Binding of G-CSF, GM-CSF, Tumor Necrosis Factor-α, and γ-Interferon to Cell Surface Receptors on Human Myeloid Leukemia Cells Triggers Rapid Tyrosine and Serine Phosphorylation of a 75-Kd Protein

By Jane P.M. Evans, Anthony R. Mire-Sluis, A. Victor Hoffbrand, and R. Gitendra Wickremasinghe

Granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), γ-interferon (γ-IFN), or tumor necrosis factor-α (TNF-α) triggered the rapid, stable phosphorylation of a 75-Kd protein (p75) when incubated with permeabilized HL60 human myeloid leukemia cells in the presence of [γ-32P] ATP. Among several chemical inducers of HL60 cell differentiation, dimethyl sulfoxide also triggered p75 labeling, but retinoic acid or 12-0-tetradecanoylphorbol-13-acetate did not elicit this response. Pretreatment of cells with G-CSF or GM-CSF for more than 30 seconds before permeabilization rendered the p75 labeling undetectable, suggesting that ligand-stimulated labeling was rapidly completed within this time in intact cells. Phosphorylation of p75 occurred on serine and tyrosine residues. This conclusion was confirmed by direct phosphoamino acid analysis. Immunoblot analysis of lysates of intact HL60 cells that had been incubated with G-CSF, GM-CSF, IFN, or TNF confirmed that tyrosine phosphorylation of a p75 also occurred in response to these cytokines in intact cells. Pretreatment of intact HL60 cells with one biologic agent or dimethyl sulfoxide abolished p75 labeling in response to incubation of permeabilized cells with a second agent, strongly suggesting that the same protein was phosphorylated in response to these treatments. p75 labeling was strictly dependent on expression of the appropriate ligand receptor. Data suggest that activation of a tyrosine kinase system is an early response to the binding of G-CSF, GM-CSF, TNF, or IFN to their respective cell surface receptors, or to the addition of dimethyl sulfoxide, and that the resulting phosphorylation event(s) may play a role in securing common elements in the biologic responses to these agents.

© 1990 by The American Society of Hematology.
TYROSINE PROTEIN PHOSPHORYLATION IN HL60 CELLS

MATERIALS AND METHODS

Cell lines. All cell lines were maintained in RPMI 1640 medium (GIBCO, Paisley, UK) supplemented with 10% fetal calf serum and antibiotics. HL60 human myeloid leukemia cells express receptors for G-CSF, GM-CSF, TNF, and IFN. KG1 cells express receptors for GM-CSF, whereas the derivative cell line KGa does not. K562 cells, established from a patient with chronic granulocytic leukemia, express receptors for TNF while the cell line KLOEK, established by Epstein-Barr virus infection of human B lymphocytes, is negative for TNF receptors (F.T. Cordingley and M.K. Brenner, personal communication, June 1988). All cells were used in the logarithmic phase of growth.

Growth factors and cytokines. All CSFs and cytokines used in this work were human recombinant materials that were purified to homogeneity and obtained from the following sources: G-CSF (10^6 U/mg) and IL-2 (2 x 10^6 U/mg), Amersham International, Amersham, UK; GM-CSF (1.1 x 10^6 U/mg) was donated by Glaxo Institute for Molecular Biology, Geneva, Switzerland; and IFN (2.3 x 10^6 U/mg) by Biogen SA, Geneva, Switzerland.

Detection of protein phosphorylation in response to stimulation of permeabilized cells. Protein phosphorylation was detected by a modification of a previously published procedure.10 All cells were harvested by centrifugation, washed twice in HBSS and lysed in a solution containing 0.25 mol/L Tris pH 7.4, 4% sodium dodecyl sulfate, 10% glycerol, and 0.5% β-mercaptoethanol, before electrophoresis on 15% SDS-PAGE gels. Lysates were transferred to nitrocellulose membranes, and probed with an antibody prepared against azobenzene phosphonate (ABP) haptenized to bovine serum albumin (BSA) as described. Lysate equivalent to 3 x 10^5 cells was loaded in each lane. Cross-reactivity of this antiserum with phosphotyrosine residues and its specificity have been documented elsewhere.

RESULTS

G-CSF and GM-CSF rapidly stimulate the stable phosphorylation of a 75-kd protein in permeabilized HL60 cells. HL60 human myeloid leukemia cells express cell surface receptors for G-CSF and GM-CSF and respond biologically to both of these agents.14 When gently permeabilized HL60 cells were incubated with [γ-32P]ATP, the addition of either 500 μU/mL of G-CSF (Fig 1, lane 3) or 500 pmol/L GM-CSF (Fig 1, lane 4) resulted in the labeling of a 75-kd protein (p75). Final concentrations of CSFs added were the reported optimum levels for their biologic actions.21,22 Addition of the vehicle alone (0.1% BSA) did not elicit p75 phosphorylation (Fig 1, lanes 1 and 5). 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a direct activator of protein kinase C,8 did not stimulate p75 labeling (Fig 1, lane 2), suggesting that the labeling of p75 stimulated by either G-CSF or GM-CSF was not dependent on the activation of kinase C. Phosphorylation of other proteins in response to treating intact HL60 cells with TPA has previously been described.23 However, these proteins were of low mol wt (17 and 22 Kd) and would not have been sufficiently well-resolved in the gel system used here. By contrast, the addition of TPA to permeabilized T lymphocytes has previously been shown to trigger the phosphorylation of a 42-kd protein, demonstrating that TPA activation of kinase C can take place in the permeabilized cell system. Retinoic acid, which promotes granulocytic differentiation of HL60 cells, presumably via its binding to a cytosolic receptor protein,14 also failed to elicit p75 labeling (data not shown). By contrast,
Phosphorylation of a 75Kd protein in response to G-CSF and GM-CSF stimulation of HL60 cells. 

Additions to the labeling reactions were as follows: lanes 1 and 5, 0.1% BSA; lane 2, 10 μmol/L TPA; lane 3, 500 U/mL G-CSF; lane 4, 500 pmol/L GM-CSF. All concentrations are the final concentrations in the labeling reactions. Molecular weights of marker proteins (in Kd) are indicated at left. Unlabeled arrow at left indicates the migration position of the 75-Kd protein (p75). Dimethyl sulfoxide, which also triggers granulocytic differentiation of HL60 cells, stimulated the rapid labeling of p75 (see Fig 7C). When permeabilized HL60 cells were incubated with [32P] ATP and G-CSF for 3 minutes followed by the addition of 5 mmol/L unlabeled ATP, further incubation for 45 minutes did not diminish the labeling of p75 (Fig 2A, lanes 2 and 3). This observation suggests that the G-CSF-stimulated labeling of p75 in the permeabilized cell system was due to a stable phosphorylation event and not to the continued stimulation of turnover of protein-bound phosphate. The identical result was obtained when GM-CSF was used as the stimulus (Fig 2A, lanes 5 and 6).

Fig 1. Phosphorylation of a 75-Kd protein in response to G-CSF and GM-CSF stimulation of HL60 cells. [32P] ATP labeling of permeabilized HL60 cells was performed as described in Materials and Methods. Additions to the labeling reactions were as follows: lanes 1 and 5, 0.1% BSA; lane 2, 10 μmol/L TPA; lane 3, 500 U/mL G-CSF; lane 4, 500 pmol/L GM-CSF. All concentrations are the final concentrations in the labeling reactions. Molecular weights of marker proteins (in Kd) are indicated at left. Unlabeled arrow at left indicates the migration position of the 75-Kd protein (p75).

Time course of phosphorylation in response to GM-CSF. Phosphorylation of p75 in response to GM-CSF binding was a rapid event. Figure 3 shows the results of an experiment in which HL60 cells were permeabilized and incubated with 500 pmol/L GM-CSF. When [32P] ATP was added simultaneously with GM-CSF, the labeling of p75 was clearly detected (Fig 3, lane 2). If [32P] ATP was added 30 seconds after addition of the CSF, p75 labeling was markedly reduced (Fig 3, lane 3). An even further reduction in labeling intensity was seen if the interval between addition of GM-CSF and [32P] ATP was increased up to 30 minutes (lanes 3 through 6). These results suggest that the rapid GM-CSF-stimulated phosphorylation of p75 (by endogenous unlabeled ATP) prevented labeling by [32P] ATP if the latter was added more than 30 seconds after growth factor addition.

The experiment shown in Fig 4 suggests that rapid stable phosphorylation of p75 also occurred in response to GM-CSF binding to intact HL60 cells. When the CSF was added to intact cells, which were permeabilized 30 seconds later in the presence of [32P] ATP, labeling of p75 was clearly detected (lane 2). However, if permeabilization was delayed for 5 to 30 minutes (lanes 3 through 5), no labeling by [32P] ATP was detectable. These observations are compatible with the rapid induction of stable p75 phosphorylation by GM-CSF treatment of intact HL60 cells, which is complete within 5 minutes. Taken together with the lack of turnover of label in p75 seen in vitro (Fig 2A), the results presented in Figs 3 and 4 suggest that p75 phosphorylation in response to GM-CSF is a rapid stable event in both intact and permeable cells.

An experiment performed using G-CSF as the stimulus and a protocol identical to that described in Fig 4 showed that p75 phosphorylation in intact cells in response to G-CSF...
TYROSINE PROTEIN PHOSPHORYLATION IN HL60 CELLS

Fig 4. p75 labeling is rapidly stimulated after GM-CSF binding to intact cells. 500 pmol/L GM-CSF was added to intact HL60 cells, which were then permeabilized and incubated with [32P] ATP at the following times: lane 2, 30 seconds; lane 3, 5 minutes; lane 4, 15 minutes; lane 5, 30 minutes. Lane 1, control, 0.1% BSA was added instead of GM-CSF, and the cells permeabilized immediately.

was also a rapid, stable event. [32P] ATP labeling of p75 was detected if cells were permeabilized 30 seconds after growth factor addition, but not if a longer time interval had elapsed (data not shown).

TNF and IFN also trigger the phosphorylation of a 75-Kd protein. Whereas GM-CSF and G-CSF trigger the differentiation of HL60 cells to cells resembling granulocytes, TNF and IFN promote the expression of macrophagelike features. Therefore, we determined whether these cytokines also stimulated the phosphorylation of p75. Data shown in Fig 5A shows that, in addition to G-CSF (lane 1) and GM-CSF (lane 3), TNF (lane 4) and IFN (lane 5) also stimulated the phosphorylation of p75 when added at doses that have been shown previously to produce the optimal biologic actions. The experiment depicted in Fig 5 was performed using an HL60 cell culture of lower passage number than that used in the previous experiments. The pair of constitutively labeled bands near 94 Kd was not observed when these cells were used.

Labeling of p75 occurs on tyrosine and serine residues. The alkali resistance of labeled p75 (Fig 2B) tentatively suggests that ligand-stimulated phosphorylation occurred at least partially on tyrosine residues. We have confirmed this conclusion by direct phosphoamino acid analysis of p75 radiolabeled in response to GM-CSF, G-CSF, TNF-α, and γ-IFN. Figure 5B shows that phosphorylation in response to each of these cytokines was on serine and tyrosine residues. Although phosphoserine appears to be the predominant phosphoamino acid, phosphotyrosine is more labile to acid hydrolysis than is phosphoserine and it is therefore likely that the proportion of phosphotyrosine is underestimated by the analysis shown in Fig 5B.

Tyrosine phosphorylation of p75 can be detected in intact cells. We have demonstrated the tyrosine phosphorylation of a 75-Kd protein in intact HL60 cells by immunoblot analysis using an antibody directed against the phosphotyrosine analogue azobenzene phosphonate. Figure 6 clearly shows that G-CSF, GM-CSF, TNF, or IFN rapidly triggered the appearance of a 75-Kd band detectable by the antibody, suggesting that each of these cytokines also stimulated the tyrosine phosphorylation of a p75 in intact cells. Stimulation of tyrosine phosphorylation of other bands was also seen, albeit at a reduced intensity compared with p75.

Pretreatment of intact cells with one cytokine abolishes the subsequent stimulation of p75 phosphorylation in response to a second stimulus. The experiments depicted in Fig 7 were performed using the lower passage strain of HL60 cells also used for the experiment shown in Fig 5. p75 phosphorylation was clearly stimulated by G-CSF, GM-

Fig 5. (A) Phosphorylation of p75 in response to stimulation of HL60 cells by different CSFs and cytokines. Labeling reactions were performed by standard procedure. Additions to the labeling reactions were as follows: lane 1, 500 U/mL G-CSF; lane 2, control (0.1% BSA); lane 3, 500 pmol/L GM-CSF; lane 4, 100 U/mL TNF; lane 5, 100 U/mL IFN; lane 6, 75 U/mL IL-2. (B) Phosphoamino acid analysis of p75 labeled in response to: lane 1, control (0.1% BSA added); lane 2, 500 U/mL G-CSF; lane 3, 100 U/mL TNF; lane 4, 1,000 U/mL IFN; lane 5, 500 pmol/L GM-CSF.
CSF, TNF, and IFN, albeit with reduced intensity compared with the reference band p67 (Fig 7A). Pretreatment of intact cells with TNF followed by stimulation of permeabilized cells with G-CSF (Fig 7A) or GM-CSF (data not shown) did not stimulate p75 phosphorylation, although the addition of G-CSF to untreated permeabilized cells triggered labeling of p75 in the same experiment. Additionally, G-CSF pretreatment of cells abolished p75 labeling stimulated by TNF (Fig 7A) or GM-CSF (not shown). Similarly, pretreatment of intact cells with IFN abolished the ability of TNF, GM-CSF, or G-CSF to elicit p75 labeling in permeabilized cells (Fig 7B). Preincubation of intact cells with GM-CSF was also shown to abolish p75 labeling in response to TNF or by G-CSF (not shown). Taken together with the observation that all four cytokines stimulate the labeling of a p75 in intact cells (Fig 6), these results strongly suggest that p75 elicited by each of the agents tested involves phosphorylation of the same p75 substrate, in both intact and permeabilized cells.

Dimethyl sulfoxide (DMSO) addition to permeabilized HL60 cells also elicited p75 labeling (Fig 7C). DMSO pretreatment of intact cells also abolished p75 labeling elicited by IFN, G-CSF, GM-CSF, or TNF, suggesting that DMSO-stimulated p75 phosphorylation also proceeded by the same pathway as that triggered by biologic agents.

Phosphorylation of p75 in response to GM-CSF or TNF is dependent on expression of appropriate receptors. The lymphokine IL-2, which is not a known growth or differentiation-inducing factor for HL60 cells, did not stimulate p75 labeling in HL60 cells (Fig 5A, lane 6), consistent with the view that ligand-stimulated p75 phosphorylation was dependent on expression of appropriate surface receptors. The KG1 human myeloid leukemia cell line expresses receptors for GM-CSF, whereas the related KG1a line does not.15

![Immunoblot analysis of phosphotyrosine-containing proteins in HL60 cells treated for 5 minutes with: lane 2, 500 U/mL G-CSF; lane 3, 500 pmol/L GM-CSF; lane 4, 100 U/mL TNF; lane 5, 1,000 U/mL IFN. Lane 1, control, no cytokines added.](image)

![Fig 7. (A through C) Pretreatment of intact cells with CSFs, cytokines, or DMSO abolishes p75 labeling elicited by CSFs or cytokines in permeabilized cells. DMSO was used at a final concentration of 1.4%. Concentrations of biologic agents were as in previous figures. Dash indicates addition of vehicle only. Agent indicated to the left of the slash was added to intact cells, and that to the right was added to permeabilized cells in the presence of [32P] ATP. (D) p75 labeling in response to GM-CSF requires expression of the appropriate receptor. KG1 cells (GM-CSF receptor positive) or the KG1a variant (receptor negative) were incubated for 3 minutes with or without GM-CSF under standard labeling conditions.](image)
Figure 7D shows that GM-CSF stimulated p75 labeling in KG1 but not in KG1a cells, verifying that the observed labeling occurred only when the appropriate receptor was expressed at the cell surface.

TNF-stimulated p75 labeling was also shown to be dependent on the expression of the appropriate receptor. K562 myeloid leukemia cells express receptors for TNF, whereas KLOEK cells do not. TNF stimulated the labeling of p75 in K562, but not in KLOEK cells in the same experiment (data not shown).

**DISCUSSION**

The results show that four hormones which trigger the differentiation of HL60 cells, G-CSF, GM-CSF, TNF-α, and γ-IFN rapidly stimulated the stable labeling of a 75-Kd protein on serine and tyrosine residues when incubated with permeabilized cells in the presence of [³²P] ATP. p75 labeling was dependent on expression of appropriate ligand receptors. All of these agents stimulated p75 phosphorylation in intact and permeabilized cells. The observation that treatment of intact cells with any one cytokine abrogated the subsequent stimulation of p75 labeling by the addition of a second cytokine after permeabilization strongly suggests that the p75 phosphorylated in response to all four cytokines tested here is the same protein. GM-CSF has also been shown to stimulate the phosphorylation of 72-, 92-, and 150-Kd proteins on tyrosine residues in murine myeloid cells, but it is unclear whether the 72-Kd protein is related to p75.

Phosphorylation of tyrosine residues by TPK is strongly correlated with the regulation of cell proliferation and differentiation, since many growth factor receptors are transmembrane proteins possessing an intracytoplasmic ligand-stimulated TPK domain. Activation by ligand binding triggers the autophosphorylation of the receptor. Additionally, about half of the known oncogene products are TPKs. Receptors for GM-CSF and G-CSF have mol wts of 130 and 150 Kd, respectively, suggesting that they are potentially large enough to include an intracellular TPK domain. However, since p75 is phosphorylated in response to four different ligands, it is most probable that it is not itself an autophosphorylated growth factor receptor but a separate protein whose phosphorylation may serve to integrate some of the biologic actions of these agents.

The binding of growth factors to their receptors on nonhematopoietic cells triggers multiple parallel cascades of phosphorylation events. Therefore, it is likely that intracellular events initiated by binding of the hematopoietic growth factors are also complex. Binding of G-CSF to murine FDC-P1 cells has been shown to stimulate the relatively slow phosphorylation of a 68-Kd protein (p68) on threonine residues, probably mediated via kinase C activation. G-CSF stimulated labeling of p75 has not previously been observed using methods involving the labeling of HL60 cells with [³²P]PO₄. However, the long incubation time required in order to label cellular ATP pools led to extensive labeling of proteins in the 75-Kd range, which would have precluded the detection of ligand-stimulated changes in labeling of p75, even though two-dimensional gels were used in this study. The procedure used here, involving the labeling of permeabilized cells with [³²P] ATP, circumvents this problem since no preincubation with radiolabel is required, thereby reducing background labeling and facilitating the detection of rapid ligand-stimulated events.

GM-CSF did not stimulate the threonine phosphorylation of p68 in FDC-P1 cells, whereas G-CSF did. By contrast, the present work suggests that tyrosine phosphorylation of p75 is an early event that occurs rapidly after the binding of either G-CSF or GM-CSF to their respective receptors on HL60 cells, and did not appear to be mediated via kinase C activation. TNF and IFN also shared this early cellular response, suggesting that the receptors for each of these hormones is a ligand-activated TPK that phosphorylates p75. Alternatively, each receptor may be linked to a nonreceptor TPK whose activation leads to p75 phosphorylation. Rapid activation of nonreceptor TPKs after stimulation of cell-surface receptors lacking intrinsic TPK activity is well-documented in T lymphocytes.

Among the chemical inducers of HL60 cell differentiation tested by us, only DMSO elicited p75 labeling. DMSO has previously been shown to stimulate TPKs, whereas it has no effect on cyclic AMP-dependent protein kinase or protein kinase C. The ability of DMSO to trigger granulocytic differentiation of HL60 cells may be attributable, at least in part, to its ability to stimulate phosphorylation of p75. By contrast, neither TPA nor retinoic acid stimulated p75 labeling, suggesting that these agents may induce HL60 cell differentiation by acting at points in signaling pathways that are distinct from or proximal to the phosphorylation of p75.

Factors shown here to elicit p75 phosphorylation have different biologic actions depending on the context set by the target cell in question. G-CSF and GM-CSF promote granulocytic differentiation of HL60 cells, but GM-CSF can also stimulate their proliferation in some conditions, and also that of KG1 cells. While both TNF and IFN promote monocytic differentiation of HL60 cells, TNF acts as a growth factor for fibroblasts, T lymphocytes, normal, and malignant B lymphocytes. This complexity makes it unlikely that the phosphorylation of p75 alone determines the biologic consequence of factor binding, and other events further in the pathway or additional signals caused by ligand binding must determine the manner in which the signal is interpreted by a particular cell. However, observed patterns of synergy between different chemical inducers of HL60 cell differentiation suggest that differentiation is at least a two-phase process. Whereas the first lineage nonspecific phase involves the cessation of proliferation and can involve synergistic action of different chemical stimuli, the second phase involves specific changes in gene expression associated with commitment to either the granulocyte or monocyte lineage and is triggered by specific chemical agents. Therefore, we suggest that the phosphorylation of p75 may be associated in HL60 with signaling early steps in the response of HL60 cells to G-CSF, GM-CSF, IFN, and TNF, rather than with commitment to a specific differentiation pathway. In other cells (eg, KG1), p75 phosphorylation may be
involved in promoting cell division. While it appears paradoxical that the same signal may be used to secure diametrically opposite biologic effects, there are several precedents. Whereas the transient induction of the c-fos protooncogene is involved in the growth stimulation pathways of many cell types,\textsuperscript{40} fos induction also appears to be involved in the TPA-induced withdrawal of HL60 cells from proliferation.\textsuperscript{41} Furthermore, many agents that are growth factors for some cell types have antiproliferative effects in other systems.\textsuperscript{42} Therefore, the same signal may be interpreted differently in various cellular contexts. Because the induction of differentiation of leukemia cells is now considered to be a serious therapeutic option,\textsuperscript{43} the unraveling of the pathways involved in its regulation is of considerable importance. Elucidation of the biochemical properties of p75 will contribute to understanding this complex process.

ACKNOWLEDGMENT

We thank Glaxo Institute for Molecular Biology S.A. and Biogen, S.A. (Geneva, Switzerland) for generous gifts of some of the growth factors used in this work; Dr Malcolm Brenner for helpful advice; and Megan Evans for invaluable assistance in typing the manuscript.

REFERENCES


30. Rubin RA, Earp HS: Solubilization of EGF receptor with
Binding of G-CSF, GM-CSF, tumor necrosis factor-alpha, and gamma-interferon to cell surface receptors on human myeloid leukemia cells triggers rapid tyrosine and serine phosphorylation of a 75-Kd protein

JP Evans, AR Mire-Sluis, AV Hoffbrand and RG Wickremasinghe