Journal of Cellular Biochemistry 53:145–155 (1993)

1,25-Dihydroxyvitamin D_3 and Macrophage Colony-Stimulating Factor-1 Synergistically Phosphorylate Talin

T. Meenakshi, F. Patrick Ross, John Martin, and Steven L. Teitelbaum

Department of Pathology and Laboratory Medicine, Jewish Hospital at Washington University Medical Center, St. Louis, Missouri 63110

Abstract Macrophage colony stimulating factor (CSF-1) and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are potent inducers of macrophage differentiation. Both appear to modulate protein phosphorylation, at least in part, through protein kinase C (PKC) raising the question as to whether they concurrently impact on macrophage-like cells. In this regard, we utilized the CSF-1 dependent murine macrophage-like line BAC 1.25F5. CSF-1 treatment of these cells for 30 min leads to particular phosphorylation of a 165 kDa protein, the putative CSF-1 receptor, and a 210 kDa moiety. $1,25(OH)_2D_3$ exposure for 24 h prior to addition of CSF-1 enhances phosphorylation of the 165 kDa species and, especially, the 210 kDa protein. Phosphorylation of the latter protein is $1,25(OH)_2D_3$ dose- and time-dependent and the molecule is specifically immunoprecipitated with a rabbit polyclonal anti-talin antibody. Experiments with okadaic acid show that the enhanced phosphorylation of talin does not result from serine phosphatase inhibition. CSF-1 and $1,25(OH)_2D_3$, alone or in combination, do not increase talin protein expression. The tyrosine kinase inhibitor, genestein, blocks $1,25(OH)_2D_3$/CSF-1 induced phosphorylation of the putative CSF-1 receptor but has no effect on talin phosphorylation which occurs exclusively on serine. In contrast to genestein, staurosporin, an inhibitor of PKC, inhibits phosphorylation of talin. Moreover, exposure of $1,25(OH)_2D_3$ pretreated cells to phorbol 12-myristate 13-acetate (PMA) in place of CSF-1 also prompts talin phosphorylation. Finally, $1,25(OH)_2D_3$ enhances ^{3}[H]PDBu binding, indicating that the steroid increases PMA receptor capacity. Thus, CSF-1 and $1,25(OH)_2D_3$ act synergistically via PKC to phosphorylate talin, a cytoskeletal-associated protein.

Key words: CSF-1, talin, macrophages, phosphorylation, vitamin D

The macrophage colony stimulating factor, CSF-1, is responsible for survival, proliferation, and differentiation of mononuclear phagocytes from bone marrow progenitor cells to mature macrophages [Tushinski et al., 1982]. It is a homodimeric glycoprotein growth factor, and its pleiotropic effects are mediated via a high affinity cell surface CSF-1 receptor [Guilbert and Stanley, 1980; Byrne et al., 1981; Bartelmez and Stanley, 1985] identical to the c-fms proto-oncogene product [Sherr et al., 1985].

Like CSF-1, $1,25(OH)_2D_3$, the biologically active form of vitamin D_3 (calciferol), promotes differentiation of transformed and nontransformed cells along a monocytic pathway [Rovera et al., 1979; Bar-Shavit et al., 1983; Mangelsdorf et al., 1984; Murao et al., 1983]. While the precise mechanisms by which the steroid prompts such maturation are unknown, it modulates expression of c-fms [Perkins and Teitelbaum, 1991], suggesting commonalities between $1,25(OH)_2D_3$ and CSF-1 mediated macrophage differentiation.

c-fms is a member of a family of growth factor receptors with tyrosine kinase activity which undergo autophosphorylation when occupied by their ligand [Yeung et al., 1987]. Exposure of macrophages to CSF-1 also prompts phosphorylation of several cytosolic proteins and ultimately leads to downregulation of its receptor, an event associated with ligand internalization and degradation [Guilbert and Stanley, 1986]. The means by which CSF-1 phosphorylates in-

Abbreviations used: $1,25(OH)_2D_3$ = 1,25-dihydroxyvitamin D_3; CSF-1 = colony stimulating factor; PKC = protein kinase C; PDBu = phorbol dibutyrate; PMA = phorbol-12 myristate-13 acetate; PY-20 = antiphosphotyrosine antibody; SDS-PAGE = sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Received March 22, 1993; accepted June 21, 1993.

Address reprint requests to Steven L. Teitelbaum, Department of Pathology, Jewish Hospital at Washington University Medical Center, 216 South Kingshighway, St. Louis, MO 63110.

tracellular proteins appears to involve, at least in part, activation of phospholipase C, thereby hydrolyzing phosphatidyl inositol 4,5-phosphate to inositol 1,4,5-triphosphate and diacylglycerol, leading in turn to increased diacylglycerol production and activation of protein kinase C (PKC) [Shurtleff et al., 1990; Veis and Hamilton, 1991; Imamura et al., 1990].

$1,25(OH)_2D_3$, acting through its receptor, has been shown to modulate membrane phospholipid metabolism, resulting in an increased synthesis of phosphatidyl serine [Matsumoto et al., 1985], activation of PKC [Martell et al., 1987; Obeid et al., 1990; Kim et al., 1991], and, ultimately, phosphorylation of endogenous proteins [Obeid et al., 1990]. Furthermore, $1,25(OH)_2D_3$-mediated macrophage differentiation is mimicked by the PKC agonist, phorbol-12 myristate-13 acetate (PMA), suggesting that activation of the kinase by the steroid may be central to the maturational process [Ways et al., 1987]. Consistent with this hypothesis, we have recently shown that $1,25(OH)_2D_3$ increases phorbol ester receptor number in bone marrow macrophage precursors (Tanaka et al., 1991).

Thus CSF-1 and $1,25(OH)_2D_3$, both of which are potent inducers of macrophage maturation, appear to modulate protein phosphorylation through PKC, raising the question as to whether they concurrently impact on macrophage-like cells and, in so doing, synergistically phosphorylate, via PKC, talin, a high molecular weight protein critical to cytoskeletal function.

MATERIALS AND METHODS

α-MEM and newborn calf serum were purchased from Gibco (Grand Island, NY). BAC 1,2F5 cells [Morgan et al., 1987] were kindly provided by Dr. R. Stanley (Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, New York, NY). $1,25(OH)_2D_3$ is supplied by Dr. Milan Uskokovic (Hoffman-LaRoche, Inc., Nutley, NJ). Genestein and PY20-antiphosphotyrosine antibody were purchased from ICN Biochemicals, Inc. (Irvine, CA), and Staurosporin was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Carrier-free [^{32}P] orthophosphate (9,000 Ci/mmol), ^{125}I-iodine and rainbow MW markers were purchased from Amersham Corporation (Arlington Heights, IL). [^{3}H] PDBu was purchased from DuPont-New England Nuclear (Boston, MA). All other reagents were from Sigma Chemical Co. (St. Louis, MO). A previously characterized [Beckerle et al., 1986] anti-chicken talin polyclonal rabbit antibody was kindly provided by Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC).

CSF-1 Purification and Labeling

CSF-1 was generated from murine L cells and purified to Stage IV as described [Stanley and Guilbert, 1981].

^{125}I-Binding to BAC 1,2F5 Cells

BAC 1,2F5 cells were maintained as described [Sengupta et al., 1988]. Purified CSF-1 was radioiodinated with retention of biological activity (as assessed by proliferation of bone marrow macrophage precursors) using carrier-free ^{125}I by a modification of Greenwood's chloramine-T method [Greenwood et al., 1963; Stanley et al., 1975]. The specific activity was approximately 2.9×10^{11} cpm/mg CSF-1. CSF-1 binding studies were performed in an assay volume of 250 μl [Perkins and Teitelbaum, 1991]. Protein determination was performed by the Bradford Protein Assay (Bio-Rad Kit Bio-Rad, Rockville Centre, NY).

Cell Labeling and Stimulation

BAC 1,2F5 cells cultured (4×10^6 cells/60 mm diameter tissue culture dishes) as described [Sengupta et al., 1988], were treated with 10^{-8} M $1,25(OH)_2D_3$ or with ethanol carrier for 25 h. Six h after the initiation of treatment, CSF-1 was removed, in the presence or absence of $1,25(OH)_2D_3$, for 16 h, to upregulate the CSF-1 receptor. Cells were incubated for 2 h in phosphate-free medium and labeled with carrier-free [^{32}P]-orthophosphate for 2 h.

After labeling, cells were cooled to 4°C for 30 min and stimulated with saturating concentrations (as determined by binding studies) of purified CSF-1 (30,000 U/ml) for 30 min at 4°C (1U CSF-1 = .44 fmol) [Stanley, 1985]. The cells were washed five times with ice-cold phosphate-buffered saline (136 mM NaCl, 3 mM KCl, 8 mmM Na_2HPO_4, pH 7.4) at 4°C and solubilized with Triton-containing buffer (10 mM Tris-HCl, 50 mM NaCl, 1% Triton X-100, 30 mM sodium phyrophosphate, 50 mM NaF, 100 μM sodium orthovanadate, 5 μM $ZnCl_2$, 1 mM phenylmethyl sulfonyl fluoride, and 0.1% BSA, pH 7.05 (1.5 ml/100 mm dish, 1 ml/60 mm dish). Cell debris was removed by centrifugation (11,000 *g*, 30 min, 4°C).

Equal amounts of cell protein extracts (500 μg) were used for immunoprecipitation with anti-phosphotyrosine antibody, PY-20. After binding for 15–18 h, the proteins were competitively eluted at 4°C in 40 μl of 1 mM phenylphosphate containing buffer [Golden et al., 1986]. The eluates were subjected to 5–15% gradient SDS-PAGE [Laemmli, 1970] and autoradiography.

Sequential Immunoprecipitation

Eluates from antiphosphotyrosine antibody (PY-20) were subjected to sequential immunoprecipitation with anti-talin antibody coupled to Sepharose-protein A. Bound proteins were washed several times in lysis buffer, eluted in boiling Laemmli buffer, and subjected to SDS-PAGE and autoradiography.

Phosphoamino Acid Analysis

The phosphoprotein of interest was eluted from the gels by digestion with 50 μg of trypsin in 1 ml of 10 mM NH_4HCO_3. Supernatants of the digestions were hydrolyzed in 6 N HCl for 2 h at 110°C and lyophilized. The samples were dissolved in 5 μl electrophoresis buffer [50:156:1796 = formic acid (88%): glacial acetic acid: H_2O] with 0.3–0.5 μg of internal phosphotyrosine, phosphoserine, and phosphothreonine as internal amino acid standards. Phosphoamino acids were separated by high voltage electrophoresis on a thin layer chromatography plate for 90 min at 2,500 V [Cooper et al., 1982]. The amino acids were visualized by staining with 0.5% (w/v) ninhydrin in 30:70% v/v acetic acid/acetone mixture and radioactive spots by autoradiography.

^{3}H-PDBu Binding

Cells cultured in 24-well plates (5 × 10_5/well) were treated with 1,25(OH)$_2$D$_3$ or carrier for 25 h in the presence of 3,000 U/ml CSF-1. The cells were washed twice with medium containing 0.1% BSA. ^{3}H-PDBu binding studies were performed as described [Tanaka et al., 1991; Jaken, 1987]. Total and non-specific binding were determined in duplicate.

RESULTS

CSF-1 Binding to BAC 1.2F5

As previously reported [Li and Stanley, 1991], purified biologically active ^{125}I-CSF-1 binds at 4°C in a concentration dependent manner to BAC 1.2F5 cells. Saturation is achieved with 24,000 U/ml (data not shown). Thus, in subsequent experiments, maintenance and saturating doses of 3,000 and 30,000 U/ml, respectively, of the cytokine were used.

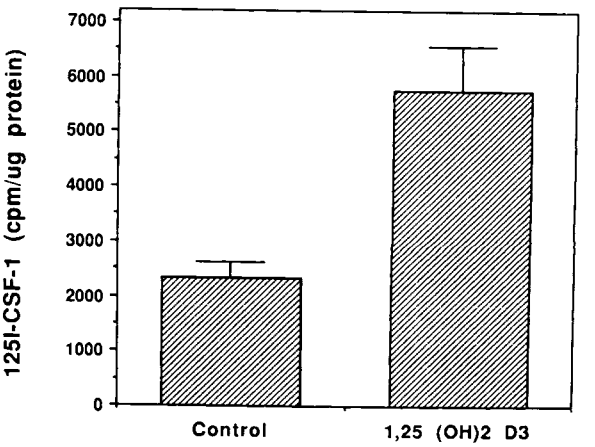

Fig. 1. Effect of 1,25(OH)$_2$D$_3$ on CSF-1 binding by BAC 1.2F5 cells. Cells were cultured in 24-well plates (5 × 10^5 cells/well) in 1 ml medium containing 3,000 U CSF-1 in the presence or absence of 1,25(OH)$_2$D$_3$ (10^{-8} M) for 24 h. After 6 h, the CSF-1 receptor was upregulated by removal of ligand and incubation continued in the presence of 1,25(OH)$_2$D$_3$ or carrier for an additional 18 h. In the presence or absence of 100 nM CSF-1, 30,000 U ^{125}I-CSF-1 was added at 4°C for 30 min and the cells were lysed in 1 N NaOH. Data expressed are specific counts bound. Each point represents a mean of triplicate cultures ± S.D.

1,25(OH)$_2$D$_3$ Upregulates the CSF-1 Receptor in BAC 1.2F5 Cells

We have previously shown that 1,25(OH)$_2$D$_3$ prompts appearance of CSF-1 receptor in murine bone marrow precursors [Perkins and Teitelbaum, 1991]. As shown in Figure 1, specific ^{125}I-CSF-1 binding by BAC 1.2F5 cells is also enhanced ($P < .001$) after 24 h exposure to 10^{-8} M 1,25(OH)$_2$D$_3$.

Effect of 1,25(OH)$_2$D$_3$ and CSF-1 on Protein Phosphorylation

Sengupta et al. [1988] have shown CSF-1 stimulation of BAC 1.25F5 cells at 4°C facilitates identification of proteins phosphorylated under the influence of CSF-1. Using this approach, we labeled 1,25(OH)$_2$D$_3$ or carrier-treated BAC 1.2F5 cells with [^{32}P] orthophosphate. The cells were then maintained at 4°C in the absence of CSF-1 or exposed for 30 min to a saturating concentration of purified CSF-1 (30,000 U/ml). They were then solubilized and phosphotryosine-containing (i.e., PY-20 reac-

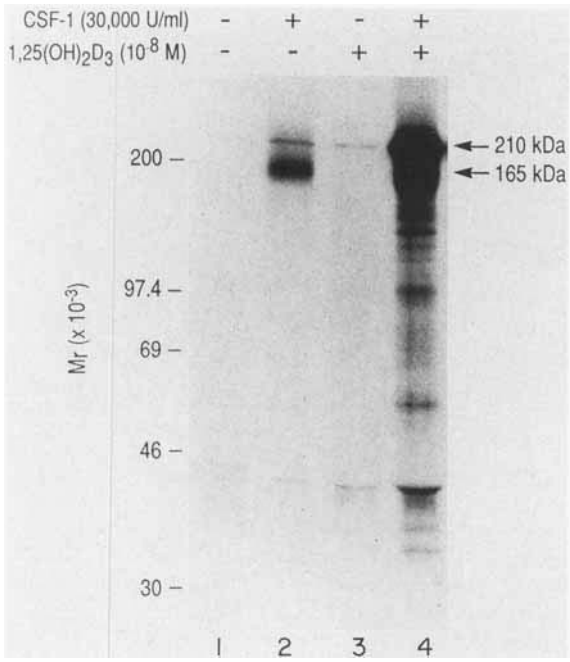

Fig. 2. Effect of $1,25(OH)_2D_3$ and CSF-1 on protein phosphorylation. Cells were treated with carrier or $1,25(OH)_2D_3$ (10^{-8} M) and labeled with ^{32}P-orthophosphate for 2 h. They were then incubated in the presence or absence of saturating concentrations of CSF-1 (30,000 U/ml), lysed and PY-20 antibody added to 500 μg protein lysates. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

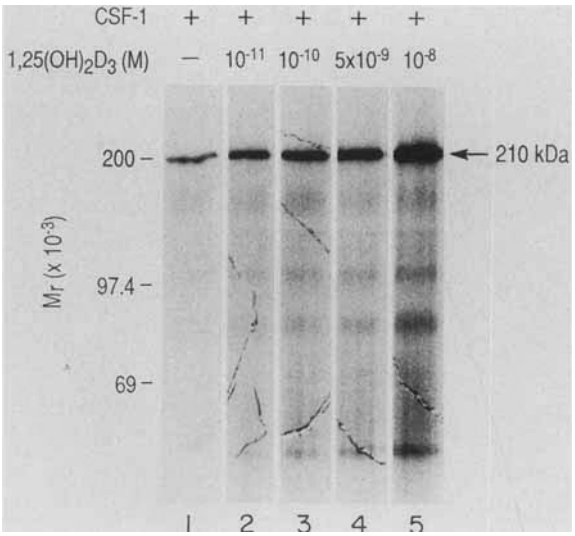

Fig. 3. Dose response of $1,25(OH)_2D_3$-induced phosphorylation. Cells were exposed to either carrier or $1,25(OH)_2D_3$ at the specified concentrations, ^{32}P-orthophosphate labeled, and incubated in the presence of a saturating concentration of CSF-1 (30,000 U/ml) for 30 min at 4°C prior to solubilization and immunoprecipitation with PY-20 antibody. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

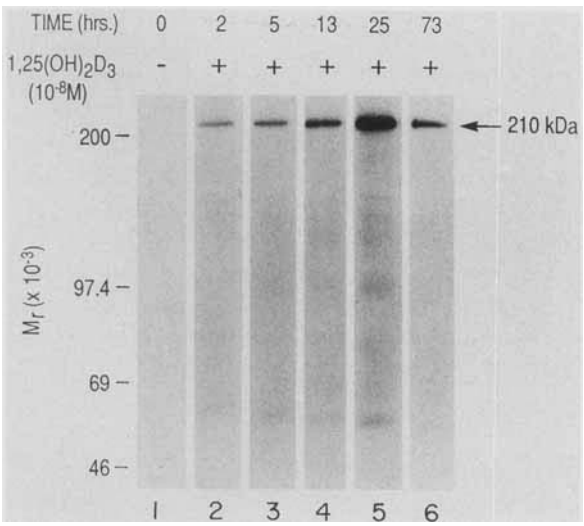

Fig. 4. Kinetics of $1,25(OH)_2D_3$-induced protein phosphorylation. Cells were pretreated with carrier or $1,25(OH)_2D_3$ (10^{-8} M) for the indicated duration, labeled with ^{32}P-orthophosphate, and incubated in the presence of 30,000 U/ml CSF-1, at 4°C for 30 min. Solubilization, immunoprecipitations, and analysis were performed as described in Figure 2.

tive) proteins immunoprecipitated and electrophoresed.

As shown in Figure 2, newly phosphorylated proteins of approximate molecular mass 210 and 165, the latter being the probable CSF-1 receptor, appear in cells treated with a saturating concentration of CSF-1. In contrast, $1,25(OH)_2D_3$ alone does not appear to phosphorylate the 165 kDa protein, but slightly phosphorylates the 210 kDa species. Even more dramatic protein phosphorylation occurs, however, when cells treated with $1,25(OH)_2D_3$ for 25 h are exposed for 30 min to 30,000 U/ml CSF-1. In this circumstance, an abundance of proteins (210, 165, 116, 97, 52, and 35 kDa), including those induced by CSF-1 alone, are phosphorylated. Consistent with the facts that $1,25(OH)_2D_3$ upregulates c-fms expression (Fig. 1) and, upon occupancy, the CSF-1 receptor undergoes autophosphorylation, the 165 kDa protein is phosphorylated to a much greater degree in response to both $1,25(OH)_2D_3$ and CSF-1 than to the cytokine alone.

$1,25(OH)_2D_3$ treatment also enhances CSF-1-mediated phosphorylation of the 210 kDa protein. The phenomenon is dose-dependent as regards $1,25(OH)_2D_3$ with increased phosphorylation appearing at 10^{-11} M (Fig. 3). As shown in Figure 4, CSF-1-stimulated 210 kDa protein phosphorylation occurs as early as two h after $1,25(OH)_2D_3$ treatment, and peaks at 25 h. Phos-

phorylation falls slightly when induced after 73 h of 1,25(OH)$_2$D$_3$ exposure but is still greater than that occurring in carrier-treated cells. It is of interest that while the 210 and 165 kDa proteins are invariably the major phosphorylated species in cells sequentially exposed to 1,25(OH)$_2$D$_3$ and CSF-1, the relative amounts of these phosphoproteins vary. For example, in light of the abundance of the 210 kDa protein, Figures 3 and 4 represent relatively underexposed autoradiograms so as to demonstrate 1,25(OH)$_2$D$_3$ dose and time dependency, respectively. In these experiments, the 210 kDa phosphorylated species was significantly greater than that of the 165 kDa protein. Furthermore, we observed that the 165 kDa phosphoprotein is sensitive to freezing and thawing, and the specimen was so treated in those experiments yielding a relative paucity of the molecule.

Characterization of 210 kDa Protein

The nature of the 210 kDa phosphoprotein was established as talin by subjecting the ^{32}P-labeled proteins to sequential immunoprecipitations. The total phosphotyrosine-containing product precipitated with the anti-phosphotyrosine antibody PY-20, was reprecipitated with anti-talin yielding a 210 kDa phosphoprotein induced by CSF-1 and 1,25(OH)$_2$D$_3$ (Fig. 5).

The enhanced ^{32}P labeling of talin may theoretically reflect either increased protein substrate or accelerated kinase activity. To resolve this issue, we estimated the quantity of PY-20 immunoprecipitated talin by silver staining. As can be seen in Figure 6, regardless of treatment, there are no differences in intensity of 210 kDa silver-stained protein band.

Mechanisms of Phosphorylation of Talin

Having identified the 210 kDa protein as talin, we turned to the mechanism by which it undergoes phosphorylation under the influence of 1,25(OH)$_2$D$_3$ and CSF-1. We first addressed the possibility that the phenomenon reflects inhibition of phosphatase activity. To this end, we utilized okadaic acid, a potent inhibitor of pp1 and pp2A [Haystead et al., 1989; Cohen et al., 1990] and found it fails to enhance talin phosphorylation (Fig. 7). Thus the 1,25(OH)$_2$D$_3$/CSF-1 effect probably does not reflect serine phosphatase inhibition.

With this information in hand, we addressed the kinase(s) responsible for 1,25(OH)$_2$D$_3$/CSF-1 stimulated talin phosphorylation. Because c-fms

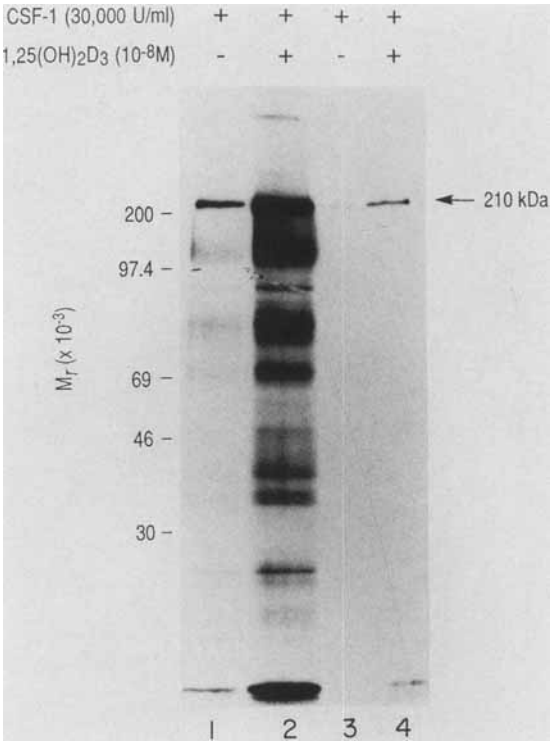

Fig. 5. Characterization of 210 kDa protein. Cells were exposed to carrier or 1,25(OH)$_2$D$_3$ (10^{-8} M) for 24 h. The CSF-1 receptor was upregulated and the cells were labeled with ^{32}P-orthophosphate for 2 h, and incubated in the presence of CSF-1 (30,000 U/ml) for 30 min at 4°C. Protein lysates (500 μg) were used for immunoprecipitation with PY-20 antibody. The PY-20 (anti-phosphoytyrosine) immunoprecipitates were eluted and reimmunoprecipitated with chicken anti-talin rabbit polyclonal antibody. The PY-30 reactive proteins (**lanes 1, 2**) and anti-talin immunoprecipitates (**lanes 3, 4**) were subjected to SDS-PAGE and autoradiography.

is a tyrosine-specific protein kinase [Yeung et al., 1987], we first explored the effect of the tyrosine kinase inhibitor, genestein [Akiyama et al., 1987; Watanabe et al., 1989]. As shown in Figure 8, this agent blocks phosphorylation of the putative CSF-1 receptor but not of talin.

In contrast to genestein, staurosporin, a potent PKC inhibitor [Tamaoki et al., 1986; Easom et al., 1989], attenuates 1,25(OH)$_2$D$_3$/CSF-1 induced phosphorylation in a concentration-dependent manner (Fig. 9). To further investigate the possible role of PKC in talin phosphorylation, cells were pretreated with 1,25(OH)$_2$D$_3$ for 24 h and labeled with ^{32}P orthophosphate for 2 h in the absence of steroid. The cells were then cooled to 4°C and stimulated with the PKC agonist, PMA (1.6×10^{-7} M). As shown in Figure 10, similar to CSF-1, PMA enhances talin phosphorylation by cells pretreated with 1,25(OH)$_2$D$_3$.

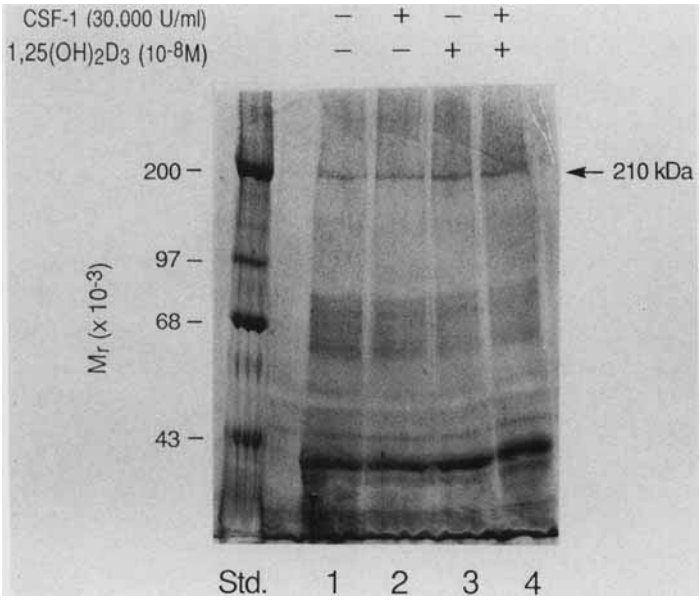

Fig. 6. Silver stained phosphoproteins. Protein lysates were prepared from control cells **(lane 1)**, or those treated at 4°C with CSF-1 **(lane 2)**, only with $1,25(OH)_2D_3$ **(lane 3)**, or with both CSF-1 and $1,25(OH)_2D_3$ **(lane 4)**. They were then immunoprecipitated with anti-phosphotyrosine antibody (PY-20). The immunoprecipitates were eluted to SDS-PAGE and silver stained.

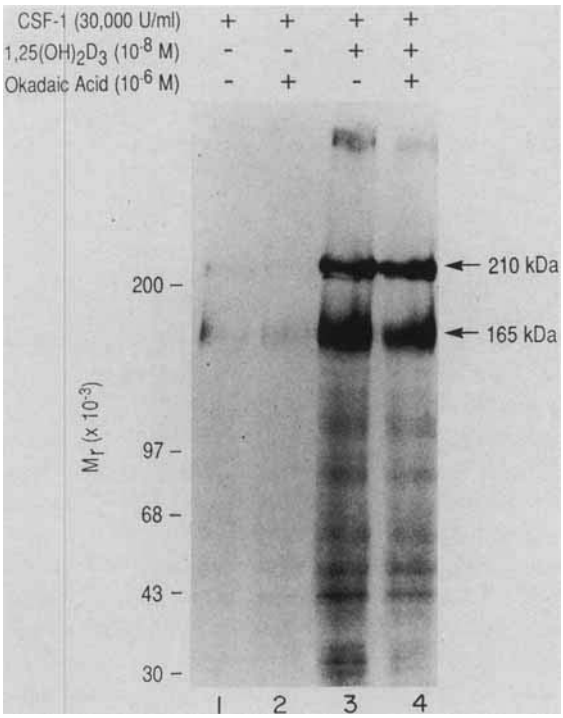

Fig. 7. Effect of okadaic acid on protein phosphorylation. Cells were treated with carrier or $1,25(OH)_2D_3$ (10^{-8} M) and labeled with ^{32}P-orthophosphate for 2 h. They were then incubated with 10^{-8} M okadaic acid at 37°C for 15 min prior to addition of a saturating concentration of CSF-1 (30,000 U/ml) for 30 min at 4°C. Solubilization, immunoprecipitation, and analysis were done as described in Figure 3.

Phosphoamino acid analysis of the ^{32}P orthophosphate-labeled talin is also consistent with PKC mediation as $1,25(OH)_2D_3$/CSF-1 induced phosphorylation occurs exclusively on serine (Fig. 11). In contrast, minimal phosphorylation occurs in cells treated only with CSF-1.

These observations are in keeping with the hypothesis that $1,25(OH)_2D_3$ enhances expression of PKC which is, in turn, activated by CSF-1. We therefore examined the effects of $1,25(OH)_2D_3$ on ^{3}[H]-PDBu binding by BAC 1.2F5 cells. As seen in Figure 12, treatment with $1,25(OH)_2D_3$ (10^{-8} M) for 25 h significantly increases specific cell-associated radioligand, indicating that in these circumstances the steroid induces expression of PKC.

DISCUSSION

The development of appropriate cell lines and the capacity to isolate homogeneous populations of monocytic precursors at various stages of differentiation has yielded major insights into agents which modulate macrophage differentiation. CSF-1 is clearly pivotal in this regard as early macrophage precursors die in the absence of the growth factor [Clohisy et al., 1987]. While not proven to be essential to macrophage survival, $1,25(OH)_2D_3$ accelerates maturation of mononuclear phagocyte precursors [Watanabe et al., 1989] and differentiates myelomonocytic

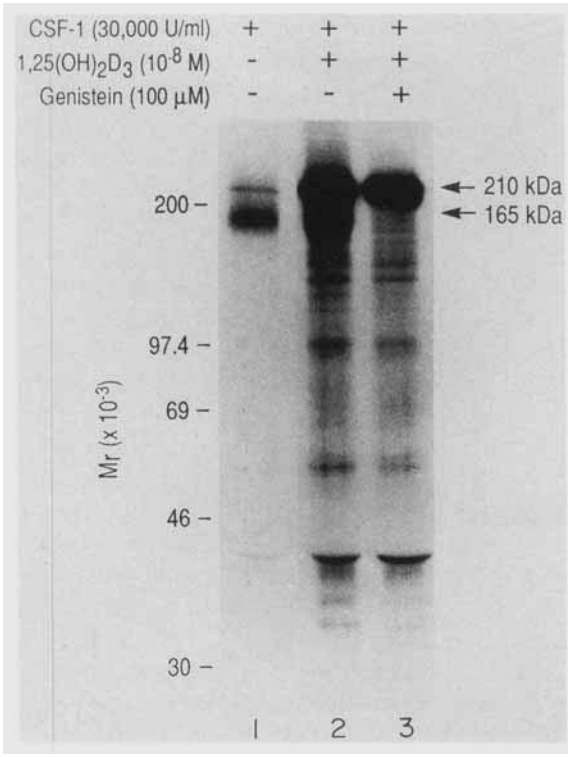

Fig. 8. Effect of genestein on protein phosphorylation. Cells were labeled with ^{32}P-orthophosphate for 2 h. They were then incubated with 100 µm genestein for 15 min at 37°C prior to addition of a saturating concentration of CSF-1 (30,000 U/ml) for 30 min at 4°C. Protein lysates (500 µg) were used for immunoprecipitations with PY-20 antibody. The immunoprecipitates were subjected to SDS-PAGE and autoradiography. Note that lanes 1 and 2 are identical to lanes 3 and 4, respectively, of Figure 2, as the experiments were performed simultaneously.

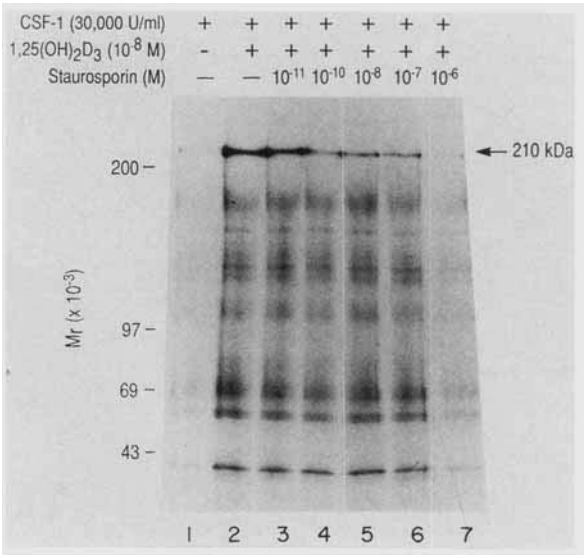

Fig. 9. Effect of staurosporin on protein phosphorylation. Cells were labeled with ^{32}P-orthophosphate for 2 h and then incubated with indicated concentrations of staurosporin for 30 min at 37°C. The saturating concentration of CSF-1 (30,000 U/ml) was then added for 30 min at 4°C. Protein lysates (500 µg) were used for immunoprecipitation with PY-20 antibody. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

leukemia cells along a monocytic pathway [Reitsma et al., 1983].

These findings suggest that CSF-1 and 1,25(OH)$_2$D$_3$ enjoy complimentary, albeit different roles in macrophage differentiation. To explore this hypothesis, we turned to a CSF-1 dependent murine line, BAC 1.2F5, known to respond to the growth factor by phosphorylation of a number of proteins, including its own receptor [Sengupta et al., 1988]. We confirmed this observation and found that consistent with our report that 1,25(OH)$_2$D$_3$ modulates the CSF-1 receptor [Perkins and Teitelbaum, 1991], the steroid augments the quantity of putative c-fms phosphorylated by its ligand.

Most importantly as regards this communication, a major species phosphorylated by CSF-1 in conjunction with 1,25(OH)$_2$D$_3$ is a 210 kDa protein. Phosphorylation of the protein is concentration-dependent as regards 1,25(OH)$_2$D$_3$ with induction apparent within the physiological range of the hormone. Moreover, despite the fact that the quantity of 210 kDa phosphoprotein maximizes after 24 h of steroid exposure, an effect obtains within two h.

Macrophage differentiation is characterized by the progressive ability of the precursor cell to attach to substrate. In fact, 1,25(OH)$_2$D$_3$ treatment of either authentic bone marrow macrophage precursors [Clohisy et al., 1987] or poorly differentiated leukemic lines, such as HL-60 [Reitsma et al., 1983], leads to rapid transition of non-adherent to adherent cells. These observations argue that vitamin-D induced differentiation may in some way alter cytoskeleton-associated proteins. We have, in this regard, recently shown that 1,25(OH)$_2$D$_3$ enhances expression of the vitronectin receptor integrin, $\alpha_v\beta_3$ by avian bone marrow macrophages [Medhora et al., 1993]. With this in mind, and given the size of the 1,25(OH)$_2$D$_3$/CSF-1 phosphorylated species, we queried if it is talin and indeed found this to be the case.

Talin is a large molecule which interacts with the β1 integrin subunit on the one hand and vinculin and actin on the other [Horwitz et al., 1986]. These proteins recognize distinct binding sites on talin [Horwitz et al., 1986] and, as they all colocalize to adhesion sites on the plasma

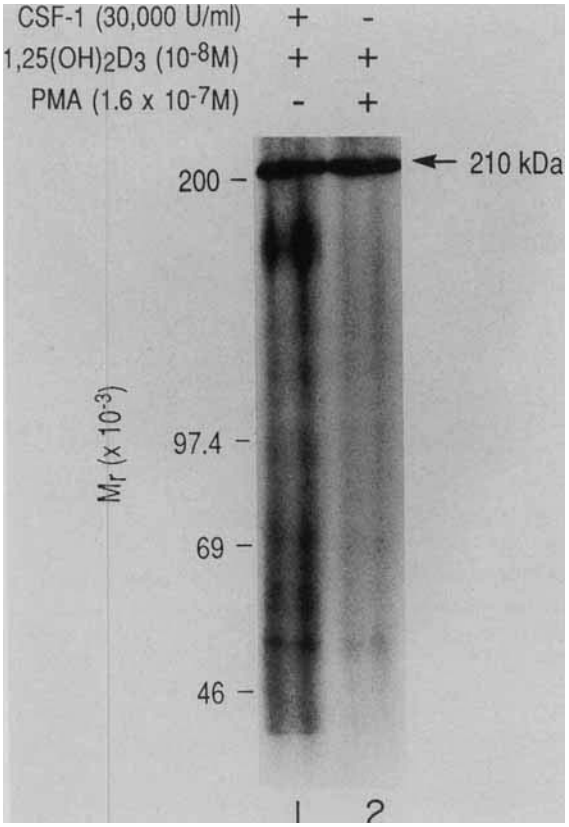

Fig. 10. Effect of PMA on protein phosphorylation. Cells were treated with $1,25(OH)_2D_3$ (10^{-8} M) for approximately 24 h and labeled with [^{32}P]-orthophosphate for 2 h at 37°C. They were then exposed to either a saturating concentration of CSF-1 (30,000 U/ml) **(lane 1)** or PMA (1.6×10^{-7} M) **(lane 2)** for 30 min at 4°C. The cells were solubilized and the phosphoproteins immunoprecipitated (PY-20), eluted, and analyzed by SDS-PAGE and autoradiography.

membrane [Burridge et al., 1990], it is likely that talin plays a pivotal role in associating matrix recognizing integrins with the cytoskeleton.

While the physiological significance of the $1,25(OH)_2D_3$/CSF-1-mediated effect is yet to be determined, talin phosphorylation is known to have biological consequences which appear cell-specific. For example, PKC-mediated talin phosphorylation of African green monkey kidney cells [Meigs and Wang, 1986] or chicken embryo fibroblasts [Beckerle, 1990] prompts actin fiber disarray and/or disappearance of focal contacts. Similarly, interleukin-1 induced talin phosphorylation is associated with retraction of periodontal ligament fibroblasts from substrate [Qwarnstrom et al., 1991]. While these observations suggest that talin phosphorylation disrupts cell-matrix attachment, induction of the phosphoprotein in lymphocytes associates it with integrins [Burn et al., 1988]. Thus, depending upon the target cell, talin phosphorylation may potentially disrupt or stabilize the cytoskeleton.

Having identified the 210 kDa protein as talin, we turned to the mechanism of its phosphorylation. We found that $1,25(OH)_2D_3$ and CSF-1 do not increase the absolute amount of talin, leading us to conclude that the mechanism must involve kinase activation or phosphatase inhibition. Eliminating serine phosphatase inhibition by the use of okadaic acid [Cohen, 1989], we explored the probability of enhanced tyrosine kinase activity, a serious consideration in light of the enzymatic activity of the occupied CSF-1 receptor [Yeung et al., 1987]. While as expected, the tyrosine kinase inhibitor genestein [Akiyama

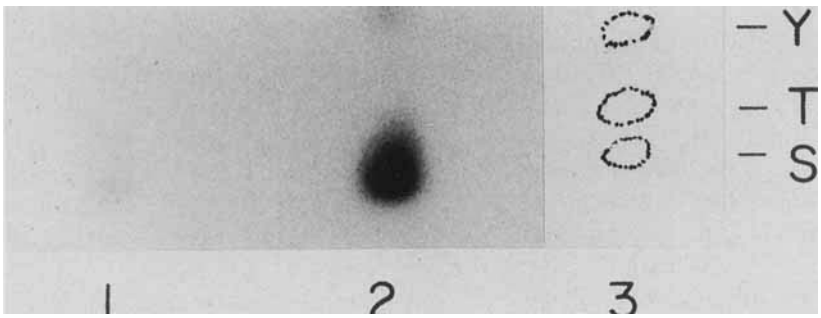

Fig. 11. Phosphoamino acid analysis of talin. The $1,25(OH)_2D_3$/CSF-1 induced 210 kDa protein excised from dried gel was extracted with trypsin and hydrolyzed in 6N HCl for 2 h at 110°C. The product was lyophilized and rehydrated in 5 μl loading buffer containing internal standards phosphoserine, phosphotyrosine, and phosphothreonine. The samples were subjected to thin layer electrophoresis and autoradiography. The dotted circles represent the positions of phosphoamino acid standards revealed by ninhydrin staining. S = phosphoserine; T = phosphothreonine; Y = phosphotyrosine.

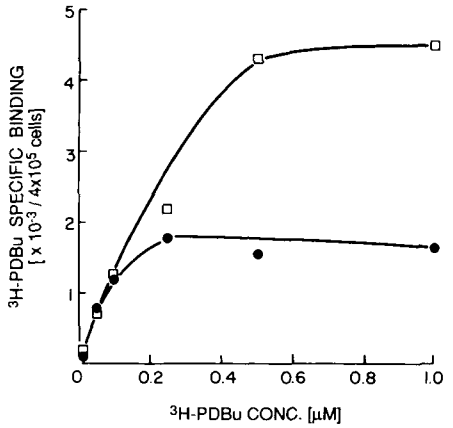

Fig. 12. Effect of $1,25(OH)_2D_3$ on [^{3}H]PDBu binding. Cells were treated with carrier (closed symbols) or $1,25(OH)_2D_3$ (open symbols) for 24 h in the presence of 3,000 U/ml CSF-1. [^{3}H]PDBu binding was then measured by incubation with radioligand for 30 min at 37°C. Non-specific binding was determined by the co-addition of 100 μM PDBu. Specific binding is illustrated which represents total minus non-specific (20–40% of total binding). Each point depicts the mean of duplicate determinations and the data are illustrative of 3 experiments.

et al., 1987; Watanabe et al., 1989] blocks phosphorylation of putative c-fms by $1,25(OH)_2D_3$/CSF-1, it has no effect on talin phosphorylation. On the other hand, talin is a known substrate for PKC [Litchfield and Ball, 1990], and we found that its phosphorylation by $1,25(OH)_2D_3$/CSF-1 is dampened by relatively low concentrations of the enzyme's inhibitor, staurosporin. Also consistent with the properties of PKC [Litchfield and Ball, 1990], serine and not tyrosine is phosphorylated in talin under the influence of the steroid and growth factor. Moreover, the PKC agonist PMA, when substituted for CSF-1 in vitamin D-treated cells, effectively phosphorylates talin.

$1,25(OH)_2D_3$ is known to directly enhance PKC transcription [Obeid et al., 1990], to activate the enzyme [Wali et al., 1990], and to translocate it to cell membranes [Simboli-Campbell et al., 1992]. While $1,25(OH)_2D_3$-induced PKC activation may [Burn et al., 1988] or may not involve stimulated diacylglycerol production [Obeid et al., 1990], regardless of mechanism, the steroid has both immediate and long-term agonistic effects on the kinase. Thus it is not surprising that while $1,25(OH)_2D_3$-primed talin phosphorylation maximizes after 1 day, it may be detected within 2 h. The mechanism of relatively short-term phosphorylation-priming remains elusive, but as evidenced by enhanced PDBu binding, the steroid ultimately appears to promote expression of PKC.

Relative to its effect when combined with CSF-1, $1,25(OH)_2D_3$ alone has little impact on talin phosphorylation, which requires short co-exposure to the growth factor. This latter finding is consistent with the known capacity of CSF-1 to activate PKC, probably by enhanced generation of diacylglycerol from phosphatidyl choline [Imamura et al., 1990]. Thus, we propose that the mechanism of talin phosphorylation by $1,25(OH)_2D_3$/CSF-1 involved increased PKC synthesis under the influence of the steroid with subsequent activation by the growth factor.

ACKNOWLEDGMENTS

This work was supported in part by NIH Grant DE05413-13 and NIH Training Grant AR32087-01.

REFERENCES

Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y (1987): Genistein: A specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 262:5592–5595.

Bar-Shavit Z, Teitelbaum SL, Reitsma PH, Hall A, Pegg LE, Trial J, Kahn AJ (1983): Induction of monocytic differentiation and bone resorption by 1,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA 80:5907–5911.

Bartelmez SH, Stanley ER (1985): Synergism between hemopoietic growth factors (HGFs) detected by their effects on cells bearing receptors for a lineage-specific HGF: Assay of hemopoietin-1. J Cell Physiol 122:370–378.

Beckerle MC (1990): The adhesion plaque protein, talin, is phosphorylated in vivo in chicken embryo fibroblasts exposed to a tumor-promoting phorbol ester. Cell Regul 1:227–236.

Beckerle MC, O'Halloran T, Burridge K (1986): Demonstration of a relationship between talin and P^{235}, a major substrate of the calcium-dependent protease in platelets. J Cell Biochem 30:259–270.

Burn P, Kupfer A, Singer SJ (1988): Dynamic membrane-cytoskeletal interactions: Specific association of integrin and talin arises in vivo after phorbol ester treatment of peripheral blood lymphocytes. Proc Natl Acad Sci USA 85:497–501.

Burridge K, Nuckolls G, Otey C, Pavalko F, Simon K, Turner C (1990): Actin-membrane interaction in focal adhesions. Cell Differ Develop 32:337–342.

Byrne PV, Guilbert LJ, Stanley ER (1981): Distribution of cells bearing receptors for a colony-stimulating factor (CSF-1) in murine tissues. J Cell Biol 91:848–853.

Clohisy DR, Bar-Shavit Z, Chappel J, Teitelbaum SL (1987): 1,25-dihydroxyvitamin D_3 modulates bone marrow macrophage precursor proliferation and differentiation. Upregulation of the mannose receptor. J Biol Chem 262:15922–15929.

Cohen P (1989): The structure and regulation of protein phosphatases. Ann Rev Biochem 58:453–508.

Cohen P, Holmes CFB, Tsukitani Y (1990): Okadaic acid: A new probe for the study of cellular regulation. TIBS 15:98–102.

Cooper JA, Sefton BM, Hunter T (1982): Detection and quantification of phosphotyrosine in proteins. Methods Enzymol 99:387–405.

Easom RA, Hughes JH, Landt M, Wolf BA, Turk J, McDaniel ML (1989): Comparison of effects of phorbol esters and glucose on protein kinase C activation and insulin secretion in pancreatic islets. Biochem J 264:27–33.

Golden A, Nemeth SP, Brugge JS (1986): Blood platelets express high levels of the pp60$^{c\text{-}src}$-specific tyrosine kinase activity. Proc Natl Acad Sci USA 83:852–856.

Greenwood FC, Hunter WM, Glover JS (1963): The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem J 89:114–123.

Guilbert LJ, Stanley ER (1980): Specific interaction of murine colony-stimulating factor with mononuclear phagocytic cells. J Cell Biol 85:153–159.

Guilbert LJ, Stanley ER (1986): The interaction of ^{125}I-colony-stimulating factor-1 with bone marrow-derived macrophages. J Biol Chem 261:4024–4032.

Haystead TAJ, Sim ATR, Carling D, Honnor RC, Tsukitani Y, Cohen P, Hardie DG (1989): Effects of the tumour promoter okadaic acid on intracellular protein phosphorylation and metabolism. Nature 337:78–81.

Horwitz A, Duggan K, Buck C, Beckerle MC, Burridge K (1986): Interaction of plasma membrane fibronectin receptor with talin—a transmembrane linkage. Nature 320: 531–533.

Imamura K, Dianoux A, Nakamura T, Kufe D (1990): Colony-stimulating factor 1 activates protein kinase C in human monocytes. EMBO J 9:2423–2428.

Jaken S (1987): Measurement of phorbol ester receptors in intact cells and subcellular fractions. Methods Enzymol 141:275–287.

Kim YR, Abraham NG, Lutton JD (1991): Mechanisms of differentiation of U937 leukemic cells induced by GM-CSF and 1,25$(OH)_2D_3$. Leuk Res 15:409–418.

Laemmli UP (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Li W, Stanley ER (1991): Role of dimerization and modification of the CSF-1 receptor in its activation and internalization during the CSF-1 response. EMBO J 10:277–288.

Litchfield DW, Ball EH (1990): Phosphorylation of the cytoskeletal protein talin by protein kinase C. Biochem Biophys Res Commun 134:1276–1283.

Mangelsdorf DJ, Koeffler HP, Donaldson CA, Pike JW, Haussler MR (1984): 1,25-dihydroxyvitamin D_3-induced differentiation in a human promyelocytic leukemia cell line (HL-60): Receptor-mediated maturation to macrophage-like cells. J Cell Biol 98:391–398.

Martell RE, Simpson RU, Taylor JM (1987): 1,25-dihydroxyvitamin D_3 regulation of phorbol ester receptors in HL-60 leukemia cells. J Biol Chem 262:5570–5575.

Matsumoto T, Kawanobe Y, Morita K, Ogata E (1985): Effect of 1,25-dihydroxyvitamin D_3 on phospholipid metabolism in a clonal osteoblast-like rat osteogenic sarcoma cell line. J Biol Chem 260:13704–13709.

Medhora M, Teitelbaum SL, Chappel J, Alvarez J, Mimura H, Ross FP, Hruska K (1993): 1α,25-dihydroxyvitamin D_3 up-regulates expression of the osteoclast integrin $\alpha_v\beta_3$. J Biol Chem 268:1456– 1461.

Meigs JB, Wang Y-L (1986): Reorganization of alpha-actinin and vinculin induced by a phorbol ester in living cells. J Cell Biol 102:1430–1438.

Morgan C, Pollard JW, Stanley ER (1987): Isolation and characterization of a cloned growth factor dependent macrophage cell line, BAC1.25F. J Cell Physiol 130:420–427.

Murao S, Gemmell MA, Callaham MF, Anderson NL, Huberman E (1983): Control of macrophage cell differentiation in human promyelocytic HL-60 leukemia cells by 1,25-dihydroxyvitamin D_3 and phorbol-12-myristate-13-acetate. Cancer Res 43:4989–4996.

Obeid LM, Okazaki T, Karolak LA, Hannun YA (1990): Transcriptional regulation of protein kinase C by 1,25-dihydroxyvitamin D_3 in HL-60 cells. J Biol Chem 265: 2370–2374.

Perkins SL, Teitelbaum SL (1991): 1,25-dihydroxyvitamin D_3 modulates colony stimulating factor-1 receptor binding by murine bone marrow macrophage precursors. Endocrinology 128:303–311.

Qwarnstrom EE, MacFarlane SA, Page RC, Dower SK (1991): Interleukin 1$_\beta$ induces rapid phosphorylation and redistribution of talin: A possible mechanism for modulation of fibroblast focal adhesion. Proc Natl Acad Sci USA 88:1232–1236.

Reitsma PH, Rothberg PG, Astrin SM, Trial J, Bar-Shavit Z, Hall A, Teitelbaum SL, Kahn AJ (1983): Regulation of *myc* gene expression in HL-60 leukemia cells by a vitamin D metabolite. Nature 306:492–494.

Rovera G, Santoli D, Damsky C (1979): Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. Proc Natl Acad Sci USA 76:2779–2783.

Sengupta A, Liu W, Yeung YG, Yeung DCY, Frackelton AR, Stanley ER (1988): Identification and subcellular localization of proteins that are rapidly phosphorylated in tyrosine in response to colony-stimulating factor 1. Proc Natl Acad Sci USA 85:8062–8066.

Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER (1985): The c-fms proton-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41:665–676.

Shurtleff SA, Downing JR, Rock CO, Hawkins SA, Roussel MF, Sherr CJ (1990): Structural features of the colony-stimulating factor 1 receptor that affect its association with phosphatodylinositol 3-kinase. EMBO J 9:2415–2421.

Simboli-Campbell M, Franks DJ, Welsh J (1992): 1,25$(OH)_2D_3$ increases membrane associated protein kinase C in MDBK cells. Cell Signal 4:99–109.

Stanley ER (1985): The macrophage colony stimulating factor, CSF-1. Methods Enzymol 116:564–568.

Stanley ER, Guilbert LJ (1981): Methods for the purification, assay, characterization and target cell binding of a colony stimulating factor (CSF-1). J Immunol Methods 42:253–284.

Stanley ER, Hansen G, Woodcock J, Metcalf D (1975): Colony stimulating factor and the regulation of granulopoiesis and macrophage production. Fed Proc 34:2272–2278.

Tamaoki T, Nomoto H, Takahashi I, Kata Y, Morimoto M, Tomita F (1986): Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. Biochem Biophys Res Commun 135:397–402.

Tanaka H, Hruska KA, Seino Y, Malone JD, Nishii Y, Teitelbaum SL (1991): Disassociation of the macrophage-maturational effects of vitamin D from respiratory burst priming. J Biol Chem 266:10888–10892.

Tushinski RJ, Olivier IT, Guilbert LJ, Tynan PW, Warner JR, Stanley ER (1982): Survival of mononuclear phagocytes depends on a lineage-specific growth factor that the differentiated cells selectively destroy. Cell 28:71–81.

Veis N, Hamilton JA (1991): Colony stimulating factor-1 stimulates diacylglycerol generation in murine bone marrow-derived macrophages, but not in resident peritoneal macrophages. J Cell Physiol 147:298–305.

Wali RK, Baum CL, Sitrin MD, Brasitus TA (1990): $1,25(OH)_2D_3$ stimulates membrane phosphoinositide turnover, activates protein kinase C, and increases cytosolic calcium in rat colonic epithelium. J Clin Invest 85:1296–1303.

Watanabe T, Shiraishi T, Sasaki M, Oishi MC (1989): Inhibitors for protein kinases, ST638 and genistein: Induce differentiation of mouse erythroleukemia cells in a synergistic manner. Exp Cell Res 183:335–342.

Ways DK, Dodd RC, Bennett TE, Gray TK, Earp HS (1987): 1,25-dihydroxyvitamin D_3 enhances phorbal ester-stimulated differentiation and protein kinase C-dependent substrate phosphorylation activity in the U937 human monoblastoid cell. Endocrinology 121:1654–1661.

Yeung YG, Jubinsky PT, Sengupta A, Yeung DCY, Stanley ER (1987): Purification of the colony-stimulating factor 1 receptor and demonstration of its tyrosine kinase activity. Proc Natl Acad Sci USA 84:1268–1271.