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Molecules and Cells

Iron-Saturated Lactoferrin Stimulates Cell Cycle Progression through PI3K/Akt Pathway

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Iron binding lactoferrin (Lf) is involved in the control of cell cycle progression. However, the molecular basis underlying the effects of Lf on cell cycle control, as well as its target genes, remains incompletely understood. In this study, we have demonstrated that a relatively low level of iron-saturated Lf, Lf(Fe^{3+}), can stimulate S phase cell cycle entry, and requires Akt activation in MCF-7 cells. Lf(Fe^{3+}) immediately induced Akt phosphorylation at Ser473, which subsequently induced the phosphorylation of two G1-checkpoint Cdk inhibitors, $p21^{Cip/WAF1}$ and $p27^{kip1}$. The Lf(Fe^{3+})-induced phosphorylation of Cdk inhibitors impaired their nuclear import behavior, thereby inducing cell cycle progression. However, the treatment of cells with a PI3K inhibitor, LY294002, almost completely blocked Lf(Fe^{3+})-stimulated cell cycle progression. LY294002 treatment abrogated Lf(Fe^{3+})-induced Akt activation, and prevented the cytoplasmic localization of $p27^{kip1}$. Higher levels of $p21^{Cip/WAF1}$ were also detected in the cytoplasmic sub-cellular compartment as a measure of cellular response to Lf(Fe^{3+}). Consequently, the degree of phosphorylation of retinoblastoma protein was enhanced in response to Lf(Fe^{3+}). Therefore, we conclude that Lf(Fe^{3+}), as a potential antagonist of Cdk inhibitors, can facilitate the functions of E2F during progression to S phase via the Akt signaling pathway.

INTRODUCTION

The mammalian cell cycle is tightly controlled, and its dysregulation induces tumor development. Cell cycle progression is generally regulated by growth factors or hormones. Therefore, investigations into the mechanism that regulates S phase cell cycle entry have focused largely on divergent mitogenic signaling pathways. Phosphoinositide 3-kinase (PI3K)/Akt is a major proliferative mediator in the signal transduction cascade activated by a variety of growth factors (Kim et al., 2004; Liang and Slingerland, 2003). Activated PI3K and Akt are sufficient to promote cell proliferation via the inhibition of $p21^{Cip/WAF1}$ and $p27^{kip1}$ by direct phosphorylation. Moreover, Akt inactivates glycogen synthase kinase-3 beta, which phosphorylates and degrades cyclin D1 (Liang and Slingerland, 2003). The inactivation of Cdk inhibitors and stabilization of cyclin D1 enhance the Cdk activity, and thereby support the G1/S transition.

Lactoferrin (Lf) is a multifunctional protein which is known to be involved in the regulation of the immune response and the modulation of inflammatory reactions found in the secondary granules of neutrophils and body fluids, including milk (Legrand et al., 2005). Lf has also been shown to regulate cell proliferation and death (Brock, 2002). Previous studies have demonstrated that Lf inhibits cell proliferation via cell cycle arrest or apoptosis (Xiao et al., 2004). However, the results from other studies have demonstrated that Lf may function as a growth factor (Huang et al., 2008; Naot et al., 2005). Therefore, the role of Lf as a regulator of cell growth remains unclear, owing primarily to ambiguous results. It is possible that the controversial results presented by different research groups may be attributable to variations in the iron saturation degree of Lf, cell lines, doses, or treatment conditions. Lf harbors an iron binding motif which sequesters two iron molecules, and its degree of iron saturation has a pivotal influence on its physiological function (Ambruso and Johnston, 1981). Recently, we demonstrated that the Lf concentration is crucial to the control of the survival and death of the cell (Lee et al., 2009). However, the mechanism underlying the regulation of the cell fate decision remains to be clearly elucidated.

In this study, we attempted to determine whether a relatively low level of Lf(Fe^{3+}) could facilitate the cell cycle, and whether the Lf(Fe^{3+})-induced cell cycle might be associated with the Akt signaling pathway. Our studies have been focused generally on the molecular mechanism underlying the role of Lf(Fe^{3+}) in cell cycle control by Cdk inhibitors in conjunction with PI3K/Akt activation. Because Akt-dependent cell cycle control involves a variety of unique responses in cells, an understanding of Lf-mediated Akt induction should provide novel insights into PI3K/Akt-mediated diseases, as well as possible rationales for the development of new therapeutic modalities.

MATERIALS AND METHODS

Cell culture and reagents

The human breast adenocarcinoma MCF-7 cells were obtained from ATCC, and maintained in RPMI 1640 media (Cambrex,

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USA) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, USA) and 100 U/ml of penicillin/streptomycin (Gibco-BRL) in a humidified incubator at 37°C and 5% CO_2. 5 days before treatment, the culture medium was changed to phenol red-free RPMI 1640 containing 10% dextran-coated charcoal FBS (DCC-FBS, Biotechnics Research, USA). Iron-saturated Lf(Fe^{3+}) were purchased from Fluka (USA). The cells were treated for 24 h with the PI3K inhibitor, LY294002 (Sigma-Aldrich), in accordance with the manufacturer's instructions at the indicated concentration.

Cell cycle analysis

MCF-7 cells were maintained for 5 d in phenol red free RPMI 1640 containing 10% DCC-FBS, and plated in 60 mm dish. After 24 h, the cells were starved in phenol red free RPMI 1640 containing 0.5% DCC-FBS for 36 h, and the medium was replaced with iron-saturated Lf and/or phenol red free RPMI 1640 containing 10% DCC-FBS. The cells were harvested at the indicated time points, then fixed for 4 h in cold 70% ethanol. The fixed cells were washed with PBS and treated for 1 h with 0.5 mg/ml of RNase A (Sigma-Aldrich) at 37°C, then stained with 10 μg/ml of propidium iodide (PI) solution (Sigma-Aldrich). Flow cytometry analysis of stained cells was conducted with a flow cytometer, using Cell Quest-pro software (Becton Dickinson, USA).

Immunofluorescence assay

MCF-7 cells, maintained with phenol red free medium containing 10% DCC-FBS, were placed on coverglass slips. After 24 h, the cells were arrested in G0/G1 via serum deprivation, and treated for 24 h with LY294002 and/or iron-saturated Lf(Fe^{3+}) in phenol red free medium containing 10% DCC-FBS. After incubation, the cells were fixed with 3.7% formaldehyde, and treated for 1 h with 5% BSA. After overnight incubation with primary antibodies, including anti-Ki-67 (Zymed Laboratories, USA) anti-p21, or anti-p27 (Chemicon International), the slides were incubated for 1 h with FITC or Texas-Red conjugated secondary antibodies (Santa Cruz Biotechnology, USA). The washed coverslips were incubated with DAPI and mounted in antifade reagent (BioRad, USA), then examined via confocal microscopy (Carl ZEISS).

Preparation of nuclear extracts, immunoprecipitation and immunoblot analysis

The cells were washed with PBS and resuspended in cytoplasm extraction buffer (10 mM Hepes, pH 7.5, 40 mM KCl, 2 mM $MgCl_2$, 10% glycerol, 1 mM NaPPi, and supplemented with protease inhibitors). After centrifugation, the pellets were resuspended in sucrose buffer (0.25 M sucrose, 100 mM $MgCl_2$, 500 mM Hepes pH 7.5), and centrifuged for 5 min. The nuclear pellet was washed briefly with nuclear lysis buffer (10 mM Hepes, 500 mM NaCl, 1% Triton-X100, 10% glycerol, 1 mM NaPPi, and protease inhibitors). For immunoprecipitation, endogenous p21 or E2F1 was immunoprecipitated with each specific antibody, followed by incubation with protein A/G plus-agarose beads (Santa Cruz Biotechnology). After washing, the complexes were subjected to immunoblotting with anti-Cdk2 or anti-Rb antibody. Immunoblotting was conducted as previously described (Lee et al., 2009). Proteins (15 μg) were loaded on SDS-PAGE gel and immunoblot analysis was conducted with the indicated antibodies, in accordance with the manufacturer's instructions (Cell Signaling Technology). Anti-phospho-Akt (S473), anti-Akt, and anti-phospho-Rb (Ser807/811) antibodies were purchased from Cell Signaling Technology, and anti-Rb, anti-E2F1, anti-Cdk2, anti-p27, anti-cyclin D1, anti-actin antibody were purchased from Santa Cruz Biotechnology, Inc. Anti-p21 and anti-phospho-p21(T156) were purchased from Ab Frontier (Korea). The immunocomplex was visualized using an ECL system (Millipore, USA).

RT-PCR

Total cellular RNAs were prepared with TRIzol (Invitrogen Life Technologies), in accordance with the manufacturer's recommendations. Reverse transcription of mRNA was conducted with oligo(dT) primer and reverse transcriptase (Promega). The gene transcripts were identified via PCR.

RESULTS

Iron-saturated Lf stimulates cell cycle progression via the PI3K pathway

To characterize the growth regulatory effects of Lf, MCF-7 cells were pre-cultured in a hormone-free DCC-FBS. The cells were starved for 36 h in the absence of hormones, treated in either regular media or media containing 100 μg/ml of Lf(Fe^{3+}), then harvested for cell cycle analysis. Figure 1A shows that S phase entry was already initiated at 16 h after incubation with Lf(Fe^{3+}), and attained the maximum values at 24 h, whereas the control cells in only media reached the highest S/G2-M population at 32 h. At 32 h after incubation with Lf(Fe^{3+}), the majority of cells moved to the G1 phase, thereby suggesting that Lf(Fe^{3+}) does indeed contribute to the stimulation of ordinary cell cycle progression. In an effort to further explore the mechanisms involved in Lf(Fe^{3+})-induced cell cycle progression, cells were exposed to a PI3K inhibitor that is known as a typical mitogen-activated kinase. We noted that the treatment of cells with 10 μM of PI3K inhibitor, LY294002, completely blocked Lf(Fe^{3+})-induced S phase entry (Fig. 1B). The effects of Lf(Fe^{3+}) on cell cycle control were confirmed via immunostaining with the anti-Ki-67 antibody (Fig. 1C). Ki-67 is a cell cycle-associated nuclear protein and is generally employed as a cell cycle marker (Kill, 1996). Typically, Ki-67 forms numerous granules scattered over the nucleus in G1 phase, and foci become limited to the nucleoli in late G1 to S phase. As compared to the cells exposed only to media, the cells treated with Lf(Fe^{3+}) evidenced significant increases in the proportion of cells with Ki-67 foci in the nucleoli, which was reflective of cells in the S phase. However, the treatment of cells with LY294002 reduced the observable differences in the proportion. These findings indicate that Akt signaling performs a crucial role in Lf(Fe^{3+})-induced cell cycle progression.

Iron-saturated Lf immediately activates Akt

In an effort to investigate more precisely the mechanisms involved in G1/S transition after treatment with Lf(Fe^{3+}), Akt and p21 levels were determined via time course experiments. As is shown in Fig. 2, Lf(Fe^{3+}) induced rapid Akt phosphorylation at Ser473, and the maximum level of phosphorylation was obtained in 1 h. This observation showed that Akt is critically associated with Lf in cell cycle control. As the p21 Cdk inhibitor is known to exert a negative effect during G1 phase and also has an Akt-specific phosphorylation site at Thr145 (Zhou et al., 2001), we have attempted to determine whether p21 would be phosphorylated at Thr145 as the consequence of Lf(Fe^{3+}) treatment. Our data showed that p21 phosphorylation occurred at approximately 4 h after treatment, although the total levels of p21 proteins were not significantly altered in the presence of Lf(Fe^{3+}). This data indicates that Lf(Fe^{3+})-induced p21 phosphorylation may be tightly correlated with Akt phosphorylation. Therefore, we concluded that Akt-mediated p21 phosphoryla-

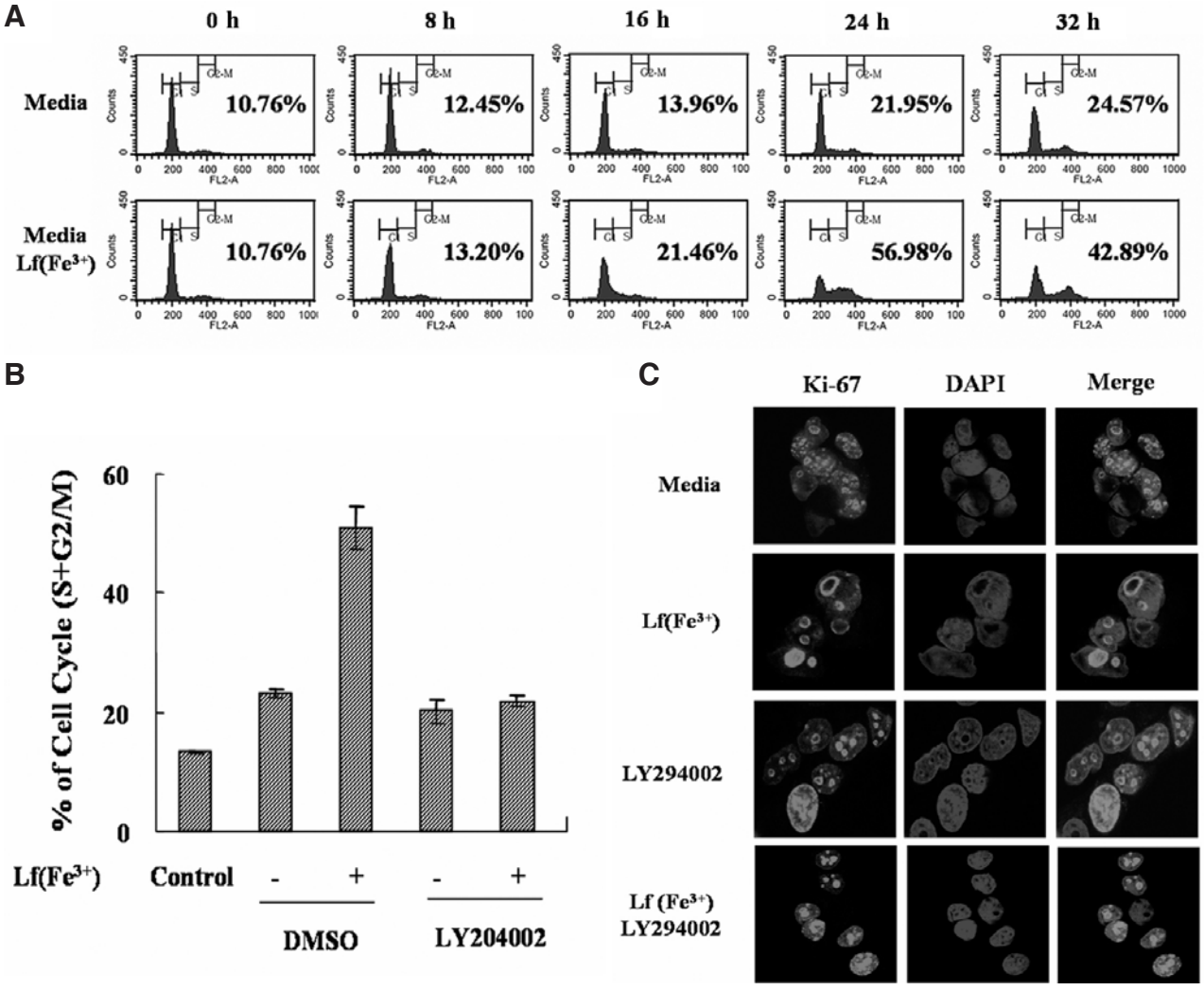

Fig. 1. Lf(Fe^{3+})-induced cell cycle progression via Akt signaling. MCF-7 cells were starved with 0.5% DCC-FBS in phenol red free RPMI (PR-RPMI) for 36 h, and treated with Lf and/or 10% DCC-FBS in phenol red free RPMI. (A) Cells were treated with 100 μg/ml of Lf(Fe^{3+}) for the indicated time, harvested, and prepared for cell cycle analysis via flow cytometry. The percentage of S + G2-M phase is indicated. (B) Synchronous cells were pre-treated for 30 min with 10 μM of LY294002 in phenol red free RPMI containing 10% DCC-FBS, and incubated with 100 μg/ml of Lf(Fe^{3+}) for 24 h. S + G2-M phase is expressed as a percentage. (C) Synchronized cells were treated with 100 μg/ml of Lf(Fe^{3+}) in the presence or absence of 10 μM of LY294002, and incubated for 24 h. The cells were co-stained with anti-Ki-67 antibody and DAPI. Localization of Ki-67 was conducted with confocal microscopy.

tion may be involved in Lf(Fe^{3+})-induced cell cycle progression.

Lf(Fe^{3+})-mediated Akt activation impairs nuclear import of Cdk inhibitors and activates genes required for S phase entry

The translocation of cell cycle factors may be crucial for its regulation. It has been previously reported that Cdk inhibitors are inactivated via cytoplasmic localization occurring as a result of Akt activation (Viglietto et al., 2002; Zhou et al., 2001). Thus, we attempted to determine whether Lf(Fe^{3+})-induced Akt activation could affect the cellular localization of Cdk inhibitors during cell cycle regulation. In order to analyze the nuclear-cytoplasmic translocation, we separated the nuclear compartment from the cytoplasm, and quantified the proportion of Cdk inhibitor proteins in each sub-cellular compartment as a measure of cellular response to Lf(Fe^{3+}). Figure 3A demonstrates that the protein levels of both p21 and p27 were higher in the cytoplasmic fraction, particularly in the presence of Lf(Fe^{3+}). In addition, we confirmed the cytoplasmic localization of endogenous p21 using anti-p21 immunofluorescence (Fig. 3B). Lf(Fe^{3+}) treatment resulted in exclusive cytoplasmic localization of p21 and impaired its nuclear import. It has been demonstrated previously that Akt-induced p21 phosphorylation causes accumulation in the cytoplasm, thereby inactivating its inhibitory effects on Cdk2 (Zhou et al., 2001). Figure 3C demonstrates that the level of p21 phosphorylation at Thr145 was increased in the presence of Lf(Fe^{3+}), but was inhibited almost completely by LY294002 treatment. This result indicated that the phosphorylation and cytoplasmic accumulation of p21 specifically resulted from Lf(Fe^{3+})-induced Akt activation. Similarly, we attempted to determine the localization of p27 via an immunofluorescence assay. Figure 3D shows the movement of fluorescently-labeled p27 protein between the cytoplasm and nucleus. The treatment of cells with Lf(Fe^{3+}) appeared to enhance the cytoplasmic

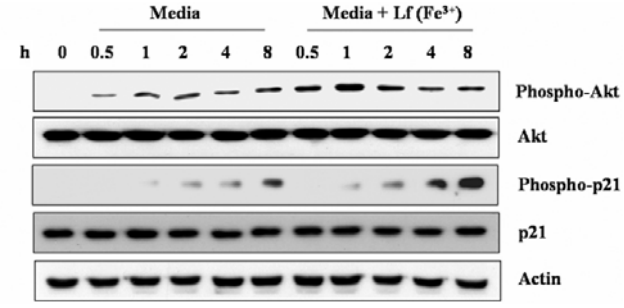

Fig. 2. Lf(Fe^{3+}) induces Akt phosphorylation. Serum-starved cells were incubated with or without 100 μg/ml of Lf(Fe^{3+}) in PR-RPMI containing 10% DCC-FBS, and incubated for the indicated time points. The cells were harvested for Western blotting using antibodies against phospho-Akt, Akt, phospho-p21, or actin.

localization of p27, but the distribution of cytoplasmic p27 was eliminated by treatment with the PI3K/Akt inhibitor LY294002. Thus, these data provided evidence that Lf(Fe^{3+})-mediated Akt phosphorylation modulates the cell cycle regulatory functions of Cdk inhibitors by interfering with nuclear translocation.

As the activated form of Cdks is located in the nucleus, only nuclear Cdk inhibitors may evidence inhibitory functions. As a result of Akt-mediated p21 inhibition, Fig. 4A demonstrates that p21-Cdk2 complex formation was decreased, whereas the total protein levels were not altered significantly in response to Lf (Fe^{3+}). It has been well understood that the cyclin E-Cdk2 complex phosphorylates Rb, and consequently releases the E2F transcription factor (Harbour and Dean, 2000), thereby allowing cells to enter the S phase of the cell cycle. Based on this fact, the inhibition of p21 and p27 by Lf(Fe^{3+}) may contribute to the activation of the cyclin E-Cdk2 complex, consequently stimulating cell cycle progression. Therefore, we attempted to determine whether the Rb-E2F1 complex could be regulated upon exposure to Lf(Fe^{3+}), since Cdk-dependent Rb phosphorylation regulates the availability of E2F. Figure 4B demonstrates that the levels of the Rb–E2F1 complex were reduced in the presence of Lf(Fe^{3+}). Lf(Fe^{3+}) treatment did not effect any changes in the level of E2F1, but did induce a marked increase in the level of phosphorylated Rb at Ser807/811. These results show that the inactivation of Rb by the Lf(Fe^{3+})-mediated inhibition of Cdk inhibitors may be tightly involved in the cell cycle process, and may send the cell cycle into the synthesis phase.

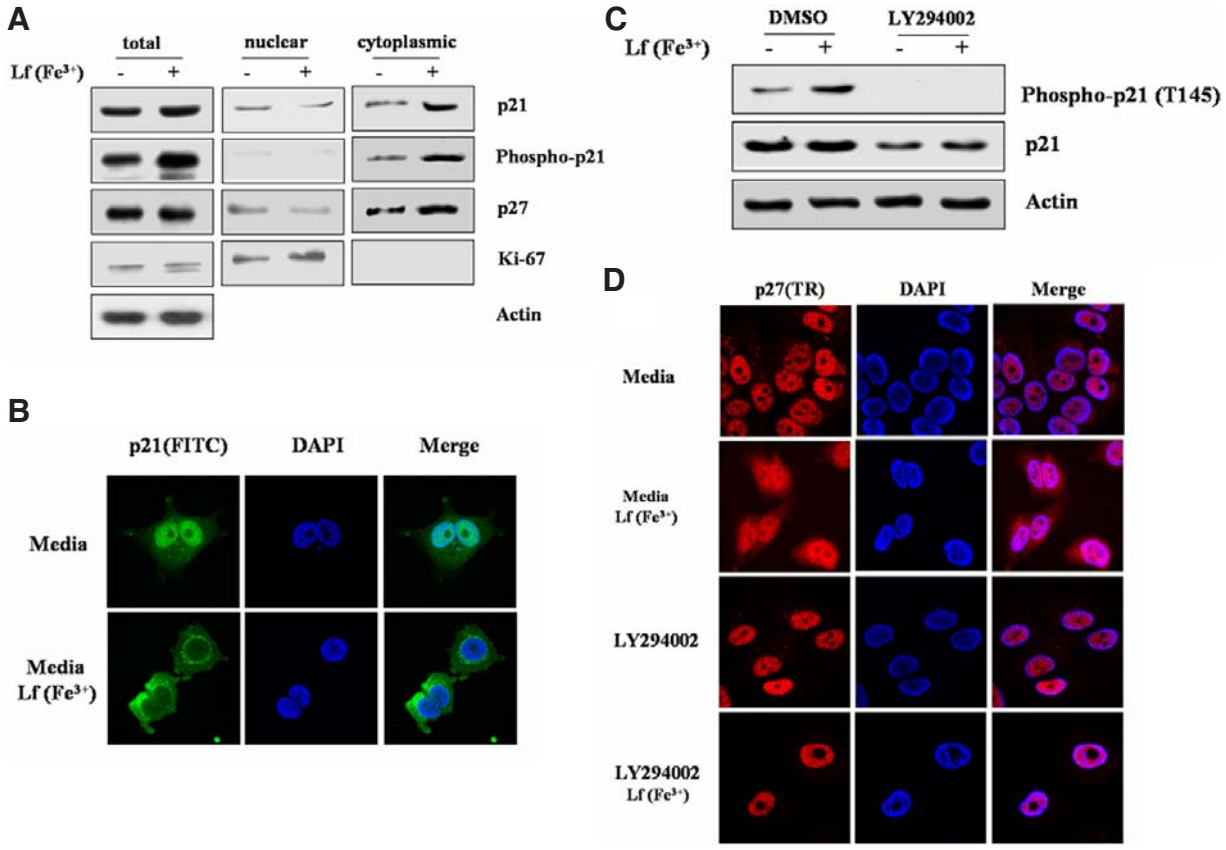

Fig. 3. Effects of Lf(Fe^{3+}) on Akt-regulated localization of p21 and p27. MCF-7 cells were starved with 0.5% DCC-FBS in PR-RPMI for 36 h, and treated with some inhibitors or Lf(Fe^{3+}) in 10% DCC-FBS in PR-RPMI. (A) The Lf(Fe^{3+})-treated cells were collected and separated into nuclear and cytoplasmic compartments, as described in "Materials and Methods". Western blotting was conducted using antibodies against p21, phospho-p21, or p27. Anti-Ki-67 antibody was utilized as a nuclear protein marker. (B) Cells were seeded on glass coverslips and treated with Lf(Fe^{3+}). The cells were then fixed and incubated with anti-p21 antibody, and stained with FITC-conjugated secondary antibody. The localization of p21 was observed under confocal microscopy. Nuclei were stained with DAPI (blue). (C) The phosphorylation level of p21 in LY294002 and/or Lf(Fe^{3+})-treated cells was analyzed via Western blotting. (D) Cells were seeded on glass coverslips and treated with Lf(Fe^{3+}). The cells were fixed and stained with anti-p27 antibody (red) and then observed under confocal microscopy. Nuclei were stained with DAPI (blue).

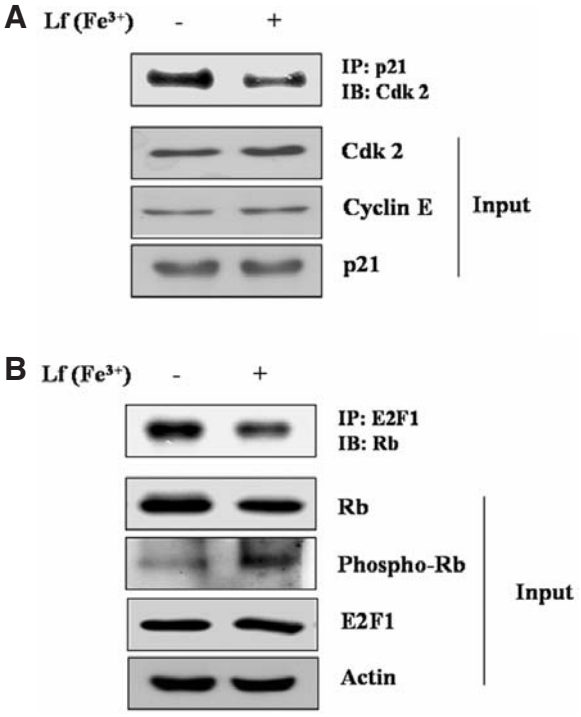

Fig. 4. Lf(Fe^{3+}) regulates binding activity of Cdk2/p21 or Rb/E2F1. MCF-7 cells were starved with 0.5% DCC-FBS in PR-RPMI for 36 h, and released with 10% DCC-FBS in PR-RPMI and/or Lf(Fe^{3+}) for 20 h. (A) Total cell lysates were immunoprecipitated with anti-p21 antibody followed by Western blotting with anti-Cdk2 antibody. Input lysates were subjected to Western blotting with antibodies against p21 and Cdk2. (B) Lysates were immunoprecipitated with E2F1 antibody, and immunoblotted with anti-Rb antibody. Input lysates were analyzed by Western blotting with antibodies against Rb, phospho-Rb (Ser807/811), E2F1, and actin.

DISCUSSION

In this study, we noted that iron-saturated human Lf stimulated cell cycle progression in MCF-7 cells. Our objective, initially, was to investigate a possible role of Lf as a proliferation effector molecule. Many previous studies have reported that Lf gene expression is correlated with cell growth and proliferation (Teng, 2006), and thus our work essentially evaluated the action of Lf on cell cycle progression. We determined that Lf itself promoted cell cycle progression to a remarkable degree in the absence of hormones. Therefore, our study focused principally on the role of Lf as a growth stimulator, and thus it might be utilized as a hormone substitute. Lf has been shown to control the proliferation of a variety of mammalian cells. In the previous study, we proposed that ectopically expressed Lf might phosphorylate the p65 NF-κB subunit via signaling pathways including the TRAFs-NIK-IKK pathway (Oh et al., 2007). On the other hand, a relatively high concentration of human Lf, with a natural 15% iron saturation, was sufficient to induce apoptosis in Jurkat leukemia T cells (Lee et al., 2009). We have observed that cell cycle progression might be correlated with the degree of iron saturation of Lf, but additionally affected by other physiological condition in a variety of cell types (data not shown). These results showed that the mode of action of Lf in controlling cell proliferation might be cell type-specific. The most remarkable observation in our study was that the cell cycle progression in MCF-7 cells was steadily induced after treatment with Lf(Fe^{3+}), which was fully saturated with iron. Lf(Fe^{3+}) facilitated entry into the S phase of the cell cycle. As we noted previously, other groups have demonstrated the coincident effect of Lf (Huang et al., 2008; Oguchi et al., 1995). This raises two possibilities; one is that Lf(Fe^{3+}) may provide the iron to the cells by releasing its iron, and the other is that Lf(Fe^{3+}) may perform a noble growth-regulatory function. It was also reported that hepatocytes take up iron from Lf via the endocytosis of the Lf-iron complex (McAbee, 1995), and thus the proliferative ability of Lf(Fe^{3+}) may be induced by iron content, which functions as a nutrient. However, we concentrated principally on the second possibility, because iron ($FeCl_3$) supplementation did not noticeably promote cell cycle progression (data not shown). In the human body, Lf is primarily secreted from neutrophils at inflammatory sites. It was proposed that Lf released at inflammatory sites might occupy iron via phagocytic vacuoles or by the microbes themselves (Ambruso and Johnston, 1981). It was reported that iron binding to Lf appears to be sufficient to cause the conformational change (Baker and Baker, 2009), thus it is believed that iron-induced conformational change of Lf would result in new characteristic feature in a human body. Therefore, Lf may be concluded to function as a suppressor of excessive inflammation, and may subsequently be expected to perform a pivotal function in tissue repair.

We also noted that cell cycle progression in response to Lf(Fe^{3+}) occurred via a classical PI3K/Akt signaling. The cell cycle is harmonized by a number of proteins, including cyclins, Cdks, and Cdk inhibitors, and many of the functions of these regulatory factors can be modulated via PI3K signaling (Liang and Slingerland, 2003). Therefore, we attempted to determine whether any correlation could be drawn between cell cycle control and Lf(Fe^{3+})-mediated PI3K/Akt signaling. The Akt requirement in the proliferative effect of Lf(Fe^{3+}) in MCF-7 cells was demonstrated using a specific PI3K inhibitor, LY294002. Multiple mechanisms involving Akt activation may play essential roles in Lf(Fe^{3+})-induced cell cycle progression. It is generally believed that Akt is involved in the phosphorylation of Cdk inhibitors, including p21 or p27, and that post-translational modification may regulate the movement of Cdk inhibitors between the cytoplasm and nucleus of the cell (Liang and Slingerland, 2003; Shin et al., 2002; Zhou et al., 2001). On the basis of these results, we attempted to determine whether Lf(Fe^{3+})-induced Akt activation affected the activity of Cdk inhibitors. When the cells were treated with Lf(Fe^{3+}), the level of phosphorylated p21 (Thr145) increased, and the cytoplasmic portions of endogenous p21 and p27 proteins were increased significantly in the Lf(Fe^{3+})-treated cells. Akt inhibition induced by LY294002 prevented p21 phosphorylation (Thr145) and p27 cytoplasmic localization. These results constitute compelling evidence that iron-saturated Lf stimulates cellular proliferation via Akt activation in MCF-7 cells. Although we clearly demonstrated that Akt induces cell cycle progression by Lf(Fe^{3+}) and is correlated with the post-translational regulation of p21 and p27, the relationship between Lf and downstream signaling molecules remains to be thoroughly elucidated. Further studies will be required in order to clarify whether Lf may require additional signaling networks to operate as an efficient effector of proliferation.

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