 2 h at 4°C. Nuclei and insoluble matter were removed by centrifugation for 15 min at 13,000g, before performing the P-Tyr immunostaining or the MAP kinase activity assays. For the P-Erk immunostaining, cells were incubated as described above but lysed in 20% SDS buffer containing β-mercaptoethanol by heating for 10 min at 100°C.

Immunoblotting

Protein concentrations were determined using the BCA protein assay reagent with BSA as a standard (Pierce), and the lysates were separated on SDS-PAGE 10% and then transferred electrophoretically onto nitrocellulose membranes.

P-Tyr immunostaining. The blots were incubated for 1 h in Tris-buffered saline (TBS : 150 mM NaCl, 10 mM Tris-HCl pH 7.6) containing 3% BSA and 0.1% Tween 20. Membranes were then incubated for 2 h at room temperature with primary anti-P-Tyr mouse monoclonal antibody (1 µg/ml) in TBS containing 0.1% BSA and 0.1% Tween 20, followed by washing in the same buffer without BSA. Blots were incubated with secondary antibody (HRP-conjugated anti-mouse IgG, 1 µg/ml) in TBS containing 0.1% Tween 20 for 1 h at room temperature, washed and then developed using the ECL system.

P-ERK immunostaining. The blots were incubated for 1 h in TBS containing 0.1% Tween 20 and 5% nonfat milk. Membranes were incubated for 2 h at room temperature with primary anti-P-Erk mouse monoclonal antibody (0.5 µg/ml) in TBS containing 0.1% Tween 20 and 1% nonfat milk, washed and then incubated with HRP-conjugated anti-

mouse IgG (0.5 μg/ml) for 1 h in the same buffer. For the MAP kinase detection, blots were incubated in the same conditions with primary anti-MAP kinase rabbit polyclonal antibody (3.1 μg/ml), followed by the HRP-conjugated anti-rabbit IgG (0.5 μg/ml). The differents blots were then washed and developed using the ECL system.

Determination of MAP Kinase Activity on MBP

Minicolumn assay. The "minicolumn" assay consisted in fractionating the cytosolic extracts on DEAE-Sepharose to estimate kinase activities. Briefly, DEAE Sepharose CL-6B resin (0.4 ml) was prepared in buffer A (50 mM β-glycerophosphate pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.1 mM Na_3VO_4), and washed with buffer A added of 0.02 M NaCl. Cytosolic extracts (0.5 ml, 8×10^6) were loaded onto the minicolumn followed by two washes of buffer A containing 0.02 M NaCl. Then MAP kinase was eluted with 0.75 ml of buffer A added of 0.22 M NaCl. Finally, aliquots (6.25 μl) of the MAP kinase fraction were incubated with 2.1 μl of MBP (2 mg/ml) and 4.15 μl of buffer B (30 mM $MgCl_2$, 4.5 mM DTT, 75 mM β-glycerophosphate pH 7.3, 0.15 mM Na_3VO_4, 6 μM PKI, 3.75 mM EGTA, 2.5 mg/ml BSA) containing 2 μCi of [γ-^{32}P] ATP. After 15 min at 30°C, an aliquot of 10 μl was spotted onto phosphocellulose paper squares and washed with 150 mM phosphoric acid. The substrate phosphorylation was quantified by β-counting (LKB, Beckman).

Immunoprecipitation of MAP kinase. The lysate (100 μl, 4×10^6) was precleared by addition of 1 μl of non-immune rabbit serum for 12 h at 4°C, followed by incubation with 50 μl of 2% protein A-Sepharose for 30 min at 4°C. After centrifugation, the supernatant (140 μl) was added to 62 μg of polyclonal anti-MAP kinase antibody, 360 μl H_2O, 500 μl of 2× immunoprecipitation buffer (1× = 1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 0.5% NP-40) and incubated for 1 h at 4°C. Protein A-Sepharose (50 μl, 2%) was then added for 30 min at 4°C. The pellet was washed two times by centrifugation with 1× immunoprecipitation buffer, one time with the minicolumn assay buffer B, and then incubated with 7.5 μl of MBP (2 mg/ml) and 12.5 μl of buffer B containing 2 μCi of [γ-^{32}P] ATP. After 15 min at 30°C the samples were heated at 100°C and the supernatants were separated on SDS-PAGE 15%. The gel was dried and submitted to an autoradiography.

RESULTS

Upregulation of CD4 Antigen by Lactoferrin

The density of CD4 surface antigen was analysed by flow cytometry. Incubation of untreated cells with anti-CD4 antibody resulted in greater levels of fluorescence (mean 379) than those detected with the isotype control (mean 356), demonstrating that Jurkat cells present a basal density of CD4 at their surface (Fig. 1A). The basal expression of this marker was enhanced in the presence of lactoferrin (mean 407) after 48 h of treatment. This effect was dose-dependent, and an optimal increase (2.4-fold) was observed with a concentration of 10 μg/ml of lactoferrin (Fig. 1B). In the same experimental conditions forskolin, which was previously shown to promote the CD4 gene transcription, was used as a positive control. We effectively observed that 10 μM of forskolin enhanced (about four-fold) the CD4 density at the cell surface (data not shown). Moreover the increase of the CD4 antigen expression was neither accompanied by a variation of the density of CD71, a marker of proliferation, nor by a modification of the cells repartition in the different phases of the cycle (data not shown).

Throughout these experiments, we demonstrated that lactoferrin induces a dose-dependent expression of the CD4 antigen at the cell surface, with a maximum observed at a concentration of 10 μg/ml, without blocking the cell proliferation.

Time-Dependence of the Tyrosine-Phosphorylation Induced by Lactoferrin

Since we observed that an optimal CD4 expression was obtained with 10 μg/ml of lactoferrin, the Jurkat T-cells were treated with this concentration of lactoferrin for various lengths of time, lysed, and P-Tyr polypeptides from total lysate were assayed by immunoblotting using an anti-P-Tyr monoclonal antibody. In untreated cells (Fig. 2, first lane), we observed the presence of polypeptides, phosphorylated on tyrosine residues: p37, p52, p55, p67, p72, and p100. The increase of tyrosine-phosphorylation was rapidly detected after the addition of lactoferrin, and was transient, reaching a maximum between 5 and 10 min (Fig. 2). In fact, the P-Tyr

proteins could be divided into three groups, based on the time at which phosphorylation reached a maximum. The first group (p47, p52, p55, p57, p59, p94, and p100) was rapidly phosphorylated, peaking between 5 and 10 min. The second group, which included the 37, 39, 61, 67, 69, 72, and 87 kDa proteins, clearly showed a maximal tyrosine-phosphorylation at 10 min. Whereas all these P-Tyr polypeptides returned to baseline levels by 20 min, the proteins of the third group (p32 and p34) were phosphorylated more slowly, reaching a maximum at about 20 min. We also observed that the phosphorylation of all the other polypeptides increased again at 30 min and decreased at 45 min.

These results indicated that lactoferrin rapidly activates a cascade of phosphorylation of numerous cytosolic proteins on tyrosine resi-
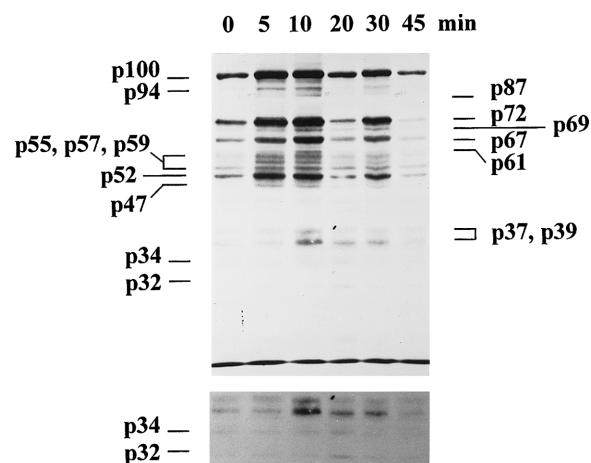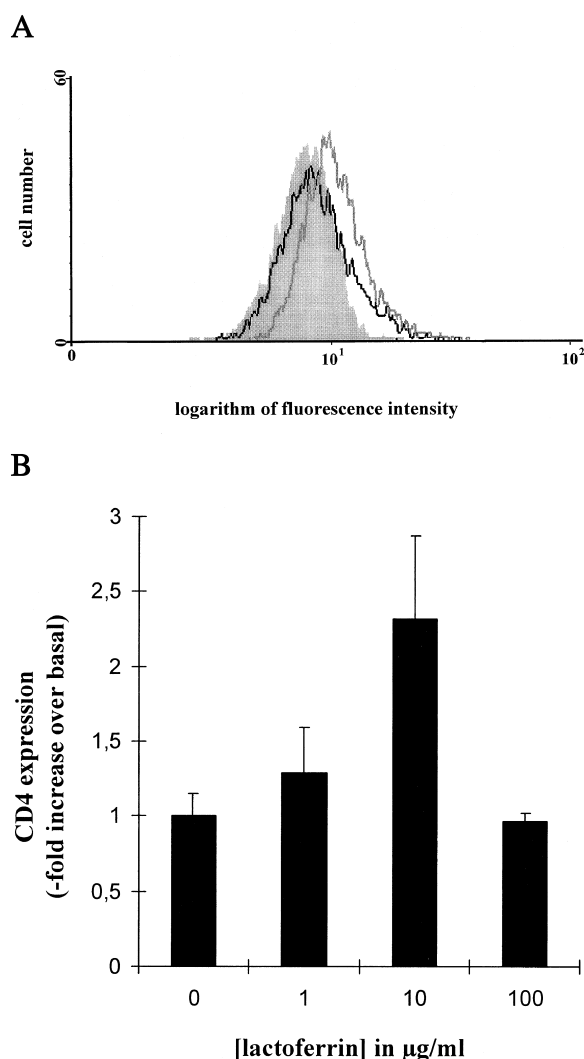
Fig. 2. Tyrosine-phosphorylation induced by lactoferrin treatment. Jurkat T-cells (1×10^6) were incubated for the indicated times with 10 μg/ml of lactoferrin. Cells were lysed and analysed by SDS-PAGE in the presence of β-mercaptoethanol using a 10% acrylamide gel. Phosphorylated proteins were detected by Western blotting using anti-P-Tyr antibody. This experiment was performed three times and we showed the most representative result, as well as the overexposure of the lower region of the blot.

dues, and that this event was time-dependent with a maximum of tyrosine-phosphorylated-proteins after 5 and 10 min of incubation with 10 μg/ml of lactoferrin.

MAP Kinase is Activated by Lactoferrin Treatment

The large number of P-Tyr polypeptides consecutive to lactoferrin treatment led us to investigate the possible activation of the MAP kinase, an enzyme involved in several transduction pathways. MAP kinase activity was

A

B

Fig. 1. Regulation of the CD4 antigen by lactoferrin at the cell surface. **A**: Effect of lactoferrin on CD4 surface density. Jurkat T cells (2×10^5) were cultured in complete RPMI medium under 5% CO_2 atmosphere, and treated with or without 10 μg/ml of lactoferrin during 48 h. The CD4 antigen was detected using flow cytometry and data were expressed as variation of logarithm of fluorescence intensity: filled peak, isotype control; open black peak, CD4 antigen on untreated cells; open grey peak, CD4 antigen on treated cells. **B**: Dose-dependent effect of lactoferrin on CD4 expression. Jurkat cells were treated for 48 h with different concentrations of lactoferrin before analysis of CD4 expression by flow cytometer. The variation of CD4 density was calculated as explained in Materials and Methods and expressed as the average fold increase (lactoferrin-treated divided by untreated) for three independent experiments. The data is statistically significative ($P < 0.05$, Student's t-test).

studied by analysing the ability of this enzyme to phosphorylate the myelin basic protein (MBP), one of its synthetic substrates.

Jurkat T cells were incubated with lactoferrin (0 to 100 μg/ml) for periods of time ranging from 0 to 15 min. At each time of lactoferrin treatment, MAP kinase enzyme was partially purified from Jurkat lysate, incubated with MBP and [γ-^{32}P]ATP, and the peptide substrate phosphorylation was measured by β-counting. The results in Figure 3A show a time- and dose-dependent phosphorylation of MBP : we observed that low (1 μg/ml) and high (100 μg/ml) concentrations of lactoferrin did not affect the kinase activity toward MBP, whereas the concentration of 10 μg/ml considerably increased the substrate phosphorylation. This effect reached a maximum after 10 min of treatment (four- to five-fold) and rapidly returned to the baseline level. Then to confirm that this kinase activity was the MAP kinase activity, the enzyme was immunoprecipitated using a specific polyclonal antibody. The complex was then incubated with [γ-^{32}P]ATP and MBP, and submitted to SDS-PAGE and autoradiography. The quantification of the radioactivity incorporated in the substrate (Fig. 3B) indicated that the activity of MAP kinase towards the MBP was enhanced and reached a maximum after 10 min of incubation with 10 μg/ml of lactoferrin.

Finally, the activated Erk isoform was identified by Western-Blotting using an antibody directed against the phosphorylated Erk1 and Erk2 isoforms of MAP kinase. These two isoforms were present at a constant level during lactoferrin treatment, Erk2 being more expressed than Erk1 (Fig. 3C, lane 1). Erk2 was also the most phosphorylated in untreated Jurkat cells (Fig. 3C, lane 2). Incubation of the cells during 10 min with the anti-CD3 antibody, as a positive control, caused a six-fold increase of the phosphorylation of the two MAP kinase isoforms, whereas lactoferrin only induced the phosphorylation of Erk2 (2.2-fold; Fig. 3C, lanes 3,4).

Taken together, these data showed that the enhanced catalytic activity of MAP kinase, which presented a maximum following 10 min of treatment with 10 μg/ml of lactoferrin, was due to the phosphorylation and activation of Erk2, one of the MAP kinase isoforms.

Implication of MAP Kinase in Upregulation of CD4 Antigen

In order to correlate tyrosine phosphorylation, MAP kinase activation and CD4 upregulation which were dose-dependent and maximal at the same concentration of 10 μg/ml of lactoferrin, we investigated the effect of genistein and PD98059 inhibitors on these events. Jurkat cells were pretreated for 45 min with inhibitors and lactoferrin was then added during 48 h for the CD4 detection or 10 min for the MAP kinase assay (Table I).

Genistein, which affects several cellular processes, presented a dose-dependent cytotoxicity towards the Jurkat cells. Indeed the viability remained maximal upon 15 μM of genistein and concentrations beyond 30 μM appeared to be toxic (data not shown). Detection of the CD4 antigen by flow cytometry at the cell surface showed that the increase of the CD4 density by lactoferrin was inhibited (77%) by pretreatment of the cells with 10 μM of genistein. At the same concentration, this inhibitor also caused 82% inhibition of MAP kinase activity (Table I).

Conversely PD98059, which is a specific MAP kinase cascade inhibitor, did not affect the cell viability whatever the concentration was (0–100 μM), and presented an inhibitory effect on the CD4 expression. We noticed that CD4 upregulation due to lactoferrin was totally abolished by a dose of 10 μM of PD98059 (Table I).

These experiments showed that MAP kinase stimulation by lactoferrin was abolished by 10 μM of genistein, and that the increase of CD4 density at the cell surface upon lactoferrin treatment was inhibited by both genistein and PD98059 at a concentration of 10 μM.

Role of Lck Kinase in CD4 Upregulation

The implication of Lck kinase, also called p56^{lck}, in CD4 upregulation and MAP kinase activation due to lactoferrin was investigated using the J.CaM1.6 cell line, a mutant derivative of Jurkat which are defective in Lck kinase expression. By flow cytometry using [HyF]-labeled lactoferrin at different concentrations, we first checked that the specific binding of lactoferrin was similar on the Jurkat and J.CaM1.6 cell lines (data not shown).

The effect of lactoferrin on the CD4 density at the surface of J.CaM1.6 was then studied. In

A

B

C
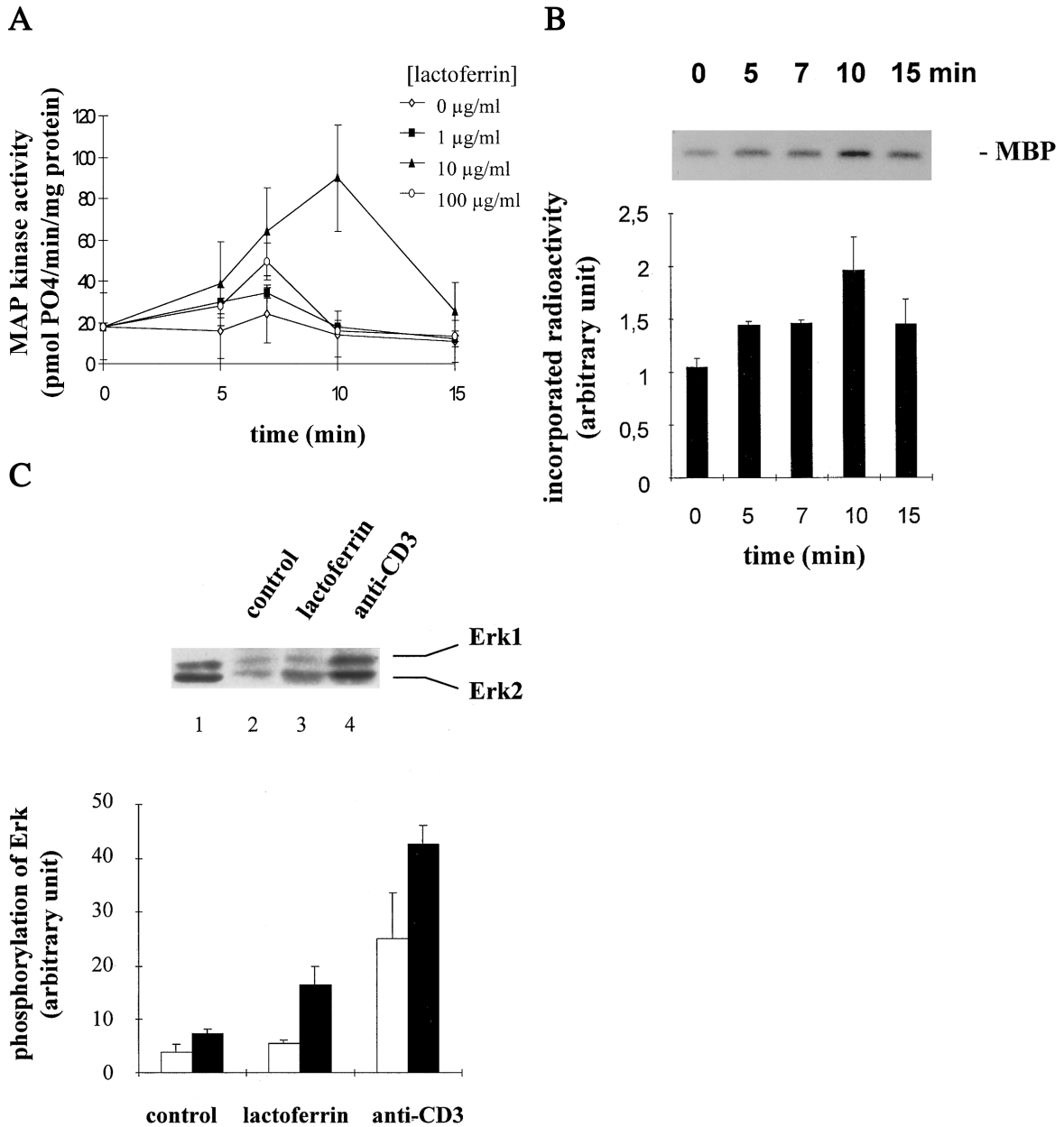
Fig. 3. Stimulation of MAP kinase activity by lactoferrin. **A**: Phosphorylation of exogenous substrate using the "minicolumn assay." Jurkat cells (8×10^6) were incubated with lactoferrin (0–100 μg/ml) for increasing periods of time and lysed. MAP kinase activity was determined by phosphate ([γ-^{32}P]ATP) incorporation into MBP using MAP kinase fractionated on DEAE Sepharose. Data were expressed as mean values of three separate experiments performed in duplicate. **B**: Immunoprecipitation of MAP kinase. After treatment of Jurkat cells (4×10^6) for the indicated times, MAP kinase was immunoprecipitated from total lysate with a polyclonal antibody, incubated with MBP and [γ-^{32}P]ATP, and subjected to SDS-PAGE using a 10% acrylamide gel. The gel was then analysed by autoradiography and the most representative experiment was shown. **Inset**: ^{32}P incorporation into the MBP substrate. The graph indicates the quantification of incorporated radioactivity expressed as the mean values of two experiments. **C**: Phosphorylation of MAP kinase isoforms. Jurkat cells (8×10^6) were incubated or not for 10 min with 10 μg/ml of lactoferrin or 0.1 μg/ml of anti-CD3. After cell lysis, proteins were separated by SDS-PAGE and detected by Western blotting using an anti-MAP kinase antibody (lane 1) or an anti-P-Erk antibody (lanes 2–4). **Inset**: The most representative data was shown, and the graph expressed the mean values of the phosphorylated-Erk1 (white columns) or -Erk2 (black columns) of two distinct experiments.

TABLE I. Effect of Inhibitors on CD4 Expression and MAP Kinase Activity Induced by Lactoferrin*

	CD4 expression				MAP kinase activity		
Lactoferrin	−	+	+	+	−	+	+
Genistein	−	−	−	+	−	−	+
PD98059	−	−	+	−	−	−	−
-Fold increase over basal	1 ± 0.15	2.32 ± 0.55	1.19 ± 0.26	1.3 ± 0.08	1 ± 0.02	2.99 ± 0.23	1.36 ± 0.12

*Jurkat cells were pretreated with either genistein or PD98059 at the concentration of 10 μM for 45 min. Lactoferrin (10 μg/ml) was then added for 48 h or 10 min for the study of the CD4 expression and the MAP kinase activity, respectively. The density of CD4 antigen was measured using flow cytometry and the data were expressed as the average fold increase for three independent experiments. The MAP kinase activity was performed using the "minicolumn assay" and the results were expressed as the mean values of two separate experiments performed in duplicate. The data presented in this table is statistically significative ($P < 0.05$, Student's t-test).
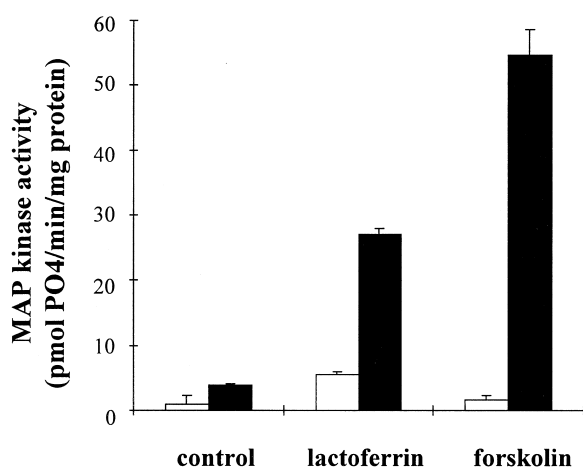
Fig. 4. Effect of lactoferrin on MAP kinase activity in J.CaM1.6 cells. Jurkat cells (black columns) and J.CaM1.6 cells (white columns) were untreated or treated with lactoferrin (10 μg/ml) or forskolin (10 μM) during 10 min. MAP kinase activity was then analysed using the "mini-column assay," and the results were expressed as the mean values of two experiments performed in duplicate.

untreated cells the fluorescent peak obtained with the anti-CD4 antibody (mean 359) was similar to those detected with the IgG1 isotype control (mean 354), showing that the J.CaM1.6 mutant cells did not present a basal density of CD4. Moreover the expression of this surface marker was not enhanced in the presence of 10 μg/ml of lactoferrin (mean 356) or 10 μM of forskolin (mean 353; data not shown). Then, analysis of MAP kinase activity in J.CaM1.6 cells showed that lactoferrin produces an insignificant effect on the enzyme, compared to experiments performed in Jurkat cells (Fig. 4). In the same way, treatment of the cells with 10 μM of forskolin did not stimulate MAP ki-

nase in J.CaM1.6 cells, whereas it greatly enhanced the kinase activity in Jurkat cells.

Taken together, these observations demonstrated that, opposing to the Jurkat cells, lactoferrin binding to J.CaM1.6 cells induced neither the MAP kinase activity nor the expression of the CD4 antigen at their surface, suggesting that p56[lck] kinase is necessary for the CD4 upregulation through the MAP kinase in response to lactoferrin stimulation.

DISCUSSION

In this investigation, we elucidated a part of the mechanism by which lactoferrin affects CD4 expression during the process of T-cell maturation. We showed that lactoferrin binding on Jurkat T-cells induces one or several transduction pathway(s) involving protein kinases, such as p56[lck] and MAP kinase, which control CD4 expression at the cell surface.

Lactoferrin has previously been described to present different effects on immature T-lymphocytes. In non-physiological conditions, in an iron-deprived medium, human lactoferrin increased the proliferation of phytohemagglutinin-stimulated lymphocytes and Jurkat cells [Mazurier et al., 1989; Bi et al., 1997]. Conversely, in the presence of 10% fetal calf serum (FCS) in culture medium, human lactoferrin has been shown to promote T-cell maturation by increasing the expression of CD4 surface antigen in double negative thymocytes and Jurkat cells [Zimecki et al, 1995; Bi et al., 1997]. In this report, using Jurkat cells as a model, we observed that the effect of lactoferrin on CD4 surface density was dose-dependent. Indeed, in a FCS-complemented medium, CD4 expression was upregulated

with an optimal response after 48 h of incubation with 10 μg/ml of lactoferrin. Moreover this event is not accompanied by an arrest of the cell proliferation, suggested by the CD71 density and cell cycle analysis.

The early stages of signal transduction leading to the CD4 expression were next investigated and in particular the protein phosphorylation, one of the fundamental intracellular messengers involved in the signaling process. Our findings showed that lactoferrin increases the tyrosine-phosphorylation of numerous intracellular polypeptides, reaching a maximum at 5–10 min. The large number of substrates which were rapidly phosphorylated indicates that protein tyrosine kinases regulate several primary events in the signal transduction mechanism induced by lactoferrin in Jurkat cells. The second peak of phosphorylation occurring after 30 min may be due to the activation of the same transduction pathways by the recycled lactoferrin after endocytosis. Indeed, the binding of lactoferrin on Jurkat cells is followed by an internalization of the ligand which is then recycled (60–70%) in the culture medium at each round of endocytosis [Bi et al., 1996].

The activity of MAP kinase, a key regulatory enzyme involved in the differentiation and proliferation process, was examined. Two complementary methods demonstrated that the treatment of the cells with lactoferrin (10 μg/ml during 10 min) leads to rapid and transient increase of the MAP kinase activity towards the MBP, one of its specific substrates. The two isoforms of MAP kinase, p44^{Erk1} and p42^{Erk2} [Boulton et al., 1991], are present in Jurkat cells, as shown by their detection using a polyclonal antibody. The activation of these Erk species occurs through phosphorylation of the Tyr202 and Thr204 residues [Seger et al., 1991]. Using a specific antibody directed against the doubly phosphorylated Tyr and Thr of Erk1 and Erk2, we demonstrated that lactoferrin only induces the phosphorylation of Erk2. Moreover, the use of genistein, an inhibitor of tyrosine kinase [Akiyama et al., 1987], indicates that the tyrosine kinases are located upstream from MAP kinase activation and CD4 expression. Finally, experiments with PD98059, a selective inhibitor of the MAP kinase-activating enzyme, MEK [Dudley et al., 1995], confirms that MAP kinase is required

for the signal transduction induced by lactoferrin, leading to the CD4 upregulation.

Our results concerning the transient stimulation of MAP kinase associated with a normal cell cycle progression, are in agreement with previous observations dealing with the importance of the duration of MAP kinase activation for cell signaling decisions [Marshall, 1995; Yaka et al., 1998]. Indeed sustained stimulation of the MAP kinase cascade is required for MAP kinase translocation to the nucleus where it initiates the gene transcription events implied in differentiation, whereas transient MAP kinase activation is associated with proliferation. Moreover, our results concerning the implication of MAP kinase in the Jurkat T-cells maturation agrees with recent papers which demonstrated that the enzyme plays a crucial role in positive thymocyte selection [Alberola-Ila et al., 1995; Sugawara et al., 1998], and particularly affects the CD4/CD8 lineage commitment by favoring the differentiation into the CD4 lineage [Sharp et al., 1997; Bommhardt et al., 1999].

We also identified a second protein involved in the CD4 expression due to lactoferrin: the p56^{lck}, a lymphocyte-specific protein kinase. Using the J.CaM1.6 cell line, a mutant cell line defective in Lck kinase expression [Straus and Weiss, 1992], we showed that binding of lactoferrin was not able to increase MAP kinase activity and cell surface CD4 density, suggesting that Lck is involved in the MAP kinase pathway leading to the maturation of Jurkat cells following lactoferrin treatment. Moreover the absence of the effect of forskolin, an activator of adenylate cyclase which is known to regulate the transcription of the CD4 gene [Flamand et al., 1998], on MAP kinase activity and on CD4 expression in J.CaM1.6 cells suggests that Lck kinase activation is not selective for lactoferrin signaling, but generally required for CD4 expression. The data concerning the implication of p56^{lck} in Jurkat T-cells maturation are in agreement with papers which demonstrated that the p56^{lck} kinase is not only involved in T-cell signaling, but also in thymocyte development. The Lck kinase is well known to be associated with the cytoplasmic tail of cell surface receptors, such as CD45, interleukine-2 receptor, CD4 and CD8, and to act on numerous substrates, including MAP kinase [reviewed by Anderson et al., 1994]. Independently of its association with the CD4

and CD8 co-receptors, p56[lck] regulates thymocyte development [Levin et al., 1993]. Moreover a model of thymocyte differentiation was recently proposed, in which Lck activation leads to the maturation into the CD4 lineage [Basson et al., 1998].

Our hypothesis of the existence of a signal transduction induced by lactoferrin in Jurkat T-cells agrees with the internalization study which suggested that native lactoferrin is not imported into the nucleus of these lymphoblastic cells [Bi et al., 1996]. We therefore proposed, for this single effect of T-cell maturation, a different mechanism to those presented by He and Furmanski [1995]. These authors demonstrated the ability of lactoferrin to specifically interact with DNA and to lead to transcriptionnal activation. This property requires the translocation of lactoferrin into the nucleus of target cells, where it acts as a transcription factor [Bauerle, 1995]. The mechanism by which lactoferrin is targeted to the nucleus is largely misunderstood. Indeed, there is no evidence that cell-surface lactoferrin receptors can transport lactoferrin either directly or via a new trafficking pathway to the nucleus. Moreover, Siebert and Huang [1997] recently identified and cloned an alternative form of human lactoferrin mRNA (ΔLf mRNA), lacking the N-terminal signal peptide and containing sequences similar to nuclear targeting signals. The transcriptional nuclear activities of lactoferrin could be due to the protein encoded by the ΔLf mRNA, whereas the role in immunomodulation and inflammation could be due to the secreted form of lactoferrin through the activation of the transduction pathway.

The molecular events that link the binding of lactoferrin, p56[lck] stimulation, MAP kinase activation and CD4 expression still remain to be elucidated. Further experiments are necessary to identify the proteins phosphorylated on tyrosine residues in the first minutes following lactoferrin binding, and to analyse the modulation of the activity of the transcription factors involved in the transcription of the CD4 gene.

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