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The Low-Density Lipoprotein Receptor-Related Protein 1 Is a Mitogenic Receptor for Lactoferrin in Osteoblastic Cells

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Lactoferrin induces osteoblast proliferation and survival *in vitro* and is anabolic to bone *in vivo*. The molecular mechanisms by which lactoferrin exerts these biological actions are not known, but lactoferrin is known to bind to two members of the low-density lipoprotein receptor family, low-density lipoprotein receptor-related proteins 1 (LRP1) and 2 (LRP2). We have examined the role(s) of these receptors in the actions of lactoferrin on osteoblasts. We show that lactoferrin binds to cultured osteoblastic cells, and that LRP1 and LRP2 are expressed in several osteoblastic cell types. In primary rat osteoblastic cells, the LRP1/2 inhibitor receptor associated protein blocks endocytosis of lactoferrin and abrogates lactoferrin-induced p42/44 MAPK signaling and mitogenesis. Lactoferrin-induced mitogenesis is also inhibited by an antibody to LRP1. Lactoferrin also induces receptor associated protein-sensitive activation of p42/44 MAPK signaling and proliferation in osteoblastic human SaOS-2 cells, which express LRP1 but not LRP2. The mitogenic response of LRP1-null fibroblastic cells to lactoferrin is substantially reduced compared with that of cells expressing wild-type LRP1. The endocytic and signaling functions of LRP1 are independent of each other, because lactoferrin can activate mitogenic signaling in conditions in which endocytosis is inhibited. Taken together, these results 1) suggest that mitogenic signaling through LRP1 to p42/44 MAPKs contributes to the anabolic skeletal actions of lactoferrin; 2) demonstrate growth-promoting actions of a third LRP family member in osteoblasts; and 3) provide further evidence that LRP1 functions as a signaling receptor in addition to its recognized role in ligand endocytosis. (*Molecular Endocrinology* 18: 2268–2278, 2004)

OSTEOPOROSIS IS A MAJOR cause of morbidity and health expenditure in ageing populations (1). Currently available therapies, the majority of which target the osteoclast to decrease bone resorption, are limited in their ability to restore bone mass, and reduce the incidence of osteoporotic fractures by 50% at best (2). Consequently, there is intense interest in the study and development of compounds that are anabolic to the skeleton by promoting osteoblastic bone formation (3), and which might induce greater increments in bone density and produce greater reductions in fracture risk than antiresorptive therapies.

We recently reported that the iron-transporting glycoprotein lactoferrin is anabolic to bone (4). Thus, *in vitro* lactoferrin stimulates mitogenesis, differentiation, and survival of osteoblasts, while also potently inhibiting osteoclastogenesis (4). Importantly, local administration of lactoferrin over the calvariae of mice potently increases new bone formation *in vivo* (4). These data suggest that lactoferrin or related compounds have potential as anabolic agents for treatment of osteoporosis and that lactoferrin may be a physiological regulator of bone cell activity.

Lactoferrin is a pleiotropic factor. It exhibits antibacterial activity (5–7) but also regulates cell growth and differentiation (8), embryonic development (9), myelopoiesis (10), endothelial cell adhesion (11), production of cytokines (12, 13) and chemokines (14), and immune function (15, 16). The molecular mechanisms by which lactoferrin acts at a cellular level are largely unknown. Among the putative lactoferrin receptors are the low-density lipoprotein receptor-related proteins (LRPs) 1 and 2 (17–20), which are multiligand members of the LRP family of endocytic receptors (21). Recent evidence suggests that LRP1 functions both as an endocytic receptor and a signaling receptor (21–23).

In the current work we investigated the role of LRP1 in transducing the proliferative actions of lactoferrin in osteoblastic cells. We report that lactoferrin binds to osteoblastic cells, that osteoblastic cells express both

Abbreviations: DiI, 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; FCS, fetal calf serum; Fwd, forward; LPA, lysophosphatidic acid; LRP, low-density lipoprotein receptor-related protein; LRP1-wt, wild-type LRP1; RAP, receptor-associated protein; Rev, reverse.

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LRP1and the related receptor LRP2/megalin, that LRP1 mediates the internalization of lactoferrin by these cells and the induction by lactoferrin of osteoblast mitogenesis and mitogenic signaling, and that LRP1-deficient fibroblastic cells exhibit a considerably blunted mitogenic response to lactoferrin compared with cells expressing wild-type LRP1. The endocytic and signaling functions of LRP1 are independent phenomena, because activation of mitogenic signaling occurs in conditions in which endocytosis is abrogated. These findings suggest the existence of a novel pathway for the regulation of osteoblast growth.

RESULTS

Lactoferrin Binds to Osteoblastic Cells

We first determined whether lactoferrin binds to osteoblastic cells, using a conventional competitive binding assay. As shown in Fig. 1, binding of [^{125}I]lactoferrin to both primary rat osteoblasts (*left panel*) and SaOS2 cells (*right panel*) was competitively inhibited by the addition of increasing concentrations of unlabeled lactoferrin. The EC_{50} for the binding of lactoferrin to primary rat osteoblasts was 6.2×10^{-7}M, consistent with published results from binding studies performed in other cell types (24–26).

LRP1 and LRP2 Are Expressed in Osteoblastic Cells

Because lactoferrin has been reported to bind to both LRP1 (17, 18, 20) and LRP2 (17, 27), we determined whether these receptors are expressed in osteoblastic cells. As shown in Fig. 2, LRP1 is expressed in each of the osteoblastic cell types examined, being primary neonatal rat calvarial osteoblasts (pROB, lane 1), the osteoblastic cell lines SaOS-2 (lane 3), UMR-106 (lane 4), MC3T3E1 (lane 5), and the ST2 stromal cell line

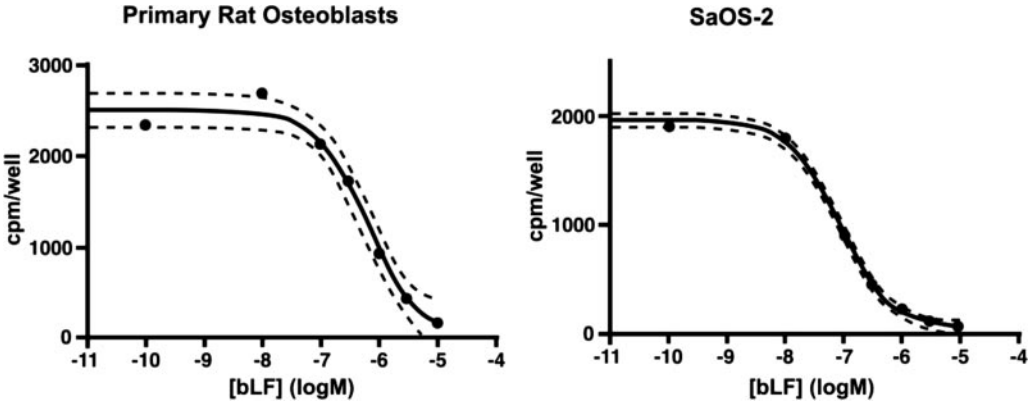

Fig. 1. Specific Binding of Lactoferrin (LF) to Osteoblastic Cells

[^{125}I]Lactoferrin was added to cultures of primary rat osteoblasts (*left panel*) or SaOS2 cells (*right panel*) at room temperature in the presence of increasing concentrations of unlabeled lactoferrin, and specific binding was assessed as described in *Materials and Methods*. The *dashed lines* represent prediction intervals. bLF, Bovine lactoferrin.

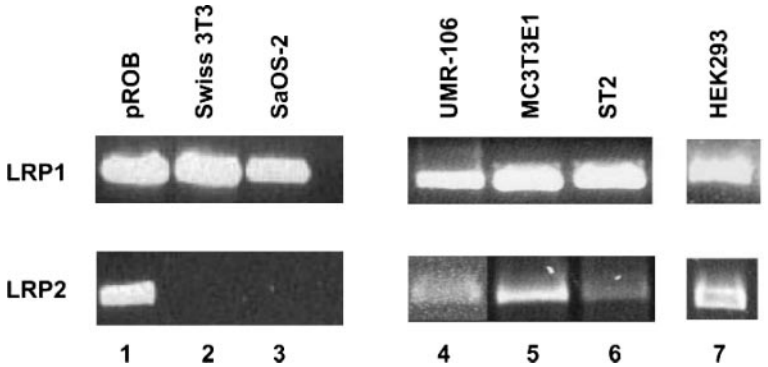

Fig. 2. Osteoblastic Cells Express LRP Receptors

RNA extracted from cultures of primary rat osteoblastic cells (pROb), the murine fibroblastic cell line Swiss 3T3, the human osteoblastic cell line SaOS-2, the rat osteoblastic cell line UMR 106–01, the murine osteoblastic cell line MC3T3E1, the murine stromal cell line ST2, and the human kidney cell line HEK293 was subjected to RT-PCR analysis for detection of LRP1 (*top panel*) and LRP2 (*bottom panel*) expression, as described in *Materials and Methods*.

(lane 6). It is also expressed in the Swiss 3T3 fibroblastic cell line (lane 2). LRP2 is expressed in primary rat osteoblasts, UMR-106 cells, MC3T3E1 cells, ST2 cells, and HEK293 cells (lane 7), but not in SaOS-2 cells or Swiss 3T3 cells. Thus, osteoblastic cells express both LRP1 and LRP2 receptors, and SaOS-2 cells represent an osteoblastic cell type that expresses LRP1 but not LRP2.

Lactoferrin Is Endocytosed by Osteoblastic Cells in a Receptor-Associated Protein (RAP)-Sensitive Fashion

LRP1 and LRP2 have primarily been studied as endocytic receptors (21). In osteoblastic UMR 106 cells, Barmina *et al.* (28) reported that LRP1 was responsible for the internalization of collagenase 3. We therefore determined whether lactoferrin is endocytosed by osteoblastic cells. As shown in Fig. 3, A and B, analysis by confocal microscopy 30 min after addition of labeled lactoferrin to primary rat osteoblastic cells revealed a vesicular pattern of cytoplasmic lactoferrin label. Costaining of cells with the membrane label DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Fig. 3C) demonstrated colocalization of the cytoplasmic lactoferrin label with intracellular vesicles (Fig. 3D). These results suggest that lactoferrin is endocytosed by osteoblastic cells. Confirmation of this observation was provided by experiments performed under conditions in which endocytosis is inhibited. Thus, no lactoferrin label was present within primary rat osteoblastic cells placed at 4 C, a temperature at which receptor binding is intact but endocytosis is inhibited, even 60 min after addition (Fig. 3F). The internalization of lactoferrin was also inhibited in the presence of hypertonic medium (400 mM sucrose, Fig. 3G), a condition under which endocytosis is also abrogated. Importantly, pretreatment with the LRP inhibitor, RAP, virtually abolished the endocytosis of lactoferrin (Fig. 3H). These data demonstrate that lactoferrin is endocytosed by osteoblastic cells via a mechanism that involves a functional LRP1 or LRP2.

To confirm the observation that RAP inhibits the internalization of lactoferrin by osteoblastic cells, we tested the ability of RAP to inhibit the intracellular accumulation of [^{125}I]lactoferrin. As shown in Fig. 4, RAP dose dependently inhibited the uptake by primary rat osteoblasts of radiolabeled lactoferrin during a 3-h incubation period. At a concentration of 500 nM, RAP inhibited cellular uptake of lactoferrin by 72%, an effect comparable in size to that produced by the addition of excess (10 μM) unlabeled lactoferrin.

The Mitogenic Actions of Lactoferrin in Osteoblastic Cells Are Mediated by LRP1

One of the striking effects of lactoferrin in osteoblastic cells *in vitro* is its potent mitogenicity. Thus, lactoferrin induces dose-dependent increases in DNA synthesis in cultures of primary rat osteoblasts (Fig. 5A, *left*).

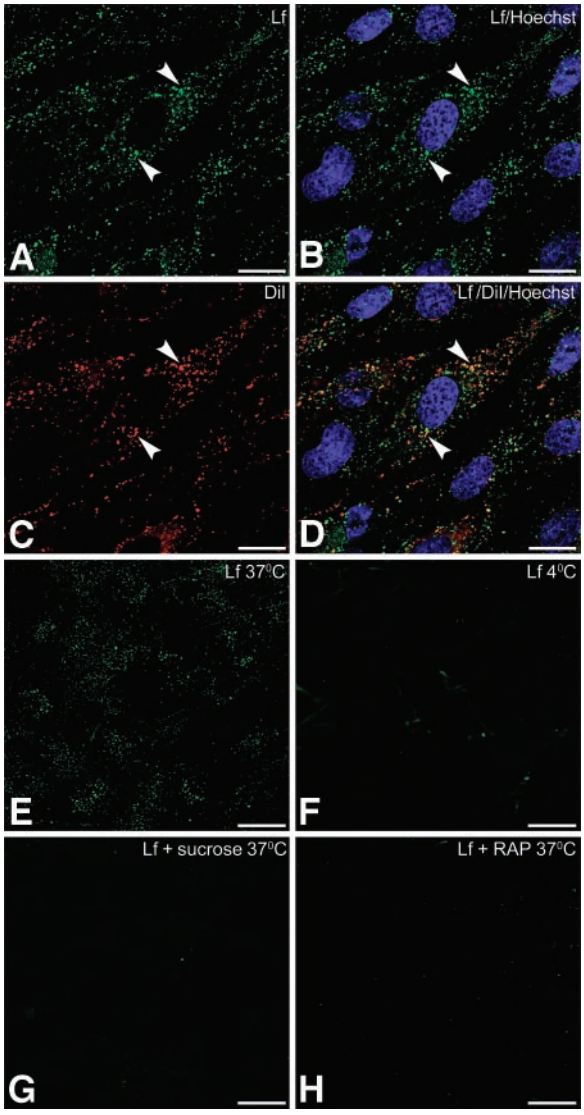

Fig. 3. Osteoblastic Cells Endocytose Lactoferrin (Lf) in a RAP-Sensitive Fashion

Upper panel, Confocal micrographs showing single optical sections of primary rat osteoblastic cells labeled with (A) Oregon Green-conjugated lactoferrin for 30 min at 37 C (panel A), Oregon Green-conjugated lactoferrin and the nuclear stain Hoechst 333252 (panel B), and the membrane stain DiI (panel C). D, Overlay of images in panels A–C. The *arrowheads* are positioned at the same point in each image and demonstrate, in panel D, colocalization of lactoferrin with cytoplasmic vesicles. *Lower panel*, Confocal micrographs showing single optical sections of primary rat osteoblastic cells labeled with Oregon Green-conjugated lactoferrin for 30 min at 37 C (panel E), Oregon Green-conjugated lactoferrin for 60 min at 4 C (panel F), (G) Oregon Green-conjugated lactoferrin for 30 min at 37 C in the presence of 400 nM sucrose (panel G), and Oregon Green-conjugated lactoferrin for 30 min at 37 C in the presence of 50 nM RAP (panel H). *Scale bars*, 20 μm (A–D); 40 μm (E–H).

Mitogenic effects of equivalent magnitude were observed in cultures of the same cells exposed to recombinant human lactoferrin (Fig. 5A, *right*), indicating

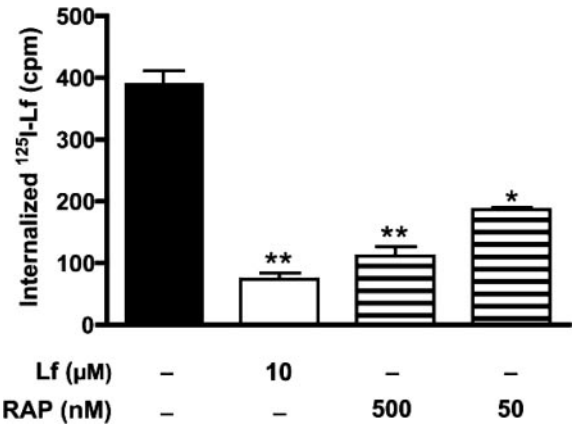

Fig. 4. Uptake of [^{125}I]Lactoferrin (Lf) by Osteoblastic Cells Is RAP Sensitive

Primary rat osteoblasts were incubated with [^{125}I]lactoferrin for 3 h at 37 C in the presence or absence of excess unlabeled lactoferrin (*open bar*), or the indicated concentrations of RAP (*hatched bars*), and intracellular accumulation of radiolabeled lactoferrin was measured as described in *Materials and Methods*. *, $P < 0.05$ *vs.* control; **, $P < 0.01$ *vs.* control.

that contaminating compounds within the purified bovine lactoferrin preparation are not responsible for its growth-promoting activity. The proliferative actions of lactoferrin in primary rat osteoblastic cells are abrogated by pretreatment of the cultures with RAP (Fig. 5B). The ability of RAP to inhibit lactoferrin-induced osteoblast proliferation is not attributable to cell toxicity, because addition of RAP alone to the cell cultures did not alter DNA synthesis, nor is it nonspecific, because RAP did not inhibit the mitogenic actions of the phospholipid growth factor lysophosphatidic acid (LPA), which signals through a G protein-coupled receptor (Fig. 5B). These data demonstrate that lactoferrin-induced osteoblast mitogenesis requires a functional LRP receptor, but do not allow discrimination between the individual contributions of LRP1 and LRP2, because both receptors are expressed in primary rat osteoblasts and RAP inhibits binding of ligands to each receptor (27). Importantly, pretreatment of primary rat osteoblastic cells with an LRP1 antibody that inhibits ligand binding (29) blocks the proliferative response to lactoferrin (Fig. 5B). In addition, lactoferrin, in a dose-dependent manner, promotes mitogenesis in SaOS-2 cells (Fig. 5C, *left*), which express LRP1 but not LRP2 (Fig. 2, lane 3). Lactoferrin-induced proliferation of SaOS-2 cells is also blocked by cotreatment with RAP (Fig. 5C, *right*). Taken together, these data strongly suggest that LRP1 functions as a mitogenic receptor for lactoferrin in osteoblastic cells.

Lactoferrin-Induced Osteoblast Mitogenesis Involves RAP-Sensitive Activation of p42/44 MAPKs

Activation of p42/44 MAPKs (ERKs) is a common feature of proliferative signals initiated by a variety of extracellular agents (30). We therefore examined whether p42/44 MAPKs are involved in lactoferrin-induced mitogenic signaling in osteoblasts. As shown in Fig. 6A, pretreatment of primary rat osteoblastic cells with either PD-98059 or U-0126, structurally unrelated inhibitors of MAPK kinase, the kinase that specifically phosphorylates and activates p42/44 MAPKs, inhibited, in a dose-dependent manner, the ability of lactoferrin to induce osteoblast mitogenesis. Lactoferrin induces phosphorylation of p42/44 MAPKs in osteoblastic cells (Fig. 6B, *left*), and this effect is inhibited by RAP (Fig. 6B, *right*, lanes 5 and 6). The inhibitory action of RAP in this assay is specific, because RAP does not inhibit LPA-induced activation of p42/44 MAPKs (lanes 3 and 4). Importantly, lactoferrin also activates p42/44 MAPK signaling in SaOS-2 cells, an effect that is blocked by RAP (Fig. 6C, lanes 3 and 4). These data suggest that lactoferrin stimulates osteoblast mitogenesis by signaling through LRP1 to the p42/44 MAPK pathway.

Proliferation of LRP1-Deficient Fibroblasts in Response to Lactoferrin Is Impaired

To confirm that lactoferrin-induced mitogenesis involves signaling through LRP1, we compared the mitogenic effects of lactoferrin in cultures of fibroblastic cells that express wild-type LRP1 (MEF-1, LRP1-wt) with those observed in LRP1-deficient fibroblastic cells (PEA13, LRP1-null). As shown in Fig. 7 (*open bars*), LRP1-wt cells proliferate vigorously in response to lactoferrin across a similar range of concentrations to that which stimulates osteoblast growth. Unusually, the mitogenic response of these cells to lactoferrin was greater at a concentration of 10 μg/ml than 50 μg/ml, although the latter concentration still induced a substantial (>2.3-fold) increase in thymidine incorporation. Importantly, at each active lactoferrin concentration, the proliferative response of the LRP1-null cells (*shaded bars*) was significantly abrogated, such that [^{3}H]thymidine incorporation was reduced by about 70% in the absence of LRP1. Further, addition of the LRP inhibitor RAP to cultures of LRP1-wt cells reduced the proliferative response to a level equivalent to that observed in the LRP1-null cells (Fig. 7, *hatched bar*). The residual mitogenic response to lactoferrin in LRP1-null cells was not sensitive to RAP (data not shown). These data provide direct evidence that LRP1 functions as a mitogenic receptor for lactoferrin.

Lactoferrin-Induced Osteoblast Mitogenesis Does Not Require Endocytosis

The experiments described above demonstrate that LRP1 mediates the endocytosis of lactoferrin by osteoblastic cells and transduces the proliferative signal activated by lactoferrin. Some evidence suggests that lactoferrin directly regulates gene transcription (31–33). We therefore determined whether endocytosis of lactoferrin is necessary for activation of mitogenic sig-

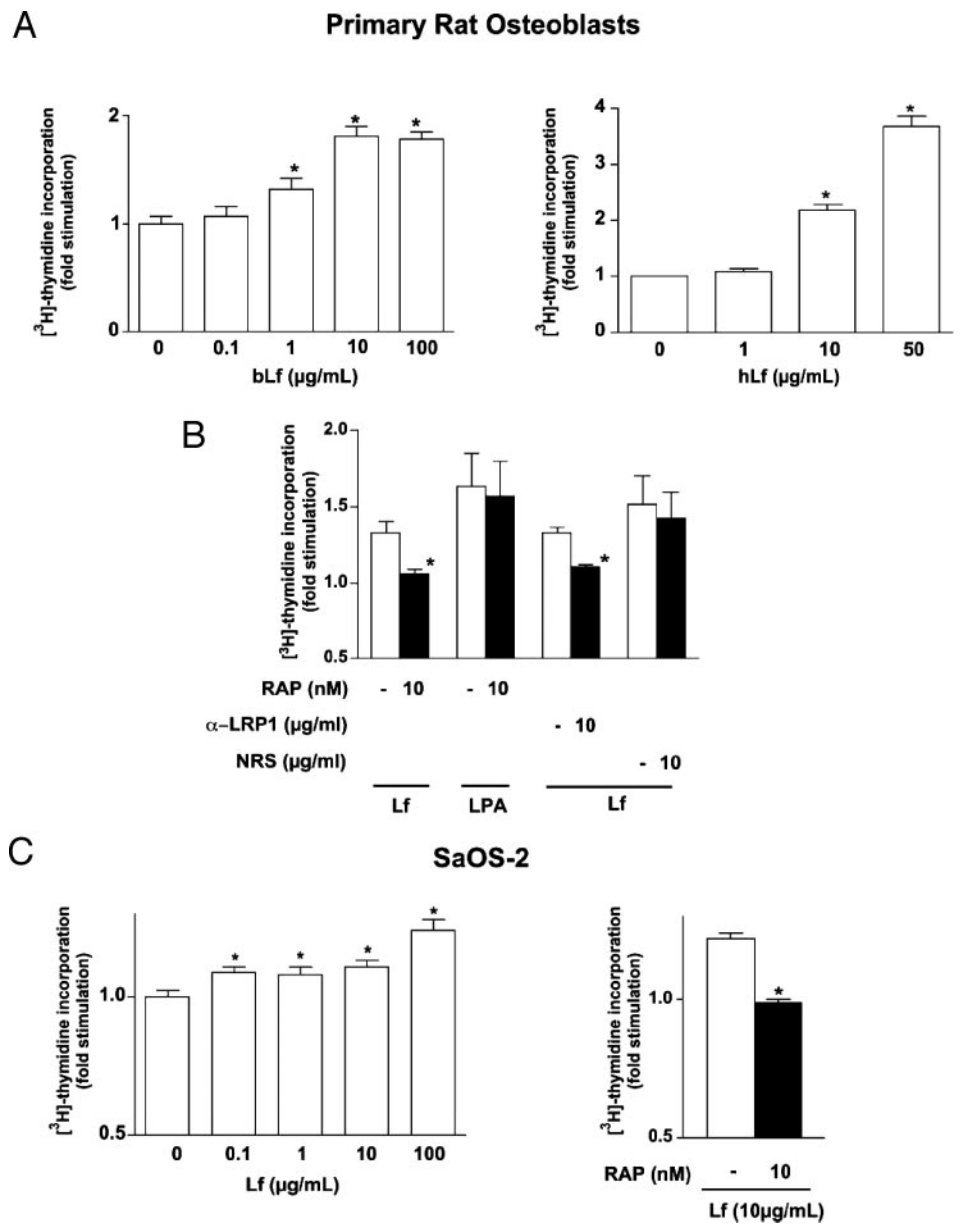

Fig. 5. Lactoferrin-Induced Osteoblast Mitogenesis Is LRP1 Dependent

A, Growth-arrested primary rat osteoblastic cells were treated for 24 h with the indicated concentrations of purified bovine lactoferrin (bLf, *left panel*) or recombinant human lactoferrin (hLf, *right panel*), and [^{3}H]thymidine incorporation was measured as described in *Materials and Methods*. *, $P < 0.05$ *vs.* control. B, Growth-arrested primary rat osteoblastic cells were treated for 24 h with either 10 μg/ml lactoferrin or 10 μM LPA in the absence (*open bars*) or presence (*shaded bars*) of 10 nM RAP, an antibody to LRP1 (α-LRP1) or nonimmune rabbit serum (NRS). Data are presented as the fold stimulation of [^{3}H]thymidine incorporation induced by lactoferrin treatment over vehicle treatment (*open bars*), or that induced by treatment with lactoferrin + inhibitor over inhibitor treatment (*shaded bars*). *, $P < 0.05$ *vs.* lactoferrin. C (*left panel*), Growth-arrested SaOS2 cells were treated for 24 h with the indicated concentrations of lactoferrin, and [^{3}H]thymidine incorporation was measured as described in *Materials and Methods*. *, $P < 0.05$ *vs.* control; *right panel*, growth-arrested SaOS2 cells were treated for 24 h with 10 μg/ml lactoferrin in the absence (*open bars*) or presence (*shaded bars*) of 10 nM RAP. Data are presented as the fold stimulation of [^{3}H]thymidine incorporation induced by lactoferrin treatment over vehicle treatment, or that induced by treatment with lactoferrin + RAP over RAP treatment. *, $P < 0.05$ *vs.* lactoferrin.

naling. Endocytosis of lactoferrin by osteoblastic cells is abrogated by placing the cells at 4 C (Fig. 3F) or in hypertonic medium (400 mM sucrose, Fig. 3G). Under each of these experimental conditions, however, lactoferrin was able to activate p42/44 MAPK signaling (Fig. 8, *top panels*). Pretreatment of osteoblasts with phenylarsine oxide (PAO), another pharmacological inhibitor of endocytosis, also failed to affect lactoferrin-

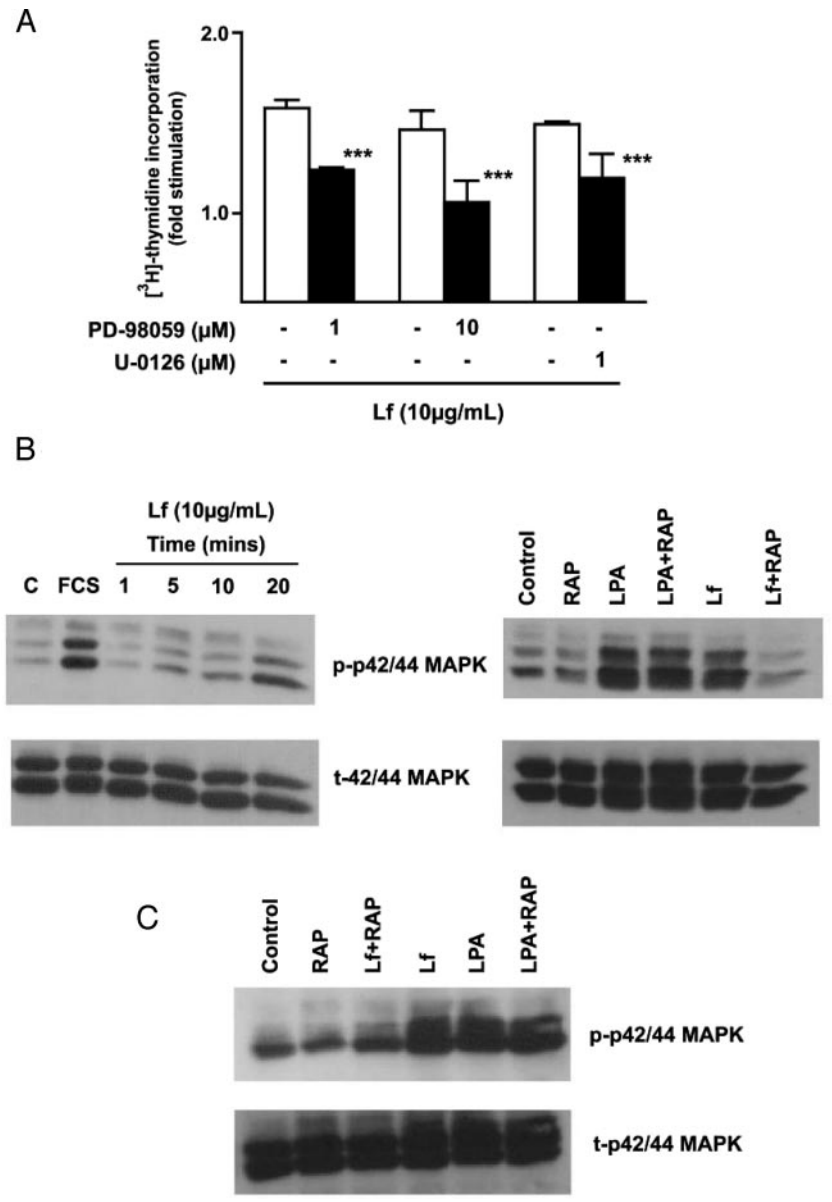

Fig. 6. Lactoferrin (Lf) Signals Osteoblast Mitogenesis via LRP1-Dependent Activation of p42/44 MAPKs

A, Growth-arrested primary rat osteoblastic cells were treated with lactoferrin 10 μg/ml in the absence (*open bars*) or presence (*shaded bars*) of the indicated MAPK kinase inhibitor. Data are expressed as the fold increase in [^{3}H]thymidine incorporation obtained in cultures of cells exposed to lactoferrin or lactoferrin and inhibitor over the values obtained in cultures of cells exposed to vehicle or inhibitor alone, respectively. ***, $P < 0.001$ *vs.* lactoferrin. B (*left panel*), Lysates of primary rat osteoblastic cells treated with 5% FCS for 10 min, or lactoferrin (Lf) 10μg/ml, for the indicated times were sequentially immunoblotted with an antibody to phosphorylated p42/44 MAPKs (*top*) and an antibody to total p42/44 MAPKs (*bottom*); *right panel*, lysates of primary rat osteoblastic cells treated with 100 μg/ml lactoferrin or 10 μM LPA in the presence or absence of 50 nM RAP were sequentially immunoblotted with an antibody to phosphorylated p42/44 MAPKs (*top*) and an antibody to total p42/44 MAPKs (*bottom*). C, Lysates of SaOS-2 cells treated with 100 μg/ml lactoferrin or 10 μM LPA in the presence or absence of 50 nM RAP were sequentially immunoblotted with an antibody to phosphorylated p42/44 MAPKs (*top*) and an antibody to total p42/44 MAPKs (*bottom*).

induced p42/44 MAPK signaling. These data suggest that the internalization of lactoferrin by osteoblastic cells is not required for activation of mitogenic signaling and that the endocytic function of LRP1 is independent of its signaling function.

DISCUSSION

The current study demonstrates that LRP1 functions as a mitogenic receptor for lactoferrin in osteoblastic cells. Although a growing body of evidence indicates

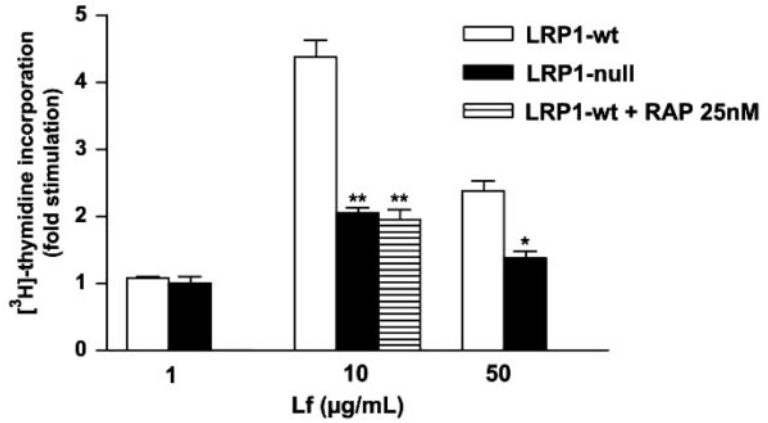

Fig. 7. Lactoferrin (Lf)-Induced Mitogenesis Is Abrogated in LRP1-Deficient Fibroblasts

Growth-arrested fibroblastic cells expressing LRP1-wt (*open bars*) or inactivated LRP1 (LRP1-null, *dark bars*) were treated with the indicated concentrations of lactoferrin for 24 h, and [^{3}H]thymidine incorporation was determined as described in *Materials and Methods.* [^{3}H]Thymidine incorporation in response to treatment with 10 μg/ml lactoferrin was also assessed in LRP1-wt cells in the presence of 25 nM RAP (*hatched bar*). *, $P < 0.05$ *vs.* LRP-wt cells; **, $P < 0.01$ *vs.* LRP-wt cells.

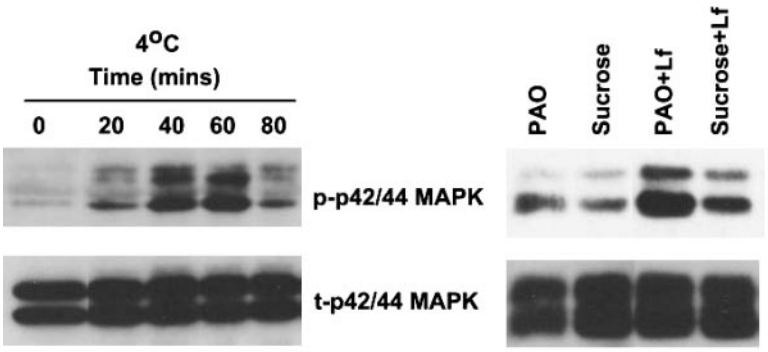

Fig. 8. Endocytosis of Lactoferrin (Lf) Is Not Required for Activation of Mitogenic Signaling

A, Primary rat osteoblastic cells were placed at 4 C for 15 min and then treated with 100 μg/ml lactoferrin for the indicated times. Whole-cell lysates were sequentially immunoblotted with an antibody to phosphorylated p42/44 MAPKs (*top*) and an antibody to total p42/44 MAPKs (*bottom*). B, Primary rat osteoblastic cells were pretreated for 10 min with 400 mM sucrose or 30 min with 5 μM phenylarsine oxide (PAO), before addition of 100 μg/ml lactoferrin for 20 min. Whole-cell lysates were sequentially immunoblotted with an antibody to phosphorylated p42/44 MAPKs (*top*) and an antibody to total p42/44 MAPKs (*bottom*).

that members of the LRP family function as both endocytic and signaling receptors (23, 34, 35), this is, to our knowledge, the first evidence that LRP1 transduces a proliferative signal. We observed that lactoferrin-induced proliferation and signaling to p42/44 MAPKs in primary rat osteoblastic cells was abrogated by RAP, which potently inhibits binding of ligands to both LRP1 and LRP2 (27). Lactoferrin binds to both LRP1 and LRP2 (17, 18, 20, 27), each of which is expressed in primary rat osteoblastic cells. That LRP1 rather than LRP2 is responsible for mediating the proliferative actions of lactoferrin is suggested by three separate pieces of evidence. First, lactoferrin-induced proliferation of primary rat osteoblastic cells was inhibited by an antibody to LRP1. Second, lactoferrin induces RAP-sensitive mitogenic effects in SaOS-2 cells, which express LRP1 but not LRP2. Third, the mitogenic response of LRP1-null fibroblasts to lactoferrin was substantially reduced compared with that observed in cells expressing a functional LRP1.

Lactoferrin exhibits diverse biological activities that include antimicrobial, immunomodulatory, antiinflammatory, and growth-modulating effects (5, 6, 8, 16). The molecular mechanisms by which these actions are mediated have not been comprehensively studied. Our data suggest that the ability of lactoferrin to induce osteoblast growth is largely mediated by signaling through LRP1 to p42/44 MAPKs. Lactoferrin promotes the maturation of T lymphocytes by activating p42/44 MAPK (36), but whether this process involves LRP1 was not examined. Recently, Takayama *et al.* (37), using antisense oligonucleotides to inhibit LRP1 expression, reported that lactoferrin-induced fibroblastic contractility was LRP1 dependent. Thus, the available evidence suggests that LRP1 plays a role in some of the cellular actions of lactoferrin. However, it

is likely that LRP1 is not the only receptor that mediates the ability of lactoferrin to stimulate cell proliferation, because there was a weak mitogenic effect of lactoferrin in LRP1-null fibroblasts that was not sensitive to RAP. Further, the role (if any) of LRP1 in mediating the antiinflammatory and immunomodulatory actions of lactoferrin remains to be determined.

Our finding that LRP1 transduces the proliferative actions of lactoferrin in osteoblastic cells suggests that this receptor mediates, at least in part, the anabolic actions of lactoferrin in skeletal tissue. Lactoferrin is a potent osteoblast growth factor *in vitro*, acting to expand the population of osteoblast precursors by promoting their proliferation and survival, and to induce a differentiated osteoblast phenotype (4). Because lactoferrin also potently inhibits osteoclastogenesis, its administration *in vivo* substantially increases bone formation (4). There is considerable interest in the development of agents that target bone formation as potential therapies for osteoporosis, a common and costly disorder of low bone mass (1, 3). The identification of LRP1 as a functional lactoferrin receptor in osteoblasts provides a mechanistic explanation for the anabolic skeletal actions of lactoferrin and suggests a novel pathway through which bone anabolism might be regulated physiologically and/or pharmacologically. It is noteworthy in this regard that LRP5 and LRP6, nonendocytic members of the LRP receptor family that are structurally related to LRP1, have been recently identified as critical regulators of osteoblast function and skeletal mass (38–40). Whether there is overlap between the skeletal functions of these two receptors and that of LRP1 is currently unclear, but worthy of further study.

The current work demonstrates that LRP1 mediates both the internalization and proliferative effects of lactoferrin in osteoblastic cells. However, these dual functions appear to be independent of each other, because inhibition of the LRP1 endocytic function did not abrogate the ability of lactoferrin to induce mitogenic signaling. These results are consistent with findings from studies of other receptor systems, such as the angiotensin II receptor (41) and the platelet-activating factor receptor (42). They are also consistent with evidence that the tyrosine moiety within the NPXY endocytic motif in the intracytoplasmic tail of LRP1 plays an important role in the receptor's ability to signal (43). It is possible that when the tyrosine residue in question is occupied by adaptor molecules subserving intracellular signaling, endocytosis is precluded, and *vice versa*. Thus, competition may exist after ligand binding between the endocytic (and presumed degradative) and signaling functions of LRP1, providing a means by which either action might be regulated.

In summary, the current findings suggest that LRP1 functions as a mitogenic signaling receptor in osteoblastic and fibroblastic cells, and likely mediates the anabolic actions of lactoferrin in skeletal tissue. Our results thus provide evidence that a third LRP family member is capable of signaling osteoblast anabolism.

MATERIALS AND METHODS

Cell Culture and Reagents

Primary rat osteoblasts were prepared from neonatal rat calvariae as previously described (44). All experiments involving animals were conducted in accordance with University of Auckland guidelines and were approved by the institutional ethics committee. Only primary cells at first or second passage were used. SaOS-2 cells were maintained in T75 flasks in 5% fetal calf serum (FCS) before subculturing for experimental procedures. Swiss-3T3 fibroblasts, ST2 stromal cells, UMR 106–01 osteoblastic cells, and HEK293 cells were cultured in T75 flasks before RNA extraction. Fibroblastic cells expressing LRP1-wt (MEF-1) and deficient in LRP1 (PEA 13, LRP1-null) were obtained from ATCC (Manassas, VA), and cultured according to ATCC recommendations.

Antibodies to phosphorylated and nonphosphorylated p42/44 MAPKs were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies and ECL detection reagents were from Amersham Pharmacia Biotech (Little Chalfont, UK). Recombinant human RAP was from Research Diagnostics (Flanders, NJ). A rabbit polyclonal LRP1 antibody (2629), which inhibits ligand binding (29), was a generous gift from Dr. Dudley Strickland (American Red Cross, Rockville, MD).

Bovine lactoferrin was isolated from fresh skim milk by cation-exchange chromatography and gel filtration. Briefly, the milk was passed through S Sepharose Fast Flow at 4 C, and the bound proteins were eluted in steps with 0.1 M, 0.35 M, and 1 M NaCl. The 1 M NaCl fraction containing lactoferrin was dialyzed and freeze-dried. The material was then dissolved in 25 mM sodium phosphate buffer, pH 6.5, and reapplied to the cation exchanger, which had been equilibrated in the above buffer. Lactoferrin was eluted by application of a salt gradient to 1 M NaCl in phosphate buffer, and the recovered material was dialyzed and freeze dried. Final purification of lactoferrin was achieved by gel filtration through Sephacryl S300 in phosphate buffer, and the protein was recovered as a dialyzed freeze-dried powder. SDS-PAGE of purified lactoferrin gave a single band at approximately 80 kDa, and purity was greater than 98% as assessed by reversed phase HPLC (45). Iron saturation was approximately 15% for the native material, and iron binding studies using ferric nitrilotriacetate titration (46–47) of the native material indicated that virtually all the lactoferrin was able to bind iron stoichiometrically. Circular dichroism analysis gave near and far UV spectra, which were typical for both native and iron-bound lactoferrin (48).

Recombinant human lactoferrin was produced in baby hamster kidney cells as previously described (49). Oregon Green-labeled human lactoferrin was from Molecular Probes, Inc. (Eugene, OR).

RT-PCR

RNA was extracted using RNeasy Mini Kit and RNase-Free DNase Set (QIAGEN, Valencia, CA). RT-PCR was performed as previously described (44). Primers for LRP1 were designed to amplify human, rat, and murine sequence, whereas species-specific primers were designed for LRP2.

LRP1: forward (Fwd), 5′-GAGTGTTCCGTGTATGGCAC-3′; reverse (Rev), 5′-GATGCCTTGGATGATGGTC-3′

LRP2 (mouse): Fwd, 5′-CGTGCCTACCTTCCCGAC-3′; Rev, 5′-CGGACTTGAGTGAGCCAGG-3′

LRP2 (rat): Fwd, 5′-GTCACCAGTTCACTTGCTCC-3′; Rev, 5′-CCACTCCCATAACCATTCC-3′

LRP2 (human): Fwd, 5′-CCAAGTTGCTATTCGTGGG; Rev, 5′-GCACCCAGTCTCCAGTCAG-3′

Cell Proliferation

Proliferation of primary rat osteoblasts and SaOS-2 cells was performed in subconfluent cell populations as previously described (44). In brief, cells were seeded in 24-well tissue culture plates at 50,000 cells/ml in MEM containing 10% FCS, grown overnight to semiconfluence, and then growth arrested in MEM-0.1% BSA for 18 h before addition of test substances. Treatments were for 24 h. [^{3}H]Thymidine incorporation was assessed by pulsing the cells with [^{3}H]thymidine (0.5 μCi/well) 4 h before the end of the experimental incubation. In experiments designed to assess the effects of pharmacological inhibitors on lactoferrin-induced cell proliferation, the inhibitors were added 30 min before lactoferrin, and an inhibitor-only treatment was included. [^{3}H]Thymidine incorporation was calculated by expressing the values obtained in cultures of cells exposed to lactoferrin or lactoferrin and inhibitor as a fold increase over the values obtained in cultures of cells exposed to vehicle or inhibitor alone, respectively.

Proliferation of LRP1-null and LRP1-wt fibroblastic cells was assessed using a modified version of the above protocol. Cells were plated in 24-well tissue culture plates at 30,000 cells/ml for 6 h, and then growth-arrested in MEM-0.1% BSA for 18 h before addition of test substances. The remainder of the assay was performed as described for the osteoblastic cells. Each proliferation experiment was performed at least twice using experimental groups consisting of at least six wells.

Cell Binding and Internalization Assays

[^{125}I]Lactoferrin was produced by incubating 200 μg of bovine lactoferrin and 1 mCi Na ^{125}I (Amersham Pharmacia Biotech) with 1 Iodo-bead (Pierce Biotechnology, Rockford, IL) for 20 min, and then adding 200 μl of a 10 mg/ml sodium metabisulfite solution for 1 min. The decanted solution was applied to a PBS preequilibrated Sephadex NAP-10 column (Amersham Pharmacia Biotech), and the [^{125}I]lactoferrin was collected as fractions 3–4 during elution with PBS.

Assessment of binding of]^{125}I]lactoferrin to cultured osteoblastic cells was performed using a competitive binding assay, as previously described (50). Analysis of binding data was performed by nonlinear regression using GraphPad 4.1 software (GraphPad Software, San Diego, CA). For measurement of internalization of [^{125}I]lactoferrin, primary rat osteoblasts were seeded (1.5×10^5 cells per well) in 12-well plates in 5% FCS-MEM overnight, and then serum deprived before the addition of [^{125}I]lactoferrin in the presence or absence of 10 μM unlabeled lactoferrin, or the indicated concentration of RAP, for 3 h. Cells were then washed with PBS and detached from the plates using PBS containing 0.5 mg/ml trypsin, 0.5 mg/ml proteinase K, and 5 mM EDTA, pH 7.4. The cell suspension was centrifuged for 2 min, the pellet washed with PBS, and the cells lysed with 0.5 M KOH. Internalized [^{125}I]lactoferrin was defined as the radioactivity associated with the cell pellet. Each experimental condition was evaluated at least twice in triplicate. Equivalence of cell numbers for each experimental condition was verified in parallel wells that did not contain radioactivity

Confocal Microscopy

Primary rat osteoblastic cells were seeded in eight-well chamber slides and grown to approximately 70% confluence. Oregon Green-labeled lactoferrin was added to the cultured cells at a final concentration of 20 μg/ml under the experimental conditions indicated, after which the cells were fixed in 2% paraformaldehyde. To determine the intracellular location of the labeled lactoferrin, cell membranes were labeled with 1 μg/ml DiI (Molecular Probes), and nuclei were labeled with 4 μg/ml Hoechst 33258 (Sigma Chemical Co., St. Louis, MO).

Labeled cells were examined using a Leica SP2 confocal laser scanning microscope with Leica Confocal Software Version 2.5.1104 (Leica Microsystems, Heidelberg, Germany). The argon laser line at 488 nm was used for excitation of Oregon Green, and the emission wavelength range selected for collection of data was 500–650 nm. The same gain and offset settings were employed for imaging each treatment to achieve comparative results. For imaging of DiI, the green helium-neon laser line at 543 nm was used for excitation, and data were collected over an emission range of 555–700 nm. Nuclei stained with Hoechst 333252 were imaged using UV laser excitation (350/364 nm) and an emission range of 400–550 nm. Optimal pinhole settings were employed, and corresponding single optical sections were collected using an image format of 512 $\times$ 512 pixels. The objective lens used was 63$\times$/1.3 numerical aperture with oil immersion.

Immunoblotting

Immunoblotting was performed as previously described (44). In experiments designed to determine the effect of inhibitors of signal transduction, the cells were pretreated with the inhibitor for 30 min before addition of lactoferrin, except where indicated in the figure legend.

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