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Interleukin 3-specific tyrosine phosphorylation of a membrane glycoprotein of M_r 150 000 in multi-factor-dependent myeloid cell lines

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Tyrosine phosphorylation of cellular proteins induced by various hematopoietic growth factors such as interleukin 3 (IL3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL4) was studied in several multi-factor-dependent myeloid cell lines. Among the growth factors, IL3 specifically induced rapid tyrosine phosphorylation of a membrane glycoprotein of mol. wt 150 kd (gpp150) in the IL3-dependent cell lines, IC2 and DA-1. The IL3-induced tyrosine phosphorylation of gpp150 was detected within 30 s, reached a maximum at 3 min and decreased thereafter. The concentration of IL3 required for half-maximum stimulation of gpp150 tyrosine phosphorylation with 2.5 × 10^6/ml cells was ~200 pM, which is the same as the dissociation constant for ^{125}I-labeled IL3 binding. gpp150 was constitutively phosphorylated on tyrosine residue(s) in growth factor independent variants, IC2Tr and DA-1Tr, derived from IC2 and DA-1 respectively. Neither variant synthesized IL3. The present findings suggest that tyrosine phosphorylation of gpp150 is a critical event involved in both IL3-dependent and -independent growth.

Key words: growth factor/hematopoiesis/interleukin/receptor/signal transduction

Introduction

Interleukin 3 (IL3) is a T-cell-derived growth factor which has been shown *in vitro* to support the growth and development of hematopoietic cells and to generate multi-lineage colonies from bone marrow cells including granulocyte, macrophage, erythroid, megakaryocyte and mast cell colonies (Dexter *et al.*, 1980; Schrader *et al.*, 1981; Tertian *et al.*, 1981; Ihle and Weinstein, 1986; Schrader, 1986). A number of cell lines which grow in response to IL3 have been established and many of these IL3-dependent cell lines also respond to another hematopoietic growth factor, granulocyte-macrophage colony-stimulating factor (GM-CSF) (Hapel *et al.*, 1984; Walker and Burgess, 1985). In addition, recent work has revealed that interleukin 4 (IL4 or BSF-1) also affects the growth of IL3-dependent cell lines (Lee *et al.*, 1986; Mosmann *et al.*, 1986; Sideras and Palacios, 1987). In accordance with these results, experiments using radiolabeled factors revealed that IL3-dependent cell lines express specific binding sites for IL3 and the other growth factors (Palaszynski and Ihle, 1984; Walker and Burgess, 1985; Nicola and Metcalf, 1986; Ohara and Paul, 1987; Park *et al.*, 1987).

We established a multi-factor-dependent cell line, IC2, which is a mast cell progenitor cell line or a P cell line (Koyasu *et al.*, 1985; 1986). IC2 cells are stimulated to initiate DNA synthesis by either IL3, GM-CSF or IL4. IC2 cells are able to start DNA synthesis in the absence of growth factors when incubated with sodium orthovanadate, a potent inhibitor of phosphotyrosine phosphatase (Swarup *et al.*, 1982; Tojo *et al.*, 1987). It has also been shown that several IL3-dependent cell lines are transformed by Abelson-MuLV, whose oncogene, v-*abl*, encodes a protein tyrosine kinase (Cook *et al.*, 1985; Pierce *et al.*, 1985). These facts raised the possibility that tyrosine phosphorylation is involved in the transduction of growth signals in IC2 cells, as it is for epidermal growth factor (EGF) and cellular transformation (Ushiro and Cohen, 1980; Ek *et al.*, 1982; Kasuga *et al.*, 1982; Jacobs *et al.*, 1983; Rubin *et al.*, 1983; Hunter and Cooper, 1985; Kadowaki *et al.*, 1987). We thus examined tyrosine phosphorylation of cellular proteins in growth-factor-stimulated IC2 cells. We report here that IL3 specifically induces tyrosine phosphorylation of a membrane glycoprotein.

Results

IC2 cells respond to multiple growth factors

Figure 1 shows that IC2 cells initiate DNA synthesis in response to either IL3, GM-CSF or IL4. IL3 and GM-CSF had a similar concentration–response curve for the induction of DNA synthesis in IC2 cells. Both growth factors stimulated 5 × 10^4/ml IC2 cells to 50% maximum proliferation at ~5 pM. IL4 affected IC2

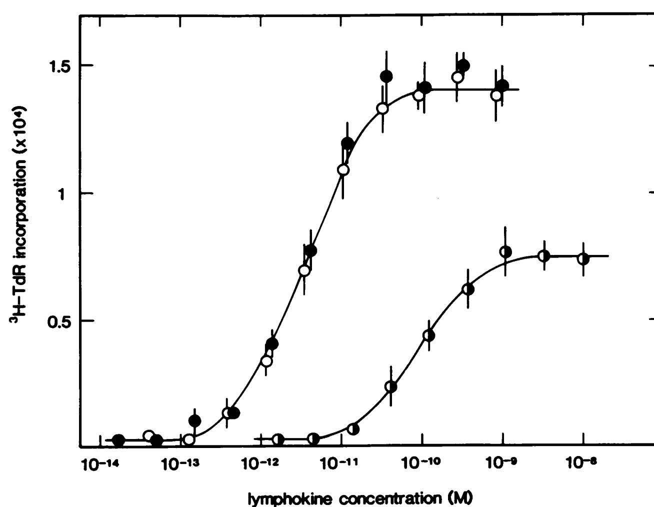

Fig. 1. DNA synthesis of IC2 cells induced by IL3 (○), GM-CSF (●) and IL4 (◑). IC2 cells (10^4 in 0.2 ml) were incubated with various concentrations of the lymphokines for 21 h followed by pulse-labeling with 1 μCi/well of [^{3}H]thymidine for 3 h as described previously (Koyasu *et al.*, 1985).

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cells in a different manner. The maximum DNA synthesis induced by IL4 at a concentration >1 nM was ~50% of that induced by IL3 or GM-CSF. Although IC2 cells express IL2 receptors, or mouse Tac molecules (Koyasu *et al.*, 1986) and insulin-like growth factor (IGF-I) receptors (unpublished data), neither IL2 nor IGF-I induced DNA synthesis in IC2 cells.

Tyrosine phosphorylation of cellular proteins

IC2 cells were labeled with [^{32}P]orthophosphate in the absence of serum and growth factors and then stimulated with various growth factors for 3 min. Phosphotyrosine-containing proteins were immunoprecipitated from extracts of these cells with rabbit antibodies directed against phosphotyrosine (anti-P-Tyr) (Kadowaki *et al.*, 1987) and analyzed by two-dimensional gel electrophoresis (Figure 2). Before stimulation, several minor background spots were observed (panels a, c and e). After stimulation with 1 nM IL3, we detected tyrosine phosphorylation in a protein of mol. wt 150 kd (pp150) in both IC2 and DA-1 cells; the latter is another IL3-dependent cell line (panels b, d and f). As shown in Figure 3, however, this tyrosine-phosphorylated protein was not detected in cells stimulated with the following agents: IL4 (panel c), GM-CSF (panel d), 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (panel e), IL2 (panel f) and IGF-I (data not shown). Phosphoproteins of 240 kd and 60–70 kd were occasionally but not reproducibly detected over the background in IL3-stimulated cells (see Figure 6A). The spot of pp150 was not detected in other cell types including various IL2-stimulated T cell clones and a T cell lymphoma, YAC-1 (unpublished data).

An area containing pp150 was cut out from the gel and subjected to phosphoamino acid analysis. Figure 4A shows that pp150 contained phosphotyrosine and phosphoserine in a ratio of ~4:6. Next, the cells were labeled with L-[^{35}S]methionine instead of [^{32}P]orthophosphate and immunoprecipitation with anti-P-Tyr was performed. As shown in Figure 4B, a ^{35}S-labeled protein which co-migrated with ^{32}P-labeled pp150 was immunoprecipitated from IL3-stimulated cells but not from unstimulated cells. These results indicate that pp150 was newly phosphorylated on its tyrosine residue(s) following stimulation with IL3.

pp150 is a membrane glycoprotein

Lysates of IC2 cells labeled with [^{32}P]orthophosphate and stimulated with IL3 were fractionated into cytoplasm and membrane fractions by centrifugation. The results of immunoprecipitation with anti-P-Tyr indicated that the membrane fraction contained pp150 (Figure 5A). pp150 was eluted from the immunocomplex by *p*-nitrophenylphosphate competition and the protein was subjected to wheat germ agglutinin (WGA)–Sepharose affinity chromatography. As shown in Figure 5B, pp150 bound to WGA–Sepharose. These results strongly suggest that pp150 is a membrane glycoprotein (denoted as gpp150 in the following sections).

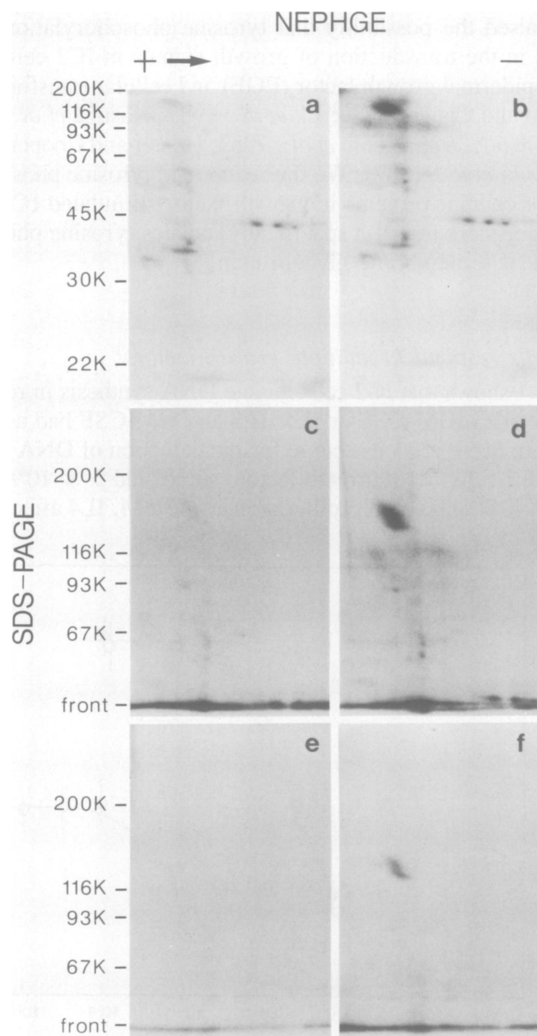

Fig. 2. Analysis of tyrosine phosphorylation of cellular proteins. IC2 (**a–d**) and DA-1 cells (**e,f**) labeled with [^{32}P]orthophosphate were incubated with saline (**a,c,e**) or 1 nM IL3 (**b,d,f**) for 3 min. Proteins immunoprecipitaed with anti-P-Tyr were analyzed by 2D-NEPHGE/SDS–PAGE. Ten percent (**a,b**) and 5% (**c–f**) acrylamide gels were used for the second dimension. Numbers on the left side indicate the positions of mol. wt markers.

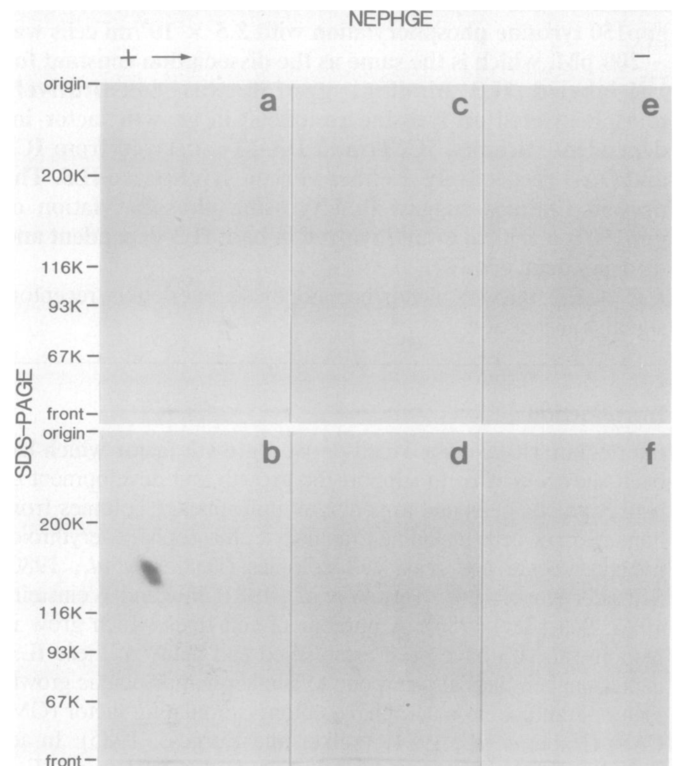

Fig. 3. IC2 cells labeled with [^{32}P]orthophosphate were incubated with saline (**a**), 1 nM IL3 (**b**), 100 nM IL4 (**c**). 1 nM GM-CSF (**d**), 10 ng/ml TPA (**e**) or 50 nM IL2 (**f**) for 3 min. Proteins immunoprecipitated with anti-P-Tyr were analyzed by 2D-NEPHGE/SDS–PAGE.

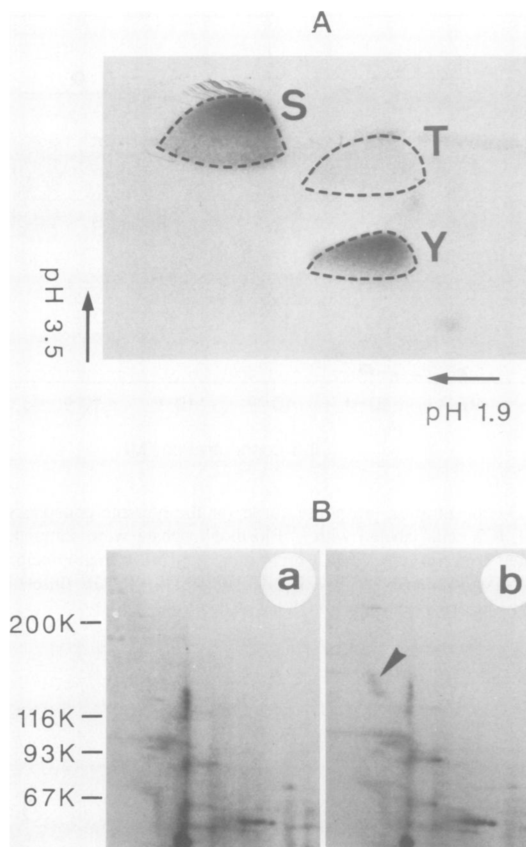

Fig. 4. (**A**) Phosphoamino acid analysis of pp150. Dotted circles indicate the positions of standard phosphoamino acids identified by reaction with ninhydrin. (**B**) IC2 cells labeled with L-[^{35}S]methionine were incubated with saline (**a**) or 1 nM IL3 (**b**) for 3 min. Proteins immunoprecipitated with anti-P-Tyr were analyzed by 2D-NEPHGE/SDS–PAGE. An arrowhead indicates pp150.

Kinetics of tyrosine phosphorylation of gpp150

IC2 cells were stimulated with 1 nM IL3 for various lengths of time after labeling with [^{32}P]orthophosphate to examine the kinetics of phosphorylation of gpp150 (Figure 6). Phosphorylation of gpp150 was detectable within 30 s, reached a maximum at 3 min and decreased thereafter (Figure 6B). gpp150 appeared to split into two adjacent spots as shown in Figure 6. The phosphopeptide maps of the two spots were similar (unpublished data). During early periods following stimulation, the higher mobility species was predominant. The lower mobility species of gpp150 increased gradually as the IC2 cells were stimulated further.

Tyrosine phosphorylation in gpp150 depended on the concentration of IL3 used in the stimulation (Figure 7). Tyrosine phosphorylation of gpp150 was detectable at 10 pM IL3 and the maximum level of phosphorylation was observed at 1 nM IL3. Half-maximum stimulation by IL3 was at 200–300 pM with 2.5 $\times$ 10^6/ml IC2 cells. The ratio of higher and lower mobility species of gpp150 appeared to be constant at any concentration of IL3 (Figure 7, insets).

Tyrosine phosphorylation of gpp150 in growth-factor-independent variants

We isolated two spontaneous transformants, IC2Tr and DA-1Tr, from IC2 and DA-1 respectively. Both of the variants could grow continuously in the absence of the growth factors, IL3, GM-CSF or IL4. Culture supernatants of these variants did not contain any detectable growth factor activity as determined by stimulation assays of the IL3-dependent IC2 cells. We also could not detect IL3 mRNA in these variants by Northern blotting analysis (unpublished data). It is thus unlikely that these variants grew in an autocrine manner. The transformants were labeled with [^{32}P]-orthophosphate without any stimulation by IL3 and phosphotyrosine-containing proteins were analyzed (Figure 8A). In these transformants, gpp150 was constitutively phosphorylated. Phospho-peptide maps of gpp150 from IL3-stimulated IC2 cells and those from IC2Tr were essentially the same (Figure 8B). Ad-

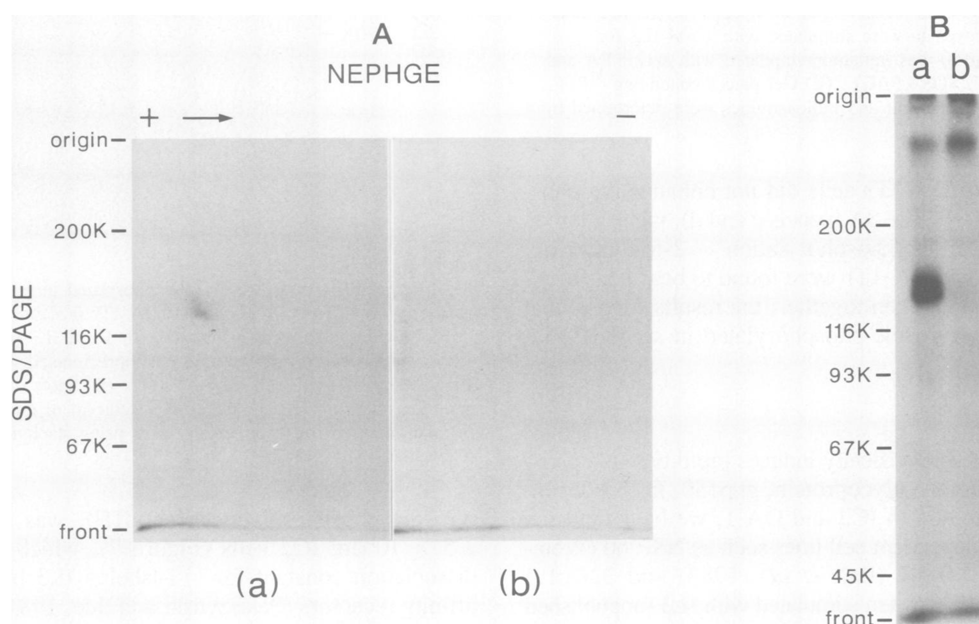

Fig. 5. (**A**) The lysates of IC2 cells labeled with [^{32}P]orthophosphate and stimulated with 1 nM IL3 were fractionated into the membrane (**a**) and the cytoplasmic (**b**) fractions. Anti-P-Tyr immunoprecipitates were analyzed by 2D-NEPHGE/SDS–PAGE. (**B**) Tyrosine phosphorylated proteins eluted from the immunocomplex by *p*-nitrophenylphosphate were subjected to WGA–Sepharose affinity chromatography. Bound (a) and unbound (b) materials were analyzed by SDS–PAGE.

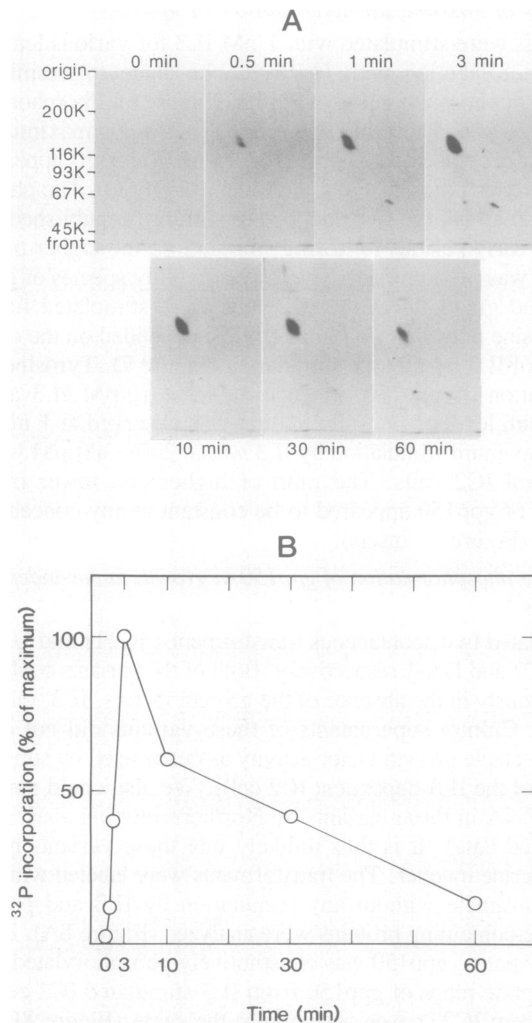

Fig. 6. Time course of tyrosine phosphorylation of gpp150. (**A**) IC2 cells labeled with [^{32}P]orthophosphate were stimulated with 1 nM IL3 for indicated periods and gpp150 was immunoprecipitated with anti-P-Tyr and analyzed by 2D-NEPHGE/SDS−PAGE. (**B**) Gel pieces containing ^{32}P-labeled gpp150 in (A) were cut out and radioactivities were determined by a liquid scintillator.

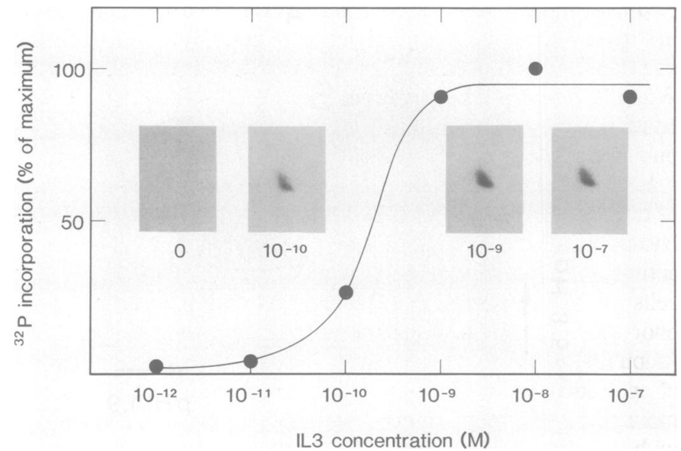

Fig. 7. IL3-concentration−response curve for the tyrosine phosphorylation of gpp150. IC2 cells labeled with [^{32}P]orthophosphate were stimulated with the indicated concentration of IL3 for 3 min. gpp150 immunoprecipitaed with anti-P-Tyr was analyzed by 2D-NEPHGE/SDS−PAGE (inset) and the radioactivity incorporated into gpp150 was determined.

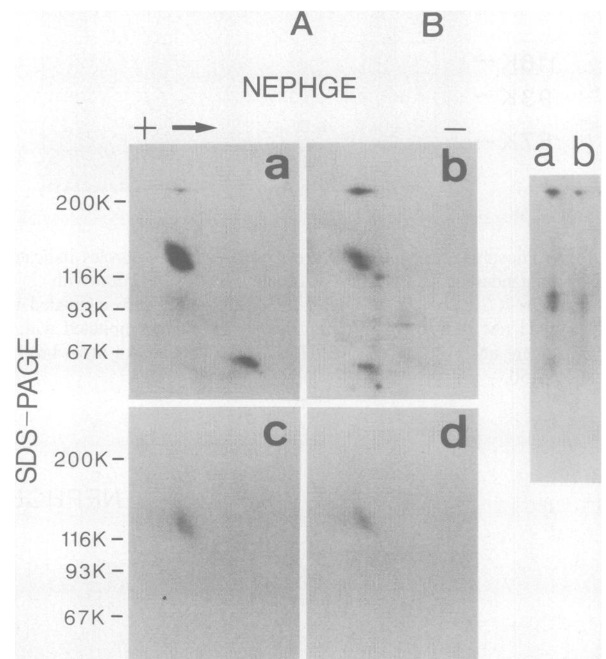

Fig. 8. gpp150 is constitutively phosphorylated in growth-factor-independent variants. (**A**) IC2 (**a**), IC2Tr (**b**) or DA-1Tr (**c,d**) labeled with [^{32}P]orthophosphate were incubated with 1 nM IL3 (a,d) or saline (b,c) for 3 min, after which gpp150 was immunoprecipitated with anti-P-Tyr and analyzed by 2D-NEPHGE/SDS−PAGE. (**B**) Spots of ^{32}P-labeled gpp150 were cut out from IL3-stimulated IC2 cells (a) or unstimulated IC2Tr cells (b) and analyzed for their peptide maps using staphylococcal V8 protease (Cleveland *et al.*, 1977).

dition of 1 nM IL3 to DA-1Tr cells did not enhance the phosphorylation of gpp150 (Figure 8A, panels c and d), while addition to IC2Tr cells enhanced the phosphorylation ~ 2-fold (data not shown). Both IC2Tr and DA-1Tr were found to bear IL3 receptors (unpublished data). Taken together, the results suggest that gpp150 is maximally tyrosine-phosphorylated in some IL3-independent variants.

Discussion

We report here that IL3 specifically induces rapid tyrosine phosphorylation of a membrane glycoprotein, gpp150, in IL3-dependent cell lines. In addition to IC2 and DA-1, we have detected gpp150 in other IL3-dependent cell lines such as NSF-60 (Weinstein *et al.*, 1986), PB-3C (Ball *et al.*, 1983) and 32Dc1.3 (Greenberger *et al.*, 1983) when stimulated with IL3 (unpublished data). Among the reagents tested including IL3, GM-CSF, IL4, IL2 and TPA, only IL3 induced tyrosine phosphorylation of gpp150. Tyrosine phosphorylation of gpp150 occurred in response to a low concentration of IL3. The EC_{50}, the concentration of IL3 required for the half-maximum stimulation of tyrosine phosphorylation of gpp150, was 200−300 pM with 2.5×10^6/ml IC2 cells (Figure 7), which is the same as the dissociation constant for ^{125}I-labeled IL3 binding to the high-affinity receptors (Palaszynski and Ihle, 1984; Park *et al.*, 1986; our unpublished data). This strongly suggests that tyrosine phosphorylation of gpp150 is induced under physiological conditions. Our previous results obtained with a human epidermoid carcinoma KB have shown that tyrosine phosphorylation of cellular proteins induced by growth factors including insulin, IGF-I and

EGF was rapid and transient (Kadowaki *et al.*, 1987). As shown in Figure 6, tyrosine phosphorylation of gpp150 was also rapidly and transiently induced in IC2 cells in an IL3-dependent manner. When IC2 cells were continuously exposed to IL3, gpp150 was continuously phosphorylated but at a relatively low level (unpublished data).

It was recently reported that IL3 induced the phosphorylation of two proteins of mol. wt 67 kd (pp67) and 20 kd (pp20). These phosphorylations occurred on threonine residues probably through activation of protein kinase C (Evans *et al.*, 1986). Using IC2 cells, we have observed that IL3 and GM-CSF induced the phosphorylation of pp67 and pp20 on serine and/or threonine residues (unpublished data). These results indicate that phosphorylation of pp67 and pp20 is not restricted to stimulation by IL3. Furthermore, phosphorylation of pp67 was reported in T cells stimulated with IL2 (Ishii *et al.*, 1986). As described above, tyrosine phosphorylation of gpp150 was not detected by incubation of IC2 cells with GM-CSF, IL4, IL2 or IGF-I. The kinetics of phosphorylation are also different. The phosphorylation of pp67 and pp20 in IL3-stimulated cells was only detectable 5 min after stimulation (Evans *et al.*, 1986). In contrast, tyrosine phosphorylation of gpp150 reached its maximum 3 min after stimulation (Figure 6). Thus, we conclude that tyrosine phosphorylation of gpp150 is one of the earliest events specifically associated with IL3-stimulation.

Chemical cross-linking studies have recently revealed that the IL3 receptor is, at minimum, a protein(s) of 60–75 kd (Nicola and Peterson, 1986; May and Ihle, 1986; Park *et al.*, 1986; Sorensen *et al.*, 1986), suggesting that gpp150 is not the receptor. Nevertheless, in addition to the 60–75 kd molecule, a high-molecular-weight species of 150–180 kd has also been detected in chemical cross-linking experiments using intact cells (Nicola and Peterson, 1986; Park *et al.*, 1986; our unpublished data). By contrast, only the 60–75 kd molecule was detected when cross-linking was performed using membrane fractions (Park *et al.*, 1986; our unpublished data). We propose several possibilities which describe the relationship between tyrosine phosphorylation of gpp150 and the IL3 binding molecule(s): (i) the IL3 binding molecule is a protein tyrosine kinase which phosphorylates gpp150 upon IL3 binding; (ii) gpp150 is a protein tyrosine kinase structurally associated with the IL3 binding molecule, and is autophosphorylated upon binding of IL3 to the receptor; (iii) IL3 stimulates a specific, but receptor-unrelated protein tyrosine kinase to phosphorylate gpp150. The second case is analogous to the insulin receptor system, which is composed of two functinal polypeptides bound together by a disulfide bond (Kasuga *et al.*, 1982). Recently, we have found that gpp150 was radioiodinated upon surface labeling of intact IC2 cells with ^{125}I (unpublished data). Appropriate chemical cross-linking experiments are needed to clarify the functional relationship between the IL3 receptor and gpp150. Furthermore, *in vitro* experiments on the protein tyrosine kinase activity will be of interest in future studies.

gpp150 was constitutively phosphorylated in IC2Tr and DA-1Tr cells which can grow in an IL3-independent manner. These variants did not synthesize IL3, suggesting that these variants did not grow in an autocrine manner and that tyrosine phosphorylation of gpp150 in IC2Tr and DA-1Tr was not caused by the binding of IL3 to its receptor. Since the activation of protein tyrosine kinases is involved in some cellular transformation events (Hunter and Cooper, 1985), it may be that constitutive tyrosine phosphorylation of gpp150 is causally related to acquisition of the ability of IC2 and DA-1 cells to grow in a growth-factor-independent manner.

Materials and methods

Materials

Female DBA/2 mice were purchased from Charles River Japan, Tokyo. RPMI 1640 medium and fetal calf serum (FCS) were obtained from Gibco; penicillin G and kanamycin were from Meiji Seika Co., Tokyo; phenylmethylsulfonylfluoride (PMSF), 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (Hepes) and aprotinin from Sigma; pansorbin (*Staphylococcus aureus* Cowan I cells) was from Behring Diagnostics; TPA was from CM Chemicals, Katonah, NY, USA; WGA–Sepharose was from Pharmacia Fine Chemicals AB, Uppsala, Sweden; [methyl-^{3}H]thymidine, L-[^{35}S]methionine and [^{32}P]orthophosphate were from Amersham International, Bucks, UK. RPMI 1640 was generally supplemented with 10 mM Hepes, 0.2% sodium bicarbonate, 5×10^{-5} M β-mercaptoethanol, 100 units/ml penicillin G and 0.2 mg/ml kanamycin.

Mouse recombinant IL3 (Yokota *et al.*, 1984) and IL4 (Lee *et al.*, 1986) were purified from serum-free culture supernatant fluids of COS-7 cells transfected with expression vectors carrying cDNAs for mouse IL3 and mouse IL4 respectively. Mouse recombinant GM-CSF (Miyatake *et al.*, 1985) was purified from culture supernatant of yeast cells as reported previously (Miyajima *et al.*, 1986). The purity of IL3 and GM-CSF was >95% and that of IL4 was ~50%. Pure human recombinant IL2 was a gift of Dr Y.Sugino, Central Research Division, Takeda Chemical Industries, Osaka, Japan.

Cells and growth conditions

Conditioned media from Con A-stimulated splenocytes and culture supernatant fluids of WEHI-3 cells were prepared as described (Koyasu *et al.*, 1985, 1986). An IL3-dependent or multi-factor-dependent P cell clone, IC2 (Koyasu *et al.*, 1985), was cultured in RPMI 1640 containing 5% FCS and 50% splenocytes conditioned medium. Another IL3-dependent cell line, DA-1 (Weinstein *et al.*, 1986), has been cultured in RPMI 1640 containing 10% FCS and 10% WEHI-3 conditioned medium. Stimulation of DNA synthesis induced by IL3 or other growth factors was determined as described previously (Koyasu *et al.*, 1985). IC2Tr and DA-1Tr, growth-factor-independent variants derived from IC2 and DA-1 respectively, were established without mutagenesis as follows. The concentration of conditioned medium was gradually decreased and surviving cells were further cultured. After a few months, growth-factor-independent variants were isolated by limiting dilution.

Analysis of tyrosine-phosphorylated proteins

Preparation of the affinity-purified anti-P-Tyr was described previously (Kadowaki *et al.*, 1987). The final concentration of the antibody was 250 μg/ml. IC2 cells were used 3 days after changing the conditioned medium. DA-1 cells were cultured for 12 h before labeling in RPMI 1640 containing 10% FCS and 1% WEHI-3-conditioned medium. Cells (2.5×10^6) were suspended in 1 ml of phosphate-free RPMI 1640 containing carrier-free [^{32}P]orthophosphate (1 mCi/ml). For labeling cells with L-[^{35}S]methionine, the cells were suspended at 2×10^5/ml in RPMI 1640 containing 10% FCS, 2% WEHI-3-conditioned medium, 10% of the normal concentration of L-methionine and L-[^{35}S]methionine (0.1 mCi/mol) and cultured for 16 h. The cells were further incubated in serum-free medium for 2 h before stimulation. Labeled cells were stimulated with or without growth factors at 37°C as indicated, centrifuged at 12 000 *g* for 1 min, and frozen with liquid nitrogen after aspiration of the medium. The cells were then thawed and solubilized immediately at 4°C in 0.4 ml of a solubilizing solution consisting of 1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium orthovanadate, 1 mM PMSF, 100 U/ml aprotinin and 50 mM Hepes, pH 7.4. Supernatants of the whole-cell extracts were prepared by homogenizing the radiolabeled cells and sedimenting the insoluble materials by centrifugation at 15 000 *g* for 15 min. After incubation of the extracts with anti-P-Tyr (1:100) at 4°C for 1.5 h, the antibodies were immobilized on pansorbin and the precipitates were washed four times with a washing solution consisting of 1% Triton X-100, 0.1% SDS, 150 mM sodium chloride, 100 mM sodium fluoride, 2 mM sodium orthovanadate and 50 mM Hepes, pH 7.4. Phosphotyrosine-containing proteins were eluted from pansorbin with a solution consisting of 10 mM *p*-nitrophenylphosphate, 0.1% Triton X-100 and 25 mM Hepes, pH 7.4, at 4°C for 1 h.

Cytosol and membrane fractions were prepared as follows. After stimulation with growth factors, the cells were pelleted by centrifugation, and frozen by liquid nitrogen as described above and suspended in the solubilizing solution without Triton X-100. The cells were homogenized, subjected to three cycles of freezing and thawing and centrifuged at 15 000 *g* for 15 min. The supernatant cytosol fraction was mixed with 1/10 vol of 10% Triton X-100. The pellets were suspended in the solubilizing solution containing 1% Triton X-100. Clear supernatants containing membrane proteins were obtained by centrifugation at 15 000 *g* for 15 min.

Phosphoamino acid analysis

The phosphoamino acid composition was determined as described previously (Haring *et al.*, 1984; Akiyama *et al.*, 1986; Kadowaki *et al.*, 1987). Dried polyacrylamide gel pieces containing phosphoprotein as identified by autoradiography

were washed for 12 h at 37°C with 20 ml of 10% methanol. The gel pieces were then dried at 70°C for 60 min and extracted with 2 ml of 50 mM NH_4HCO_3 containing 100 μg of TPCK-trypsin for 24 h at 37°C. The extracts were lyophilized after centrifugation and hydrolyzed in 0.3 ml of 6 N HCl for 2 h at 110°C. The sample was diluted with 2 ml of water, lyophilized, dissolved in 0.5 ml of water and lyophilized again. The lyophilized materials were finally dissolved in water containing 1 mg/ml each of phosphotyrosine, phosphoserine and phosphothreonine, and applied onto cellulose thin-layer plates. Electrophoresis was performed at pH 1.9 (formic acid/acetic acid/water, 25:78:897 by vol.) at 1000 V for 110 min in the first dimension and at pH 3.5 (pyridine/acetic acid/water, 1:10:89 by vol.) at 1000 V for 70 min in the second dimension. The standard phosphoamino acids were detected by reaction with ninhydrin, and the radioactive amino acids were detected by autoradiography.

Miscellaneous

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and two-dimensional (2D)-nonequilibrium pH gradient (NEPHGE)/SDS–PAGE were performed as described previously (Laemmli, 1970; O'Farrell *et al.*, 1977). Peptide mapping analysis with *Staphylococcus aureus* V8 protease was performed according to the method described previously (Cleveland *et al.*, 1977). WGA–Sepharose affinity chromatography was performed according to the methods described previously (Ek and Heldin, 1984; Kadowaki *et al.*, 1987).

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