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Lactoferrin Inhibits G1 Cyclin-Dependent Kinases During Growth Arrest of Human Breast Carcinoma Cells

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Abstract Lactoferrin inhibits cell proliferation and suppresses tumor growth in vivo. However, the molecular mechanisms underlying these effects remain unknown. In this in vitro study, we demonstrate that treatment of breast carcinoma cells MDA-MB-231 with human lactoferrin induces growth arrest at the G1 to S transition of the cell cycle. This G1 arrest is associated with a dramatic decrease in the protein levels of Cdk2 and cyclin E correlated with an inhibition of the Cdk2 kinase activity. Cdk4 activity is also significantly decreased in the treated cells and is accompanied by an increased expression of the Cdk inhibitor $p21^{CIP1}$. Furthermore, we show that lactoferrin maintains the cell cycle progression regulator retinoblastoma protein pRb in a hypophosphorylated form. Additional experiments with synchronized cells by serum depletion confirm the anti-proliferative activity of human lactoferrin. These effects of lactoferrin occur through a p53-independent mechanism both in MDA-MB-231 cells and other epithelial cell lines such as HBL-100, MCF-7, and HT-29. These findings demonstrate that lactoferrin induces growth arrest by modulating the expression and the activity of key G1 regulatory proteins. J. Cell. Biochem. 74:486–498, 1999.

Key words: lactoferrin; breast cancer cells; cell cycle; cyclin-dependent kinases; $p21^{WAF1/CIP1}$

Lactoferrin also called lactotransferrin [Montreuil et al., 1960] is an iron-binding glycoprotein synthesized by epithelial cells and polymorphonuclear cell precursors. Lactoferrin is mainly found in external secretions such as breast milk and in neutrophil secondary granules [Masson et al., 1969]. Recently, some data have suggested a possible anti-tumor activity for lactoferrin. This effect remains however highly controversial. In fact, it has been reported that, in vitro, lactoferrin promotes the proliferation of the BALB/c 3T3 cell line [Azuma et al., 1989], has no effect on lymphocyte proliferation [Djeha and Brock, 1992], or inhibits mammary cell growth [Hurley et al., 1994; Damiens et al., 1998]. Furthermore, Campbell et al. [1992] showed that lactoferrin may be downregulated in some forms of cancers such as human breast carcinoma suggesting that lactoferrin might regulate cell proliferation. Bezault et al. [1994] also demonstrated that lactoferrin suppresses, in vivo, the growth of tumor cells and inhibits experimental metastasis in mice. However, in this case, they suggest that the anti-tumor activity of human lactoferrin is mediated in part by natural killer (NK) cells. In fact, as regards immunological functions of lactoferrin, it has been shown that lactoferrin activates NK cells [Bezault et al., 1994; Shau et al., 1992] and lymphokine activated killer cells [Shau et al., 1992]. These observations suggest that the anti-proliferative effect of lactoferrin may be mediated by two distinct mechanisms, on one hand, a modulation of NK cell activity and on the other hand, a direct effect on tumor cell growth.

In eukaryotic cells, each phase of the cell division cycle is controlled by the sequential activation of various cyclin-dependent kinases (Cdks). These kinases are known to phosphorylate various proteins whose activity is critical for cell cycle progression [Morgan, 1995]. Among

Abbreviations used: Cdks, cyclin-dependent kinases; CKIs, cyclin-kinase inhibitory proteins; LF, lactoferrin; NK cells, natural killer cells; pRb, retinoblastoma protein.

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these proteins is the retinoblastoma tumor suppressor protein (pRb), which plays an essential role in the progression of cells from G1 to S through its regulation of the transcription factor E2F [Weinberg, 1995]. In vitro, Rb is phosphorylated by several Cdk-cyclin complexes including cyclin Ds- Cdk6 or Cdk4, cyclin E-Cdk2 and cyclin A-Cdk2 [Dowdi et al., 1993; Soucek et al., 1997]. Cyclin Ds with their catalytic partners Cdk6 and Cdk4 regulate events in the G1 phase of the cell cycle while cyclin E-Cdk2 complex operates at the G1 to S transition [Scherr and Roberts, 1995; Resnitszky and Reed, 1995]. However, the role of these complexes and their interactions in cell cycle progression remains to be determined.

The cell cycle is subject to various controls which arrest cell cycle in the events of unfavorable conditions. Controls operate at major points of restriction termed checkpoints localized at the G1 and G2 phases of the cell cycle. These regulatory mechanisms include: alteration of Cdk levels, variations in cyclin abundance [Scherr, 1994], positive and negative-acting phosphorylation of the kinase subunit [Morgan, 1995], and the actions of cyclin dependent kinase inhibitory proteins (CKIs). The CKIs appear to be the most diverse and flexible regulators. Among them, $p21^{CIP1}$ binds to all the G1 and G1 to S Cdk complexes but preferentially inhibits the ones containing Cdk2 [Harper et al., 1995]. The major non-redundant function identified for $p21^{CIP1}$ in vivo, appears to be in regulating p53 mediated growth arrest following DNA damage [Dulic et al., 1994]. However, recent studies indicate that $p21^{CIP1}$ is also regulated by p53-independent mechanisms such as during cell differentiation [Steinman et al., 1994; Macleod et al., 1995].

In this study, we have analyzed the effects of lactoferrin on the growth of human breast carcinoma cell lines and particularly on the MDA-MB-231 cell line. We demonstrate that treatment of MDA-MB-231 cells with lactoferrin results in growth arrest at the G1 to S transition of the cell cycle. To further investigate the molecular mechanisms associated with the effects of lactoferrin, we analyzed the influence of lactoferrin on the proteins involved in the G1 to S transition. We demonstrate that lactoferrin induces an increase of the CKI $p21^{CIP1}$ protein level by a p53-independent mechanism. The induction of $p21^{CIP1}$ is associated with a decrease of the Cdk4 kinase activity without modification of cyclin D1 and Cdk4 protein levels. This is accompanied by a great decrease of Cdk2 and cyclin E protein levels, thereby reducing Cdk2 kinase activity. Thus lactoferrin appears to exert its anti-proliferative effect via a modulation of key proteins which regulate the G1 to S transition.

MATERIALS AND METHODS

Cell Culture

The human breast cancer epithelial cells MDA-MB-231 and MCF-7 were from the ATCC. The SV-40 transformed epithelial cells HBL-100 were also used. They were grown in Eagle's minimal essential medium (Gibco-BRL, Gaithersburg, MD) containing 10% FCS, 1% non-essential amino acids (Eurobio), 2 mM L-glutamine, 5µg/ml insulin (Endopancrine, Organon), penicillin, and gentamicin (Gibco-BRL) at 37°C, 5% CO_2. The colon adenocarcinoma HT29–18-C1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2 mM L-glutamine and gentamycin at 37°C, 10% CO_2. For the starvation-synchronization assays, MDA-MB-231 cells were grown to 50% confluence and placed in serum-free medium for 24 h. Cells were then stimulated to grow by the addition of medium containing 5% FCS.

Lactoferrin

Lactoferrin was prepared from a single human lactoserum by ion-exchange chromatography and iron saturated as previously described [Mazurier and Spik, 1980; Spik et al., 1982]. The degree of purity (>99%) of human lactoferrin was checked by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and high pressure liquid chromatography (HPLC). Lipopolysaccharides were removed by chromatography through a Detoxi-gel column (Pierce Chemicals, Rockford, IL). Lactoferrin prepared in PBS was added directly into the medium to a final concentration of 50µg /ml for cell cycle analysis, immunoprecipitation, and kinase assays.

Thymidine Uptake

Breast epithelial cancer cells were cultured in a 12 well plate at a cell density of 4.10^4 cells per 500µl of medium per well. Cultures were then incubated in the presence of lactoferrin at concentrations ranging from 0 to 100µg/ml. A control was also realized with an irrelevant

protein in order to eliminate a nonspecific effect of lactoferrin. In this control experiment, lactoferrin was replaced by lysozyme (Calbiochem, San Diego, CA) at a concentration of 50µg/ml. After 24 h, 2µCi of (methyl- ^{3}H) thymidine (specific activity 50 Ci/mMol; ICN Biomedical, Costa Mesa, CA) were added to each well for 4 h at 37°C. The cells were washed with PBS, and treated with cold 5% trichloroacetic acid for 45 min at 4°C. Cells were rinsed with water and solubilized with 0.3 M NaOH for 1 h at 37°C. The radioactivity of the cells was measured with a beta counter (Beckman, Fullerton, CA).

Cell Cycle Analysis

The adherent cells (1.10^{6}) were trypsinized, and fixed in cold 70% ethanol for 4 h. Fixed cells were washed with PBS and incubated with 5µg of RNAse A (Sigma Chemical Co., St. Louis, MO) per ml and stained with 25µg/ml of propidium iodine (Aldrich Chemie, Milwaukee, WI) for 1 h at 37°C. The stained cells were analysed on a FACScan cytofluorimeter using the cellFIT Software program (Becton Dickinson Immunocytometry Systems, Braintree, MA).

Immunoprecipitation and Kinase Assays

For immunoprecipitation, cells were washed once in ice-cold PBS and lysed for 2 h at 4°C in lysis buffer containing (155 mM NaCl, 10 mM Tris, 1% Triton X-100, 1% deoxycholic acid, 1 mM EGTA) supplemented with phosphatase and protease inhibitors (10 mM NaF, 200µM sodium orthovanadate, 200µM aprotinin, 50µg/ml leupeptin, 1 mM phenylmethyl sulfonyl fluoride [PMSF]). Cellular debris were removed from soluble extracts by centrifugation at 10,000*g* for 15 min at 4°C. Total lysates (500µg total protein) containing endogenous Cdk4 complexes were immunoprecipitated using a mouse monoclonal anti-human Cdk4 for 1 h at 4°C. Immunoprecipitates were washed twice with immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM PMSF, 0.5% NP-40), and twice with glutathione S-transferase-(GST)-Rb kinase buffer (60 mM glycerophosphate, 30 mM nitrophenylphosphate, 25 mM MOPS, pH 7.0, 5 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 0.1 mM sodium orthovanadate) and then resuspended in 10µl of GST-Rb kinase buffer.

The kinase activity associated with anti-Cdk4 immunocomplexes was assayed with purified retinoblastoma protein (fused with glutathione-S-transferase) prepared as previously described [Vesely et al., 1994], in the presence of 15µM [γ-^{32}P] ATP (4500 mCi/mMol) (Amersham, Arlington Heights, IL), in a final volume of 30µl. After a 15 min incubation at 37°C, 30µl 2× Laemmli sample buffer was added. The phosphorylated substrate was resolved by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by autoradiography by exposure to Scientific Imaging Film Biomax MR (Kodak, Rochester, NY).

For Cdk2 immunoprecipitation, cells were processed as described above and immunoprecipitated for 1 h at 4°C with a mouse monoclonal anti-human Cdk2. Immunoprecipitates were washed twice with immunoprecipitation buffer and twice with H1 kinase buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 15 mM p-nitrophenylphosphate, 15 mM $MgCl_2$, 5 mM EGTA, 1mM sodium orthovanadate, 1 mM dithiothreitol), and then resuspended in 20µl of histone H1 kinase buffer.

Histone H1 kinase assay was performed with 20µg histone H1 (Upstate Biotechnology, Lake Placid, NY) in the presence of 10µCi of [γ-^{32}P] ATP in a final volume of 40µl histone H1 kinase buffer. After 10 min incubation at 30°C, 25µl aliquots of supernatant were spotted onto pieces of Whatman p81 phosphocellulose paper. Then, the filters were washed ten fold in a solution of 0.75% phosphoric acid. The wet filters were transferred into plastic scintillation vials, 3.5 ml scintillation fluid (Zinsser Analytic) were added and the radioactivity measured in a gamma counter (LS 3801 Beckman). Activities were calculated as a percentage of maximal activity.

Western Blot Analysis

Protein concentrations were determined using the BioRad protein assay reagent with bovine serum albumin as a standard. Fifty-microgram and for Rb 200 mg samples of total cell lysates were resolved by 10–12% SDS/PAGE and transferred onto polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH). Cdk4, cyclin D1, Cdk2, cyclin E, and $p21^{CIP1}$, $p16^{INK4a}$, $p27^{KIP1}$, p53, and pRb were detected with the ECL system (Amersham) after incubation with the appropriate monoclonal mouse antibodies anti-human Cdk2, anti-human Cdk4, anti-human cyclin D1, anti-human $p21^{CIP1}$, anti-human p53 (Transduction Laboratories), mono-

clonal rabbit antibodies anti-human cyclin E (Upstate Biotechnology Incorporated), monoclonal mouse antibodies anti-human pRb, anti-human $p16^{INK4a}$, anti-human $p27^{KIP1}$ (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS

Effect of Lactoferrin Treatment on Tumor Epithelial Cell Proliferation

The concentration of lactoferrin used was determined according to the dissociation constants calculated from Scatchard plot analysis and to the lactoferrin concentrations that can be found in vivo at the inflammatory site. We thus analyzed the effect of lactoferrin at concentrations ranging from 0 to 100µg/ml on cell proliferation.

Human lactoferrin reduced ^{3}H-thymidine incorporation in a dose-dependent manner in MDA-MB-231 and MCF-7 cells (Fig. 1). We did not observe any effect at 5µg/ml, while the effect was important at 50µg/ml which corresponds to the constant dissociation of the total binding sites on these cells. As compared to the control (medium alone) ^{3}H-thymidine incorporation was decreased by about 45 to 50% at a lactoferrin concentration of 50 µg/ml. Furthermore, a control realized in the presence of an irrelevant protein, the lysozyme, did not show any variation of the cell proliferation suggesting a specific inhibitory effect of lactoferrin on these mammary tumor cell line growth. As we previously described, similar results were obtained for the HBL-100 cell line [Damiens et al., 1998].

Since lactoferrin inhibits epithelial cell line proliferation, we investigated the effects of lactoferrin on cell cycle progression. Exponentially growing MDA-MB-231 and MCF-7 cultures exposed to 50µg/ml lactoferrin rapidly showed growth inhibition as indicated by the lack of cell number increase after 24 h and 48 h. After 48 h treatment of the cells with 50µg/ml lactoferrin, the cell number is about 3.1-fold weaker as compared to the control cell number (data not shown). FACS analysis revealed that compared to untreated cells, a 24 h and a 48 h treatment by lactoferrin resulted in a higher proportion of

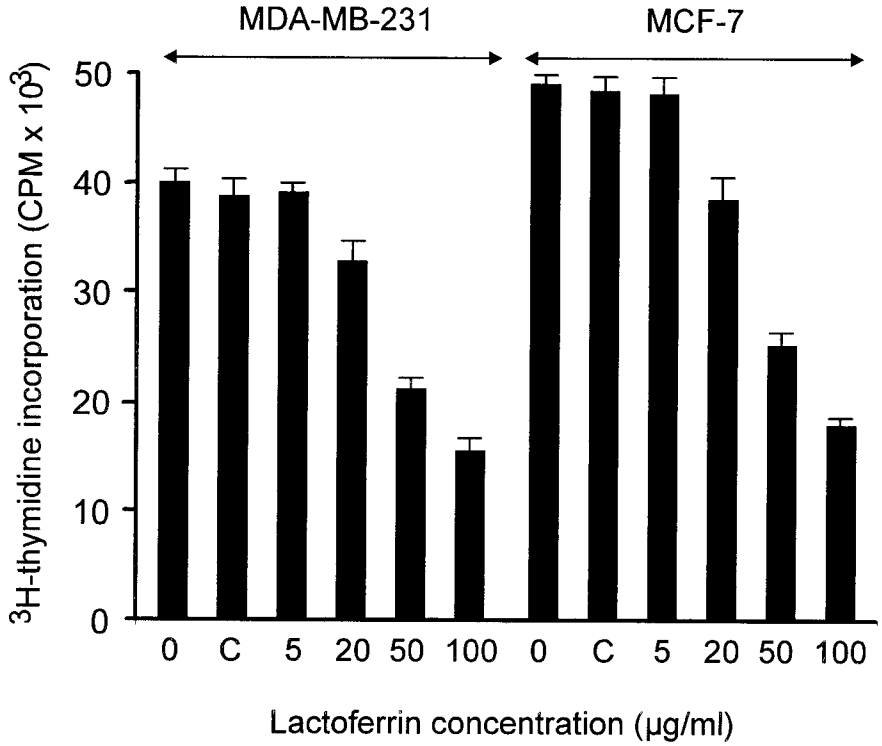

Fig. 1. Effect of lactoferrin on ^{3}H-thymidine incorporation in MDA-MB-231 and MCF-7 cell lines. Subconfluent cells were cultured with lactoferrin (concentration ranging from 0 to 100µg/ml) in 12-well plates for 24 h or with 50µg/ml of lysozyme (C) as an irrelevant protein. After incubation with methyl ^{3}H-thymidine for 4 h, the cells were harvested and incorporated methyl ^{3}H-thymidine was counted. Each point represents the mean ± SEM of three experiments.

cells in G1 consistent with a G1 arrest (Fig. 2). This effect was further observed when cells were presynchronized by serum-starvation (data not shown). These results confirm the inhibition of proliferation of tumor epithelial cells at the G1 level. An arrest of the cell cycle progression was also observed in the presence of lactoferrin for the HBL-100 breast epithelial transformed cells [Damiens et al., 1998]. In the following results, we essentially presented the potential mechanism of the lactoferrin anti-proliferative activity observed on MDA-MB-231 cells.

Protein Levels and Activity of Cdk4/Cyclin D1 in MDA-MB-231 Cells Following Lactoferrin Treatment

Western blot analysis was used to determine the effect of lactoferrin on the expression of cyclins and kinases regulating G1 progression. We first examined the protein levels of Cdk4 and cyclin D1, two components of the Cdk-cyclin complex which is active in early G1. Cyclin D1 and Cdk4 protein levels in asynchronous MDA-MB-231 cells were found to be unaltered by lactoferrin treatment as compared to the control (Fig. 3). The expression of Cdk4 and cyclin D1 was further monitored in synchronized MDA-MB-231 cells and similarly, we did not detect any variation in the Cdk4 and cyclin D1 protein levels, as compared to the untreated cells (data not shown).

The effect of lactoferrin on Cdk4 kinase activity was also examined in the synchronized cells to determine if modifications in Cdk4 kinase activity might contribute to the G1 arrest. Cdk4 was immunoprecipitated with anti-Cdk4 monoclonal antibodies and the kinase activity associated with the immunocomplex was evaluated using GST-Rb as a substrate. The GST-Rb kinase activity associated with Cdk4 immunocomplex remained extremely low in lactoferrin-

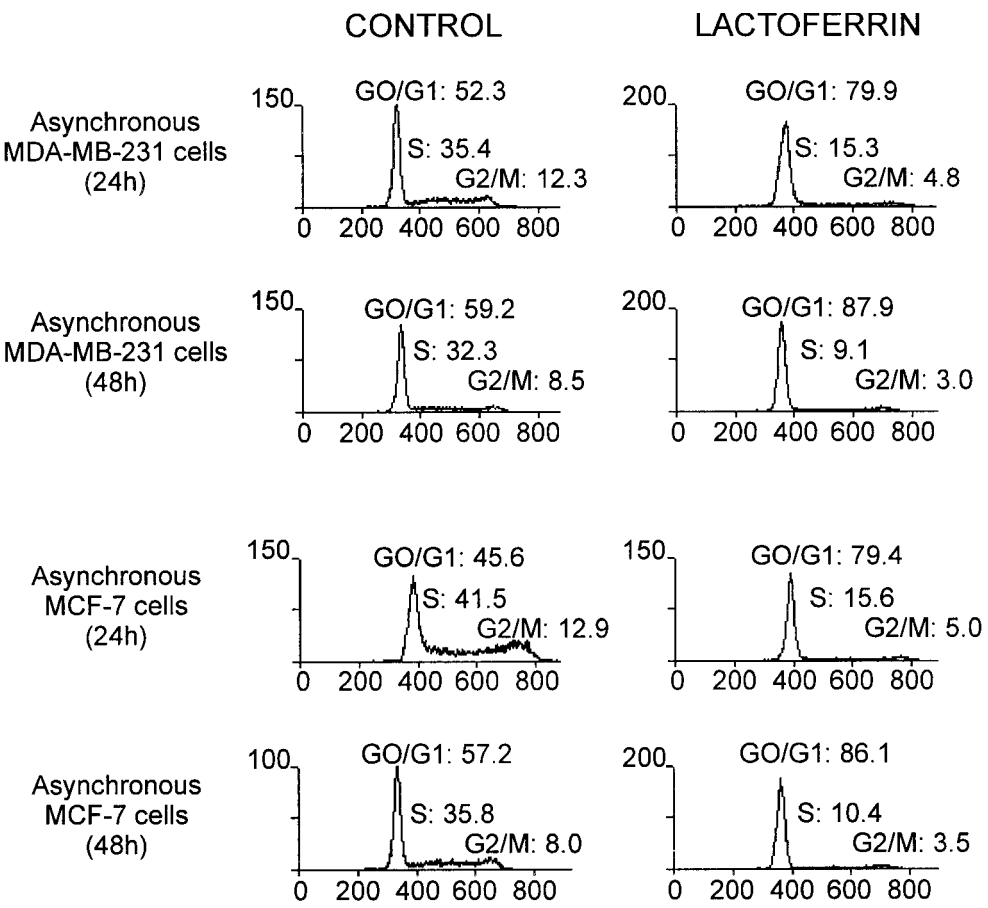

Fig. 2. FACScan analysis of untreated (control) or lactoferrin (50µg/ml)-treated MDA-MB-231 and MCF-7 cultures. Cells were treated for 24 h and 48 h prior to fixation and FACS analysis as described in Materials and Methods. Numbers indicate the percentage of cells in the different phases of the cell cycle.

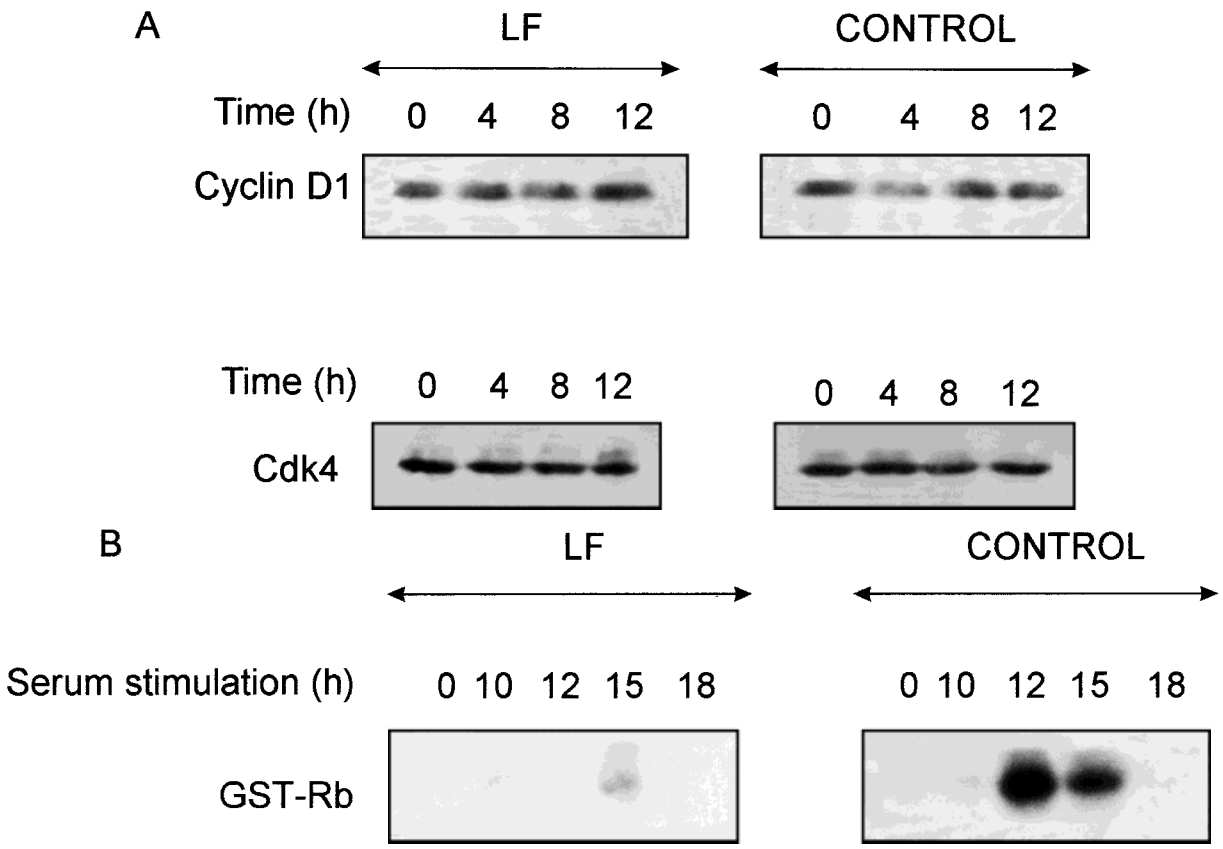

Fig. 3. **A**: Effect of lactoferrin on the protein levels of Cdk4 and cyclin D1. Cells untreated (control) or treated with 50μg/ml lactoferrin for 4 h, 8 h, and 12 h were analyzed for the determination of Cdk4 (33 kDa) and cyclin D1 (36 kDa) protein levels by Western blot analysis as described in Materials and Methods. **B**: The kinase activity of Cdk4 was determined following immunoprecipitation of the enzyme in untreated and lactoferrin-treated synchronized cells serum stimulated for the indicated times. The kinase activity associated with the Cdk4 immunocomplexes was assayed with GST-Rb as a substrate. ^{32}P-labeled GST-Rb is shown.

treated MDA-MB-231 cells throughout the entire time course of the experiment. In the absence of lactoferrin, the GST-Rb kinase activity increased in a time-dependent manner with a maximal incorporation at 12–15 h following the release from serum starvation (Fig. 3). This result is consistent with a decrease of G1 cyclin/Cdk complex activities and the growth arrest of the lactoferrin-treated cells at the G1 to S transition of the cell cycle.

Protein Levels and Activity of Cdk2/Cyclin E in MDA-MB-231 Cells Following Lactoferrin Treatment

We also examined by Western blot analysis the effect of lactoferrin on the expression of proteins implicated in control of the restriction point at the G1 to S transition. In this context, we analyzed the protein levels of Cdk2 and cyclin E. Interestingly, Cdk2 and cyclin E protein levels decreased dramatically in asynchronous lactoferrin-treated cells (>80% decline by 12 h of treatment) as compared to the control cells, in absence of lactoferrin (Fig. 4). The decrease of Cdk2 and cyclin E protein levels in lactoferrin cells was confirmed in synchronized cells (data not shown).

The effect of lactoferrin on Cdk2 kinase activity was also investigated in asynchronous and synchronized cells. Cdk2 containing complexes from untreated and lactoferrin asynchronous treated cells were immunoprecipitated by anti-Cdk2 mAbs and assayed with histone H1 as a substrate. We observed that Cdk2 kinase activity was high in control cells and markedly decreased following exposure to lactoferrin (Fig. 4). This inhibitory effect of lactoferrin on Cdk2 kinase activity was confirmed on synchronized cells. Histone H1 kinase activity was very low during the experiment for lactoferrin-treated

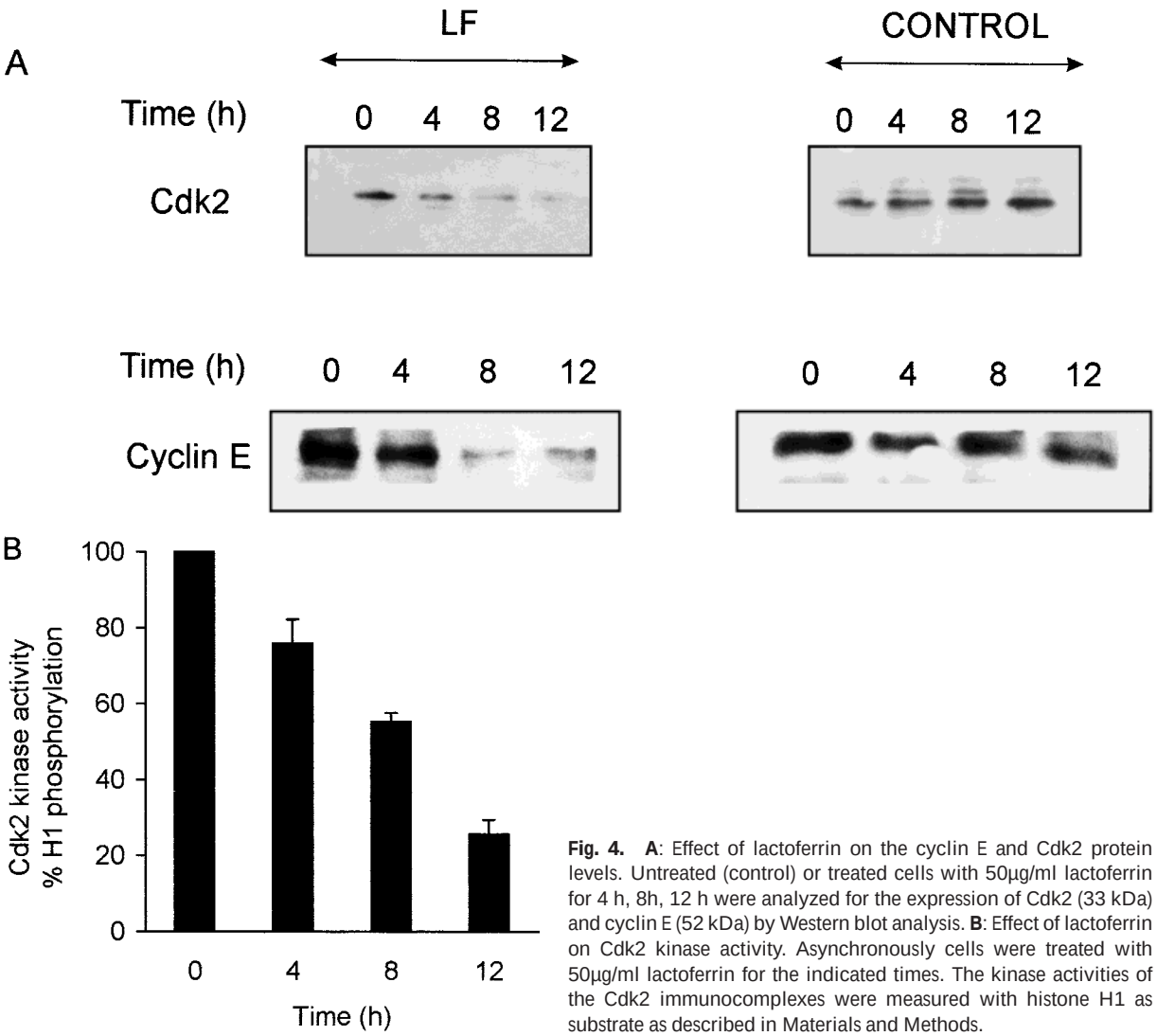

Fig. 4. **A**: Effect of lactoferrin on the cyclin E and Cdk2 protein levels. Untreated (control) or treated cells with 50µg/ml lactoferrin for 4 h, 8h, 12 h were analyzed for the expression of Cdk2 (33 kDa) and cyclin E (52 kDa) by Western blot analysis. **B**: Effect of lactoferrin on Cdk2 kinase activity. Asynchronously cells were treated with 50µg/ml lactoferrin for the indicated times. The kinase activities of the Cdk2 immunocomplexes were measured with histone H1 as substrate as described in Materials and Methods.

cells while it increased in a time-dependent manner with a maximum at 15–18 h following serum stimulation in the absence of lactoferrin (data not shown). This result together with our observation for Cdk4 kinase activity are consistent with the model of cell cycle regulation and progression whereby activation of Cdk4 precedes that of Cdk2 in association with cyclin E.

Protein Levels of the Cyclin-Dependent Inhibitors $p16^{Ink4a}$, $p21^{CIP1}$, $p27^{KIP1}$ in MDA-MB-231 Cells Following Lactoferrin Treatment

Cdk inhibitors (CKIs) mediate cell cycle arrest in response to various anti-proliferative signals. Protein levels of different Cdk inhibitors such as $p21^{CIP1}$, p16, and $p27^{KIP1}$ known to act in G1 phase, were evaluated in lactoferrin-MDA-MB-231 treated cells. In order to investigate the involvement of these CKIs in the lactoferrin-induced inhibition of cyclin E/Cdk2 and cyclin D1/Cdk4 kinase activity, we first determined by immunoblotting the effect of lactoferrin on their expression. As can be seen in Figure 5, no change in p16 and $p27^{KIP1}$ expression was observed in lactoferrin-MDA-MB-231 treated cells. In contrast, lactoferrin induces a great elevation in $p21^{CIP1}$ protein level in asynchronous MDA-MB-231 cells.

Effect of p53 Status on the Induction of $p21^{CIP1}$ in Response to Lactoferrin Treatment in Breast and Colon Cell Lines

The expression of $p21^{CIP1}$ can be induced by p53 or independently of p53. The increase of $p21^{CIP1}$ appears to be mediated through a p53-independent mechanism in the MDA-MB-231

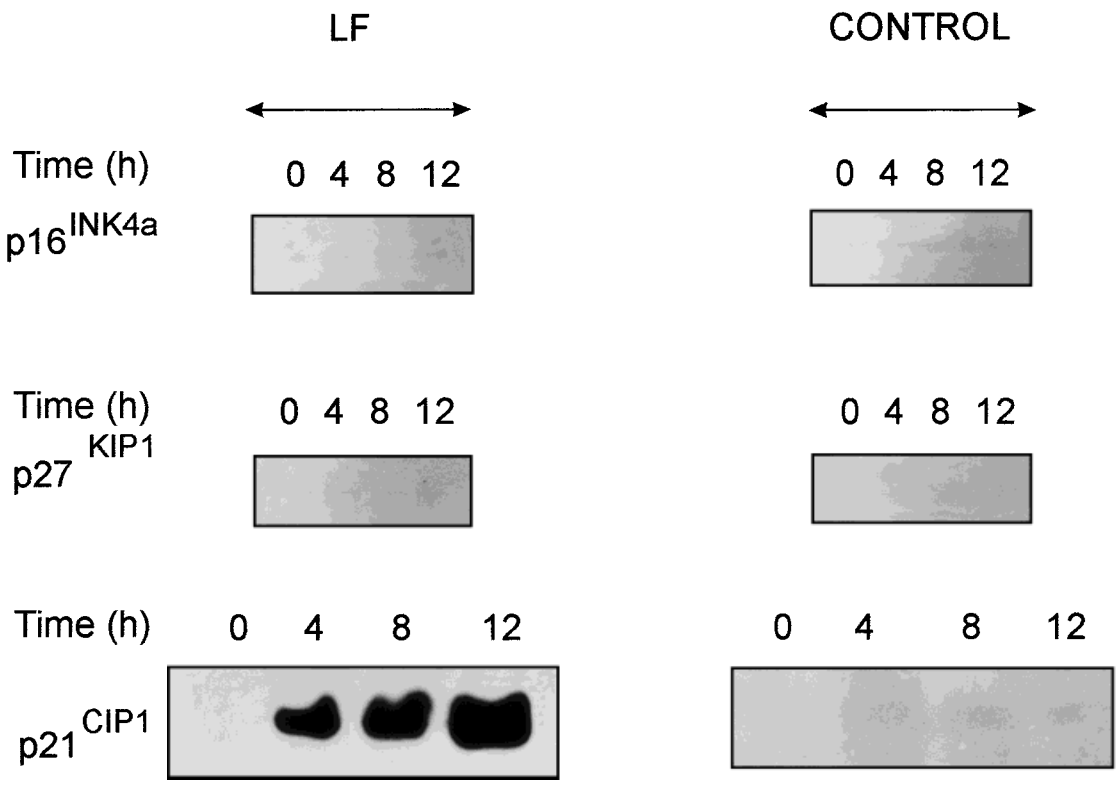

Fig. 5. Effect of lactoferrin on p16, $p21^{CIP1}$ and $p27^{KIP1}$ protein levels. MDA-MB-231 cells untreated (control) or treated with 50µg/ml lactoferrin for 4 h, 8 h, 12 h, were analyzed for expression of p16 (16 kDa), $p21^{CIP1}$ (21 kDa), and $p27^{KIP1}$ (27 kDa) by Western blotting.

cell line. Indeed, MDA-MB-231 is a particular cell line which expresses a mutant form (non-functional) of p53 [Sheikh et al., 1994]. To determine whether the growth arrest observed on the two other breast cell lines (MCF-7 and HBL-100) is mediated by $p21^{CIP1}$ independently of p53, we examined by Western blot their $p21^{CIP1}$ and p53 protein levels in the absence and presence of lactoferrin. Indeed, when p53 is involved in such phenomenon, its protein level increases rapidly in the cell before its activation as a transcription factor for genes such as $p21^{CIP1/WAF1}$. Furthermore, in order to evaluate whether this mechanism of growth arrest is specific of breast cell lines or can be found in an other carcinoma cell line, we investigated the modulation of these protein levels in the non-breast epithelial cancer cell line HT29–18-C1 (functional p53) which cell cycle progression is arrested by lactoferrin in the G1 phase [Damiens et al., 1998]. We demonstrated that exposure of the various breast and colon asynchronized cells to lactoferrin resulted in a significant increase of $p21^{CIP1}$ protein level (Fig. 6). In contrast, p53 protein levels in either breast or colon carcinoma cells were found to be unchanged by lactoferrin treatment as compared to untreated cells (Fig. 6). These results suggest that p53 function is not required for $p21^{CIP1}$ induction by lactoferrin in cells with both functional and non-functional p53.

Association of $p21^{CIP1}$ With Cdk2 and Cdk4 in MDA-MB-231 Treated Cells

As $p21^{CIP1}$ was expressed in the lactoferrin-treated cells, we sought to determine if $p21^{CIP1}$ was associated with Cdk2 and/or Cdk4. We immunoprecipitated Cdk2 and Cdk4 complexes from extracts of asynchronously 4 and 12 h lactoferrin-treated cells and untreated cells. The amount of $p21^{CIP1}$ and Cdk2 or Cdk4 in these immunoprecipitates was measured by immunoblotting. Co-immunoprecipitation analysis showed that $p21^{CIP1}$ is associated with Cdk2 and Cdk4 in cells (Fig. 7). Although no enrichment of Cdk2 was seen in 12 h-lactoferrin treated cells, lactoferrin was also associated with Cdk2. The absence of enrichment may be

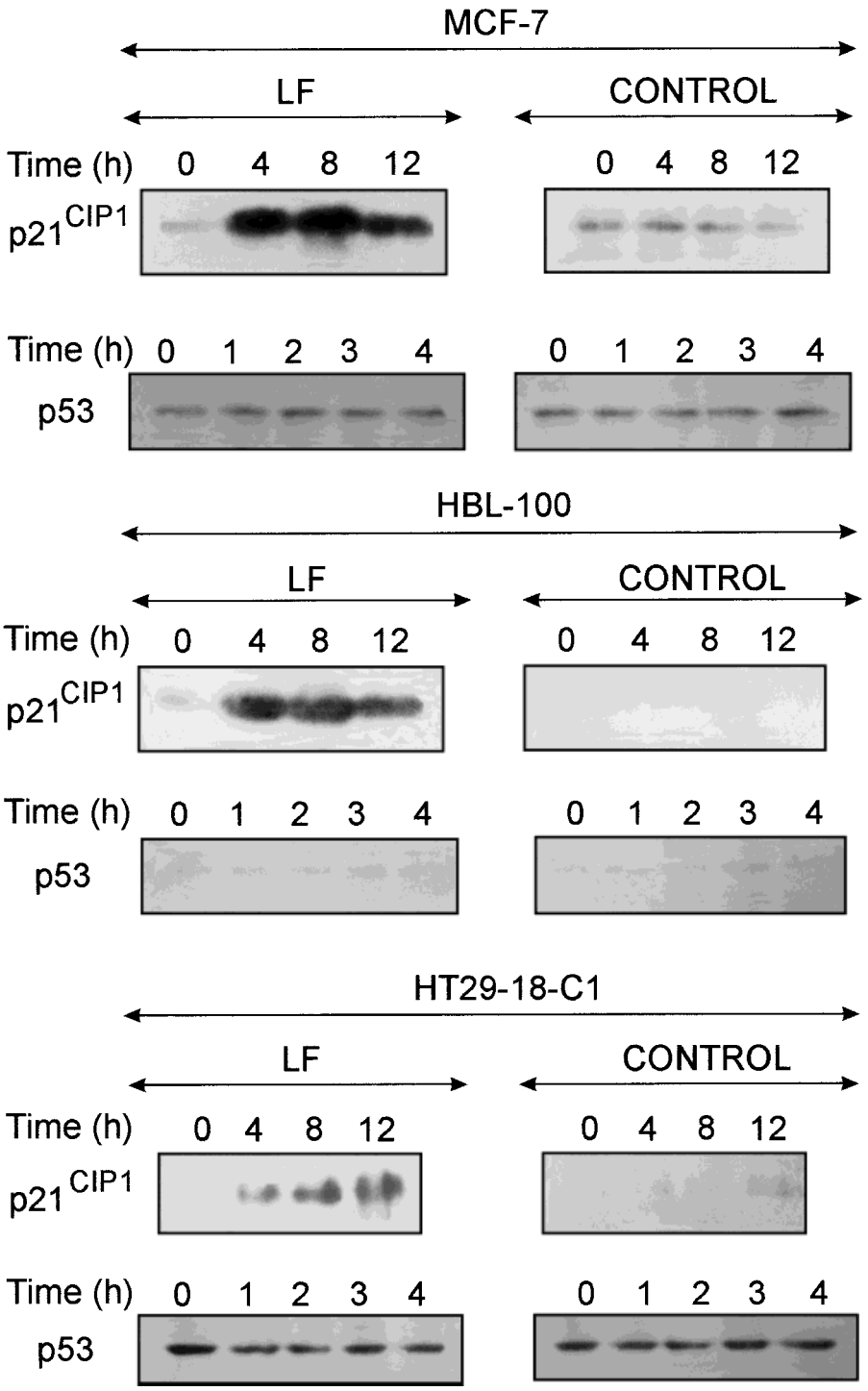

Fig. 6. Effect of p53 status on the induction of $p21^{CIP1}$ in response to the lactoferrin treatment. P21 (21 kDa) and p53 (53 kDa) protein levels of various breast and colon asynchronized cells were determined by Western blot analysis in the absence or presence of 50µg/ml lactoferrin.

explained by the decline of Cdk2 protein level following 12 h-lactoferrin treatment. Indeed, co-immunoprecipitation analysis of extract from asynchronously 4 h-lactoferrin-treated cells showed that $p21^{CIP1}$ was associated with Cdk2.

DISCUSSION

In this report, we used lactoferrin at a concentration corresponding to that found at the inflammatory site in cancer to study its role in mammary cancer cell growth. We demonstrate

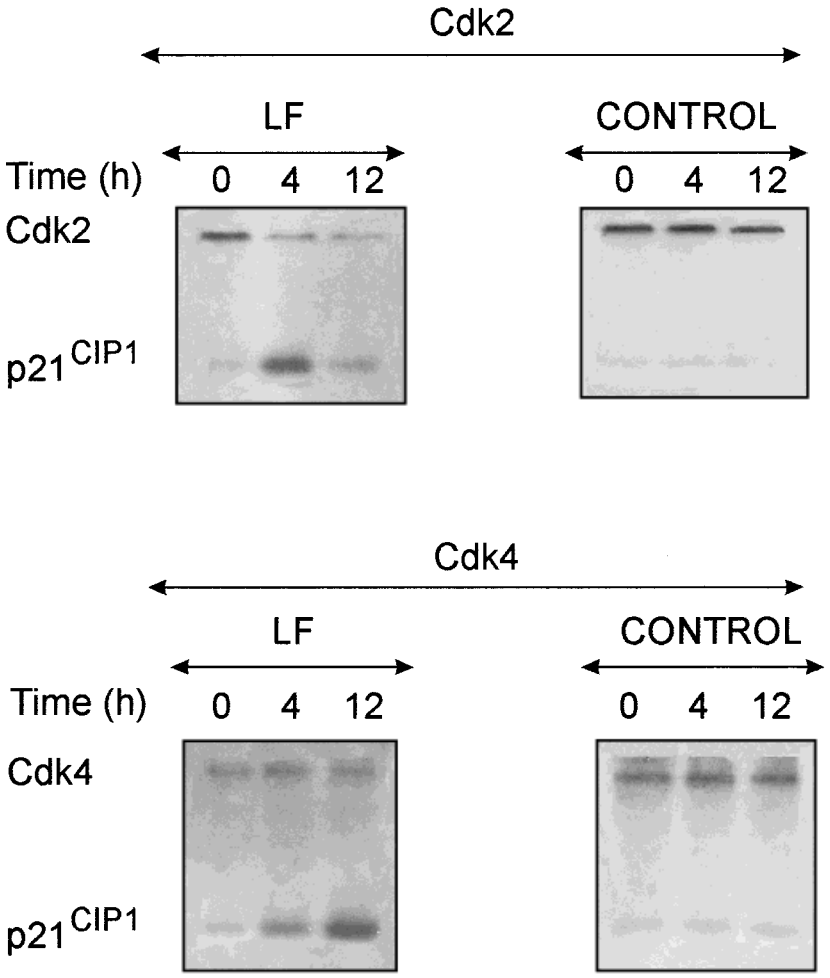

Fig. 7. Association of $p21^{CIP1}$ with Cdk2 and Cdk4 in untreated (control) and lactoferrin-treated cells for 12 h. Cell extracts containing 500µg protein were immunoprecipitated with anti-human Cdk2 or anti-human Cdk4 antibodies. The immunoprecipitates were separated on a 12% polyacrylamide gel, and the level of $p21^{CIP1}$ associated with immunoprecipitated Cdk2 or Cdk4 was determined by Western blot analysis and ECL.

that human lactoferrin is inhibitory to growth of various human mammary epithelial cell lines. Moreover, we found that lactoferrin inhibits the proliferation of these cells by an arrest in the cell cycle progression at the G1 to S transition. Our results are in agreement with previous data on the antiproliferative effect of lactoferrin on mammary cell lines [Rejman et al., 1992; Hurley et al., 1994]. This effect seems to be cell type specific as observed for other biomolecules such as growth factors and chemokines. Indeed, it has been described that lactoferrin does not influence proliferation of the hematopoietic K-562 cell line [Yamada et al., 1987] and stimulates various human lymphocytic cell line growth [Hashizume et al., 1983]. Moreover, we have observed in a previous study that lactoferrin sensitizes epithelial tumor cells to NK cell lysis while it did not on several hematopoietic cell lines reinforcing the cell type specificity of lactoferrin effect [Damiens et al., 1998]. Investigating the mechanism of the lactoferrin effect in particular on the MDA-MB-231 cell line, we showed that the growth arrest is associated with an inhibition in the activities of both Cdk4 and Cdk2. These results are in agreement with the current model of cell cycle regulation whereby activation of Cdk4 and Cdk2 by cyclin D and cyclin E respectively, plays a key role in the progression through G1 and at the G1 to S transition [Quelle et al., 1993; Resnitszky et al., 1994]. Moreover, in the view of the results, we show that the cellular mechanisms leading to the reduction of these two kinases activities differ quite widely. In the case of Cdk4/cyclin D1, protein levels are unaffected by lactoferrin treatment. It appears that Cdk4 inactivation occurs concomitantly with the increase of the

Cdk inhibitor $p21^{CIP1}$ protein level. In contrast, strong reduction of Cdk2 kinase activity appears to be due to a dramatic decrease of both cyclin E and Cdk2 protein levels following treatment with lactoferrin.

Examination of Cdk activity reveals that kinase activities appear to be correlated with the phosphorylation profile of the retinoblastoma protein pRb. We have shown that in lactoferrin-treated cells pRb remains hypophosphorylated throughout the entire time course of the experiment. In contrast, in control cells, pRb appears to be phosphorylated by the cyclin Ds-Cdk4 complexes by 12 to 15 h serum addition. pRb is phosphorylated thereafter with a peak phosphorylation at 15 to 18 h induced by cyclin E-Cdk2 complex. These findings are in agreement with the current view that the phosphorylation of the retinoblastoma gene product pRb is an essential event required for cell proliferation [Weinberg, 1995]. Our experimental data are also consistent with the notion that the cyclin Ds dependent kinases are responsible for triggering the phosphorylation of Rb thereby releasing free E2F, a transcription factor essential to initiate the cell cycle [DeGregori et al., 1995]. Furthermore it seems that pRb is the major substrate of cyclin E-Cdk2 and cyclin A-Cdk2 [Soucek et al., 1997]. The role of these complexes may be to maintain Rb in a phosphorylated state throughout the remainder of the cell cycle.

It remains to be determined how treatment by lactoferrin decreases the expression of cyclin E and Cdk2. Cyclin E mRNA is known to be regulated through changes in its rate of transcription. In fact, the cyclin E promoter contains E2F sites and activation of E2F leads to cyclin E overexpression [Geng et al., 1996]. With respect to the regulation of cyclin E expression, we can emit the hypothesis that the extensively hypophosphorylation of Rb induced by lactoferrin treatment keeps E2F inactive and inhibits cyclin E expression in MDA-MB-231 cells. Regarding Cdk2, the mechanism of expression control is unknown. However, Reynisdottir et al. [1995] suggest that the transforming growth factor (TGF-β) blocks the cell cycle by invoking the actions of Cdk inhibitory proteins (CKIs) to arrest cells, a subsequent mechanism down-modulating Cdk levels. The induction of $p21^{CIP1}$ by lactoferrin may act similarly on the protein level of Cdk2 in the various breast cell lines analysed in this study.

We have shown that the growth inhibitory response to lactoferrin treatment induces the increase of the $p21^{CIP1}$ protein level. Transcription of the $p21^{CIP1}$ gene is activated by p53-dependent and -independent mechanisms. Indeed, the major non-redundant function identified for $p21^{CIP1}$ in vivo appears to be in regulating p53-mediated growth arrest following DNA damage [Dulic et al., 1994]. Nevertheless, p53-independent induction of p21 transcription has been observed with anti-mitogenic agents such as staurosporine or prostaglandin A_2 (PGA_2) in the mammary epithelial cancer cell line MCF-7 [Jeoung et al., 1995; Gorospe et al., 1996] as well as with TGF-β treatment [Elbendary, 1994]. Furthermore, it has been described that a synthetic retinoid (AHPN) is able to induce G1 arrest with simultaneous upregulation of $p21^{CIP1}$ in the human breast epithelial cell line MDA-MB-231 that contains mutant p53 [Shao et al., 1995]. Furthermore, it has been found that growth arrest induced by serum-starvation as well as DNA-damaging agents did not increase p53 levels but did significantly increase WAF1/CIP1 mRNA levels carrying mutant p53 [Sheikh et al., 1994]. Incubation with lactoferrin did not result in elevated p53 levels in both HBL-100 and MCF-7 breast cell lines as well as non-breast HT29–18-C1 adenocarcinoma cells. Thus, it appears that the lactoferrin-mediated increase of the $p21^{CIP1}$ level in MDA-MB-231 cells and presumably in the other breast and colon epithelial cell lines must occur through a p53-independent mechanism.

In conclusion, the present study demonstrates that lactoferrin inhibits both tumor mammary and colon epithelial cell proliferation by induction of G1 growth arrest. The growth inhibitory response to lactoferrin appears to involve activation of the downstream effector $p21^{CIP1}$ in a p53-independent manner. This is accompanied by a reduction of Cdk4/cyclin D1 activity and a decrease of both Cdk2/cyclin E protein level and activity. A better knowledge of how lactoferrin acts to alter the expression of specific protein implicated in the G1 progression and the G1 to S transition to arrest proliferation could be beneficial in devising new treatment and combination with other agents acting in the different stages of the cell cycle. Such information could be of practical significance for the treatment of cancers lacking functional p53.

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