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Hematopoietic Growth Factors Activate the Tyrosine Phosphorylation of Distinct Sets of Proteins in Interleukin-3-Dependent Murine Cell Lines

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By immunoblotting with antibodies for phosphotyrosine, we have demonstrated that the hematopoietic growth factors interleukin-2, interleukin-3, interleukin-4, and granulocyte-macrophage colony-stimulating factor stimulate the tyrosine phosphorylation of specific sets of proteins in murine hematopoietic progenitor cell lines. The stimulation of tyrosine phosphorylation is a receptor-dependent transient event. The effect of these hematopoietic growth factors on protein tyrosine phosphorylation was not mediated through protein kinase C.

The growth and differentiation of hematopoietic cells is controlled by a number of glycoproteins known as interleukins or colony-stimulating factors (CSFs) (for reviews, see references 4 and 23). The actions of these factors are mediated through specific receptors present on the surface of hematopoietic cells of specific lineages and stages of differentiation (4, 23). Many of the genes encoding these growth factors and a few of their receptors have been cloned; however, the intracellular mechanisms of action of these factors are largely unknown. Activation of protein tyrosine phosphorylation is an integral response in many different mitogenic systems (2). The receptors for several peptide growth factors, e.g., the epidermal growth factor and CSF-1, contain tyrosine kinase domains which reside in the intracellular portions of the receptor proteins (2). The receptor tyrosine kinase activity is essential for the transduction of mitogenic signals (3, 14). In contrast to the tyrosine kinase receptors, the two interleukin-2 (IL-2) receptors are smaller (55 and 70 kilodaltons [kDa]), and the 55-kDa receptor has been shown to have only a 13-amino-acid intracellular domain (6, 8, 19, 30, 35–37). By chemical cross-linking with radioactive ligands, putative receptors for IL-3 and for granulocyte-macrophage CSF (GM-CSF) have been found to have apparent subunit molecular weights of 60 to 75 and 51 kDa, respectively (29, 31, 38); these receptors may also contain small intracellular domains. Although the receptors for growth factors such as IL-2, IL-3, and GM-CSF may not contain tyrosine kinase domains, it does not rule out the possibility that these growth factors also activate protein tyrosine phosphorylation in their signal transduction pathways.

To examine the effects of IL-2, IL-3, and GM-CSF on protein tyrosine phosphorylation, we used two murine hematopoietic cell lines, IC-2.9 and 32Dcl5, which were originally established as IL-3-dependent cell lines. IC-2.9 is a clone of the mast cell line IC-2 (16), which can proliferate on IL-3, IL-4, or GM-CSF (17). 32Dcl5 (32D [12]) is a basophilic cell line which expresses low levels of IL-2 receptors. Le Gros et al. found that the number of IL-2 receptors in 32D cells can be increased by culturing the cells in IL-2 alone and that the resulting 32D/IL-2 cells can proliferate on either IL-3 or IL-2 (18). Exponentially growing IC-2.9 or 32D/IL-2 cells were washed free of serum and growth factors and incubated in starvation medium (RPMI 1640–0.075% bovine serum albumin–50 μM Na_3VO_4) for 30 min at 37°C to free the receptors of their ligands. The starved cells were then stimulated with homogeneous preparations of IL-2, IL-3, IL-4, or GM-CSF for 5 min at 37°C, lysed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and analyzed by immunoblotting with antibodies for phosphotyrosine (Ptyr), as previously described (26, 34, 39). These affinity-purified anti-Ptyr antibodies are specific for tyrosine-phosphorylated proteins and do not cross-react with phosphoserine, phosphothreonine, phosphohistidine, or tyrosine sulfates, as previously demonstrated (21, 26, 39).

Stimulation of IC-2.9 cells with purified IL-3 (2 nM) caused an increase in the tyrosine phosphorylation of proteins with M_rs of 160, 95, 90, 70, and 55 kDa (Fig. 1A, lane 3). Under the same conditions, stimulation with purified IL-4 (0.1 nM) induced an increase in the tyrosine phosphorylation of proteins with M_rs of 170 and 110 kDa (Fig. 1A, lane 4). When cells were stimulated with purified GM-CSF (1.8 nM), small but reproducible increases in Ptyr were detected in proteins with M_rs of 150, 92, and 72 kDa (Fig. 1A, lanes G and GL [longer exposure]). Purified IL-2 had no effect on protein tyrosine phosphorylation in IC-2.9 cells which do not respond to IL-2 and probably contain no IL-2 receptors (Fig. 1A) (16).

The effect of IL-2 on protein tyrosine phosphorylation was examined in 32D/IL-2 cells which proliferate in response to either IL-2 or IL-3. Stimulation of 32D/IL-2 cells with IL-2 (4 nM) caused a small but detectable increase in the tyrosine phosphorylation of proteins with M_rs of 80 to 85 kDa (Fig. 1B, lane 2). This increase in Ptyr was not found in the parent 32D cells (data not shown), which indicates that pretreatment with IL-2 is required for the induction of IL-2-stimulated Ptyr increase in this myeloid cell line.

As with IC-2.9 cells, IL-3 caused a similar increase in the Ptyr of the characteristic 95-, 90-, 70-, and 55-kDa (95- to 55-kDa) proteins; however, stimulation of the 160-kDa Ptyr protein was not detected in 32D/IL-2 cells (Fig. 1B, lane 3). Interestingly, the 160-kDa protein was stimulated by IL-3 in another mast cell line, MC/9 (28), but not in another IL-3-dependent myeloid cell line, FDC-P1 (7). In both MC/9 and FDC-P1 cells, the characteristic Ptyr proteins (95 to 55 kDa) were stimulated by IL-3, and these bands were not found in

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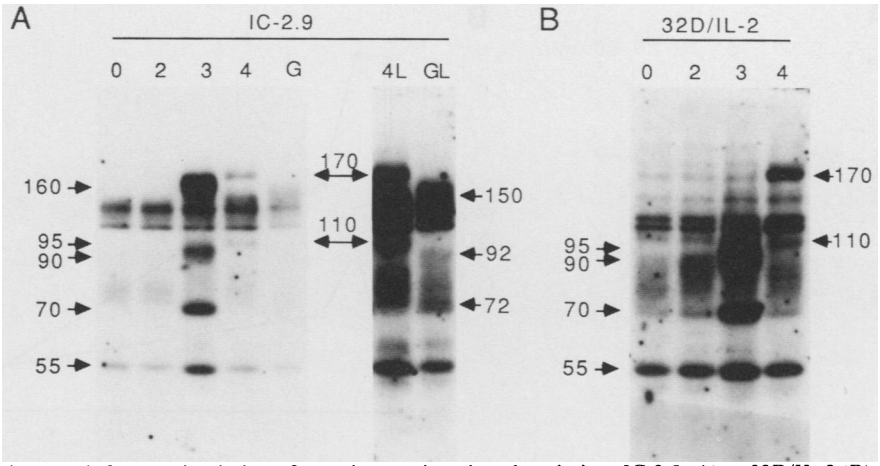

FIG. 1. Hematopoietic growth-factor stimulation of protein tyrosine phosphorylation. IC-2.9 (A) or 32D/IL-2 (B) cells were stimulated for 5 min with the following concentrations of purified growth factors: IL-2, 4 nM (lane 2); IL-3, 2 nM (lane 3); IL-4, 0.1 nM (lanes 4 and 4L); and GM-CSF, 1.8 nM (lanes G and GL). Lane O is a sample of unstimulated cells. Equal amounts of total cellular protein (as determined by the method of Lowry et al. [20]) for each sample were electrophoresed and immunoblotted with antibodies for Ptyr as previously described (26, 34, 39). Exposure times of the blots were 8 h for panel A, lanes 0 to G; 48 h for panel A, lanes 4L and GL; and 48 h for panel B, with intensifying screens at −80°C. Arrows mark the positions of the Ptyr proteins stimulated by each growth factor. Murine IL-2 was purified from *Escherichia coli* (17a), and murine IL-4 was a 50% pure preparation derived from supernatants of COS-7 cells transfected with an IL-4 cDNA clone. Murine IL-3 was purified from the media of transfected COS-7 cells as described by Miyajima et al. (25), and GM-CSF was purified from yeast as described by Miyajima et al. (24). Sizes are given in kilodaltons.

IL-3-independent murine cell lines (data not shown). It is possible that the 160-kDa protein is a mast-cell-specific protein or that tyrosine phosphorylation of that protein is not under the control of IL-3 in the myeloid cell lines.

Surprisingly, IL-4 stimulated the tyrosine phosphorylation of the characteristic 170- and 110-kDa proteins in 32D/IL-2 cells, although these cells cannot grow on IL-4 (Fig. 1B, lane 4). The Ptyr increase without a mitogenic effect suggests that the stimulation of protein tyrosine phosphorylation by IL-4 is a proximal event following receptor occupation and does not necessarily lead to proliferation.

The results of our experiments demonstrate that IL-2, IL-3, IL-4, and GM-CSF stimulate the tyrosine phosphorylation of distinct sets of proteins in cells responsive to these growth factors. Growth-factor-specific activation of distinct Ptyr proteins has also been observed in human epidermoid carcinoma KB cells, in which insulin, insulinlike growth factor I, and epidermal growth factor stimulate the tyrosine phosphorylation of common as well as distinct sets of proteins (15).

Of the growth factors and cell lines tested, IL-3 consistently caused the largest increase in protein tyrosine phosphorylation, so this response was further characterized. A time course experiment showed that an increase in tyrosine phosphorylation was detectable within 1 min after the addition of IL-3, became maximal after 5 min, and gradually decreased thereafter (Fig. 2). This rapid and transient response is typical of the activation of tyrosine phosphorylation by other growth factors, e.g., in platelet-derived growth-factor-activated tyrosine phosphorylation (11). The extent of IL-3-stimulated tyrosine phosphorylation is dependent on the IL-3 concentration. In dose-response experiments with IC-2.9, 32D, FDC-P1, and MC/9 cells, the half-maximal concentration of IL-3 required to stimulate tyrosine phosphorylation was 200 to 700 pM for all of the Ptyr proteins. The apparent K_d of the IL-3 receptor was 80 to 230 pM in the four cell lines, as measured by equilibrium binding of [^{125}I]IL-3 to intact cells at 4°C. This was in close agreement with previously published values for the K_d of the IL-3 receptor (29, 31, 32). The dose-response curve for the stimulation of the 70-kDa Ptyr protein and the binding curve for the IL-3 receptor in 32D cells are shown in Fig. 3. The extent of tyrosine phosphorylation is proportional to the extent of receptor occupancy; however, a 10-fold-higher concentration of IL-3 is required for the maximal stimulation of tyrosine phosphorylation (Fig. 3). The half-maximal concentration of IL-3 required for the growth of 32D cells, on the other hand, was approximately 5 pM (Fig. 3). Thus, the occupancy of only a very small fraction of the IL-3 receptors is sufficient for the stimulation of cell growth, but the stimulation of a detectable increase in tyrosine phosphorylation requires the occupancy of a substantial fraction of the receptors. These results suggest that either these Ptyr proteins are not involved in mitogenesis or the phosphorylation of a very small percentage of the Ptyr proteins is sufficient for the stimulation of cell growth. Alternatively, it may be that IL-3 is required only at a certain point in the cell cycle. Cells at that point would have high-affinity IL-3-responsive elements, so that the increase in tyrosine phosphorylation could be efficiently stimulated by picomolar levels of IL-3; the rest of the population, representing the majority of the cells in our experiments, could respond only to higher concentrations of IL-3 because of the lower sensitivity of their IL-3-responsive elements.

As with IL-3, stimulation of protein tyrosine phosphorylation with IL-4 was also dose dependent. The half-maximal concentration of IL-4 required to stimulate tyrosine phosphorylation in 32D and 32D/IL-2 cells was approximately 10 pM, with maximal levels of stimulation at 100 pM. This was in close agreement with the concentration of IL-4 required for half-maximal receptor occupancy, which was 40 pM (19a).

IL-2 and IL-3 have been shown to activate protein kinase C (PKC) (9, 10, 42). Since the activation of PKC was found to stimulate protein tyrosine phosphorylation in human U937 monocytes (13), we used two experiments to test whether

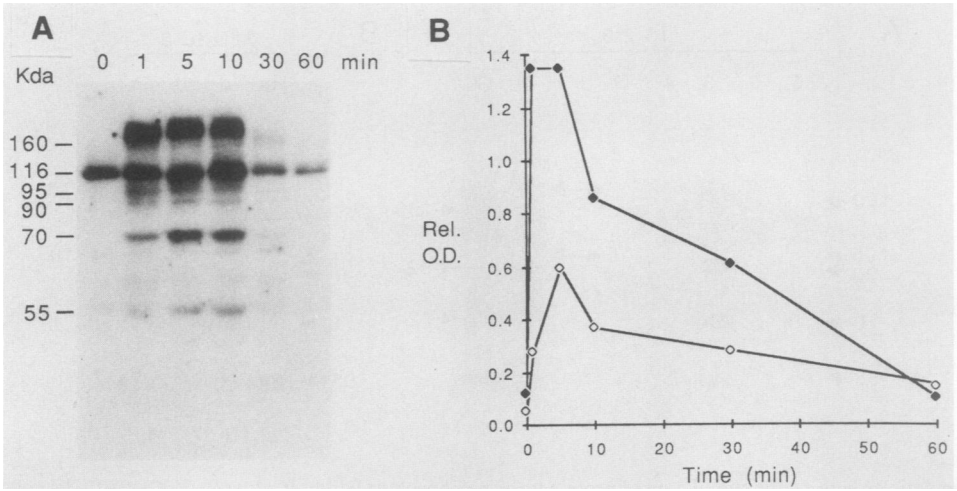

FIG. 2. Time course of IL-3-stimulated protein tyrosine phosphorylation. (A) Immunoblot with anti-Ptyr antibodies. Starved IC-2.9 cells were stimulated with 2 nM IL-3 for the length of time indicated above each lane. Immunoblots such as the one shown in panel A were exposed at room temperature and then scanned with a laser densitometer. (B) Quantitation (◆, 160-kDa protein; ◇, 70-kDa protein). Each point is the average of two independent experiments. Rel. O.D., relative optical density.

the observed Ptyr increases were mediated through the activation of PKC. In the first experiment, starved cells were treated with 100 ng of *o*-tetradecanoylphorbol 13-acetate (TPA) per ml to activate PKC, and no detectable increase in protein tyrosine phosphorylation was observed in either 32D or IC-2.9 cells (Fig. 4A, lane T). In the second experiment, these cells were cultured in the presence of 100 ng of TPA per ml for 48 h to downregulate PKC; they were then stimulated with IL-3, IL-4, or GM-CSF. The steady-state level of PKC was decreased 10-fold by this chronic TPA treatment, as determined by immunoblotting with anti-PKC antibodies (gift of H. T. Huang, National Institutes of Health, Bethesda, Md.). The stimulation of protein tyrosine phosphorylation by IL-3 and IL-4 (Fig. 4B) and GM-CSF was not affected in cells containing low levels of PKC. These data demonstrate that the effect of IL-3, IL-4, and GM-CSF on protein tyrosine phosphorylation is not mediated through PKC.

Immunoblotting with our anti-Ptyr antibodies did not detect all possible substrates of IL-3-stimulated tyrosine

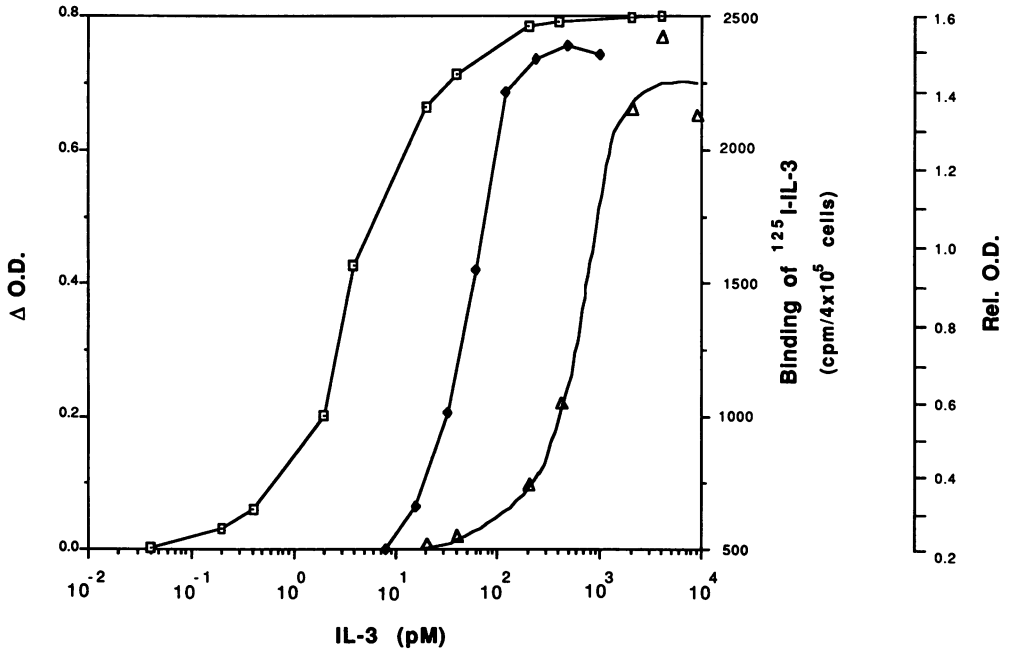

FIG. 3. IL-3 dose response for proliferation, receptor binding, and 70-kDa protein tyrosine phosphorylation. 32D cells were cultured in various concentrations of IL-3, and proliferation was quantitated by the MTT (tetrazolium) assay as described by Mosmann (27); ΔO.D. (⊡) refers to the optical density (570 nm − 630 nm) of the proliferation assay. Identical growth response curves were obtained in serum-containing and serum-free media. The specific binding of [^{125}I]IL-3 (◆) to whole cells was measured as a function of IL-3 concentration. The amount of Ptyr increase (△) in the 70-kDa protein after a 10-min stimulation with various concentrations of IL-3 was quantitated by densitometric scanning of immunoblots (Rel. O.D.). Each point is the average of two experiments.

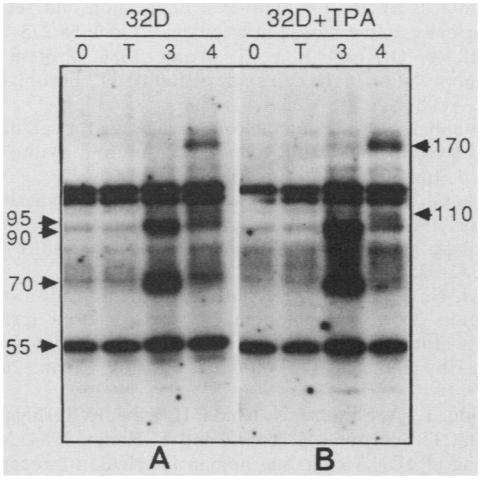

FIG. 4. Effect of TPA on hematopoietic growth-factor-stimulated tyrosine phosphorylation. Control untreated cells (A) and 32D cells cultured with 100 ng of TPA per ml for 48 hours (B) were stimulated for 10 min with either TPA (100 ng/ml; lane T), IL-3 (2 nM; lane 3), or IL-4 (0.1 nM; lane 4). Arrows indicate positions of growth-factor-stimulated Ptyr proteins. Exposure was for 17 h at −80°C with an intensifying screen. Sizes are given in kilodaltons.

kinases. By immunoprecipitating ^{32}P-labeled proteins with their anti-Ptyr antibodies, Koyasu et al. found that IL-3 activated the tyrosine phosphorylation of a membrane glycoprotein with an M_r of 150 kDa in both myeloid and mast cell lines (17; personal communications), but these workers did not consistently detect the other IL-3-stimulated Ptyr proteins described here. The 160-kDa protein we found in mast cells is a soluble protein (A. O. Morla, unpublished results); it is therefore different from the 150-kDa membrane protein described by Koyasu et al. The antibodies used by Koyasu et al. also did not detect an increase in protein tyrosine phosphorylation in response to IL-4 or GM-CSF in IC-2 cells (17). Thus, the detection of Ptyr proteins by anti-Ptyr antibodies appears to be limited by the specificity of the different antibodies and perhaps also by the methods of detection.

Our studies demonstrate that the hematopoietic growth factors IL-2, IL-3, IL-4, and GM-CSF do indeed stimulate protein tyrosine phosphorylation and that they do so in the absence of any other serum factors. As described above, the putative receptors for IL-2, IL-3, and GM-CSF are in the 50- to 75-kDa range, suggesting that they may not contain a cytoplasmic tyrosine kinase domain. If so, these receptors must be coupled to some intracellular tyrosine kinases. Many nonreceptor tyrosine kinases have been found in mammalian cells by their homologies to a group of retroviral oncogenes such as *src*, *fps*, and *abl* (1). It is believed that these kinase activities are regulated; however, the mechanisms of their regulation are not understood. It is possible that these intracellular tyrosine kinases are coupled to different receptors such as those for IL-2, IL-3, or GM-CSF. Several mechanisms can be envisioned for the coupling of receptors to intracellular tyrosine kinases. The kinase may be a component of the receptor complex, which is activated by a direct protein-protein interaction or through a coupling protein such as the G proteins. The occupied receptor may stimulate the production of a diffusible second messenger which in turn activates the kinase. Alternatively, the binding of the receptor may alter the conformation or the location of substrate proteins so that they can become phosphorylated. The observation that IL-2, IL-3, IL-4, and GM-CSF act on distinct sets of Ptyr proteins suggests that these hematopoietic growth factors activate different tyrosine kinases in their signal transduction pathways. Whatever the mechanisms for the coupling of the receptors to tyrosine kinases, the hematopoietic growth factor receptor system may serve as a model for the studies of activation of intracellular tyrosine kinases by membrane receptors.

It has been shown that infection of IL-3-dependent murine cells with the tyrosine kinase oncogenes v-*src*, v-*abl*, and v-*fms* allows the isolation of cells which no longer depend on IL-3 for growth, and this abrogation of IL-3 dependence occurs by a nonautocrine mechanism (5, 22, 33, 40, 41). Using temperature-sensitive tyrosine kinase mutants of v-*abl*, Kipreos and Wang have recently demonstrated that the v-*abl* tyrosine kinase is required continuously to abrogate the dependence of FDC-P1 cells on IL-3 (15a). The demonstration that IL-3 stimulates tyrosine phosphorylation raises the possibility that the v-*abl*, v-*src*, or v-*fms* kinases abrogate IL-3 dependence by directly phosphorylating one or more of the IL-3-stimulated Ptyr proteins. Evidence for this mechanism awaits further investigation.

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