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TPO/C-MPL LIGAND INDUCES TYROSINE PHOSPHORYLATION OF MULTIPLE CELLULAR PROTEINS INCLUDING PROTO-ONCOGENE PRODUCTS, VAV AND C-CBL, AND RAS SIGNALING MOLECULES[1]

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SUMMARY: Thrombopoietin (TPO)/c-mpl ligand is a hematopoietic growth factor that stimulates proliferation and maturation of megakaryocytes. To analyze the signaling pathway downstream of the c-mpl product, we used a human megakaryoblastic cell line, Mo7e, that has been proved to be responsive to TPO in terms of DNA synthesis. In this study, we found that TPO treatment resulted in tyrosine phosphorylation of Jak-2 kinase. Moreover, it was revealed that several functional molecules involved in the Ras signaling pathway, Shc and Sos, were phosphorylated by treatment with TPO. Finally, tyrosine phosphorylation of the proto-oncogene products, Vav and c-Cbl, has been proved to be induced by TPO. These results suggest that TPO could activate several signaling pathways including the Jak/Stat pathway, the Ras pathway and possibly another pathway involving the c-Cbl proto-oncogene product.

Megakaryopoiesis and platelet production have been thought to be regulated by humoral factors.[1] So far, these factors are divided into two groups, megakaryocyte-colony stimulating factors (Meg-CSFs) and thrombopoietin (TPO) based on their activities.[2] Meg-CSF is the factor that stimulates proliferation of megakaryocytes, and TPO stimulates the maturation of them.[2] These activities are included by several known factors such as interleukin(IL)-1,[3] IL-3,[4] IL-6,[5] IL-11,[6] granulocyte-macrophage colony-stimulating factor (GM-CSF),[7] erythropoietin (EPO),[8] c-kit ligand/stem cell factor (SCF),[9] and leukemia inhibitory factor (LIF).[10] However, critical factors that elicit these activities on megakaryopoiesis and platelet production have not been identified.

Recently, purification and molecular cloning of the humoral factor that stimulates megakaryopoiesis and platelet production have been reported by several groups. The groups of Bosselman, Eaton and Foster have biochemically purified and molecularly cloned the ligand for c-mpl, and have found c-mpl ligand to have the activity of megakaryopoiesis and platelet

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production.[11-14] Simultaneously, purification and molecular cloning of the humoral factor that stimulates both proliferation and maturation of megakaryocytes by following its biological activity have been reported by the group of Miyazaki.[15,16] Surprisingly, all the factors mentioned above have been revealed to be identical, suggesting that the ligand for c-mpl has activities of both Meg-CSF and TPO. In this manuscript, therefore, we use "TPO" or "c-mpl ligand" as the factor that stimulates megakaryopoiesis and platelet production.

C-mpl is the cellular homolog of the v-mpl oncogene,[17] and is a member of the cytokine receptor superfamily.[18] The signal triggered by TPO should be transmitted through the c-mpl product and into the signaling pathway downstream of the c-mpl product in target cells. Although it is essential for understanding the mechanisms of the biological activities of TPO to investigate the signaling pathway downstream of c-mpl, little is so far known about the c-mpl-mediated signaling pathway. In this report, using a human megakaryoblastic cell line, Mo7e, we demonstrate that TPO induces tyrosine phosphorylation of multiple cellular proteins including proto-oncogene products, Vav and c-Cbl, and signaling molecules involved in the Ras pathway.

MATERIALS AND METHODS

Cell lines and culture. The GM-CSF dependent human megakaryoblastic cell line, Mo7e, was obtained from Dr. Stieve Clark (Genetics Institute, Cambrige, MA).[19] Mo7e cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10 ng/ml of recombinant human GM-CSF (rhGM-CSF).

Growth factors. Recombinant human GM-CSF and TPO were kindly supplied by Kirin Brewery Co. Ltd. (Tokyo).

Antibodies. Polyclonal anti-Grb-2/Ash, anti-Vav and anti-c-Cbl antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit antibody against Sos was generously provided by Dr. K. Kaburagi (University of Tokyo) and used for the immunoblotting of Sos. Anti-Jak-2 polyclonal antibody and anti-Grb-2/Ash monoclonal antibody were purchased from MBL (Nagoya, Japan). A mouse monoclonal antibody against phosphotyrosine (anti-Ptyr) 4G10 was purchased from UBI and used for the immunoblotting of phosphotyrosine-containing proteins.

[^{3}H]thymidine incorporation. Mo7e cells were washed and resuspended in RPMI 1640 medium supplemented with 10% FCS and 50 ng/ml GM-CSF or 50 ng/ml TPO at the concentration of 2 x 10^5 cells/ml. Cells were seeded at 96-well plate (100 µl/well) and incubated for 60-70 hr. Following the incubation, [^{3}H]thymidine was added to each well to a final radioactivity of 10 µCi/ml and incubated for 3 hr at 37 °C. Each sample was subjected to counting [^{3}H]thymidine incorporation by a liquid scintillation counter.

Preparation of GST fusion proteins. Four types (A-D) of GST-Cbl fusion proteins were produced as follows. A 1325 bp SacI fragment containing the N-terminal region of c-Cbl (nucleotides 175-1499, Type A), and a 859 bp SacI-SspI fragment containing the proline rich region of c-Cbl (nucleotides 1499-2357, Type B) were blunt-ended using T4 DNA polymerase and separately subcloned into the SmaI site of the pGEX-1 and pGEX-3X vectors, respectively. A 257 bp SspI-StuI fragment of c-Cbl (2357-2613, Type C) was subcloned into the SmaI site of the pGEX-3X vector. A 1115 bp SacI-StuI fragment containing the proline rich region and the flanking C-terminal region of c-Cbl (1499-2613, Type D) was blunt-ended and subcloned into the SmaI site of the pGEX-3X vector. These plasmids were used for transformation of BL21 strain of *Escherichia coli*. Bacterial cultures containing pGEX and the series of pGEX-Cbl plasmids were independently treated with 0.1mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 20 hr at 25 °C for induction of each protein. The resulting GST and GST-Cbl fusion proteins were purified using glutathione agarose and used for affinity precipitation.

Preparation of cell lysates. Serum- and factor-depleted Mo7e cells were incubated for 60-70 hr and were stimulated by 50 ng/ml GM-CSF or 50 ng/ml TPO at 37 °C for 5 min in the presence of 100 µM Na_3VO_4. Cells were washed with ice-cold phosphate buffered saline (PBS) containing 100 µM Na_3VO_4 and resuspend in the lysis buffer containing 20 mM Tris/HCl (pH8.0), 1% Nonidet P-40 (NP-40), 1 mM phenylmethylsulfonyl fluoride (PMSF), 500 U/ml

aprotinin, 2 mM EDTA, 50 mM NaF, and 1 mM Na_3VO_4. The solubilized fractions were collected by centrifugation at 15000 x g at 4 °C for 10 min.

Immunoprecipitation. Polyclonal antibody against Jak-2, Grb-2/Ash, Vav, or Shc was independently added to 500 µl lysate obtained from 1 x 10^7 of Mo7e cells. Polyclonal antibody against c-Cbl was added to 500 µl lysate obtained from 5 x 10^7 of Mo7e cells. After incubation for 2 hr at 4 °C, 20 µl of protein A-Sepharose was added and incubated for 1 hr. Immune complexes were collected by centrifugation at 5000 x g for 30 sec. Beads were washed by the lysis buffer and resuspended in 40 µl of Laemmli's sample buffer. Immune complexes were eluted by heating at 100 °C for 5 min and subjected to SDS-PAGE.

Immunoblotting. Electrophoresed proteins were transferred onto polyvinylidene difluoride microporous (PVDF) membranes using an electro-transfer apparatus (BIO-RAD). PVDF membranes were blocked with the blocking buffer containing 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 % bovine serum albumin, and 0.05 % Triton X-100. The membranes were then incubated with TNB buffer containing 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 5 % bovine serum albumin and primary antibodies as indicated for 2 hr followed by washing with TNX buffer containing 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, and 0.05 % Triton X-100 for twice and TN buffer containing 10 mM Tris/HCl (pH 7.4), and 150 mM NaCl for once. Subsequently, the membranes were incubated with TNB buffer containing alkaline phosphatase-conjugated anti-mouse or anti-rabbit antibody (Promega), and washed with the same procedure as primary antibodies. For the detection of respective proteins, color reaction was performed by exposing the membranes to nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate according to the manufacture's protocol (Promega).

Affinity precipitation using GST fusion proteins. Lysates from 1 x 10^7 of Mo7e cells were independently mixed with 40 µg of the series of GST-Cbl fusion proteins noncovalently coupled to glutathione agarose (Sigma) and incubated for 2 hr at 4 °C. Subsequently, beads were washed intensively and resuspended in 100 µl of Laemmli's sample buffer. The proteins precipitated from the lysates of Mo7e cells with GST or GST-Cbl fusion proteins were eluted by heating at 100 °C for 5 min and subjected to SDS-PAGE.

RESULTS

TPO induces DNA synthesis in a human megakaryoblastic cell line, Mo7e. Factor-starved Mo7e cells were treated with recombinant human TPO or GM-CSF, and assayed for [^{3}H]thymidine incorporation into the cells as described in MATERIALS AND METHODS. Both human TPO and GM-CSF treatment increased [^{3}H]thymidine incorporation 13-14 fold when compared with no treatment control (Fig.1). These results demonstrate that TPO could induce DNA synthesis to the similar level induced by GM-CSF on which Mo7e cells are dependent.

TPO treatment induces tyrosine-phosphorylation of Jak-2 in Mo7e cells. Jak-2 tyrosine kinase is reported to be tyrosine-phosphorylated by several growth factors and cytokines, including GM-CSF.[20] As shown in Fig. 2, Jak-2 kinase was inducibly tyrosine-phosphorylated by the stimulation with TPO or GM-CSF, suggesting that TPO also activates Jak-2 kinase in Mo7e cells.

TPO induces phosphorylation of the signaling molecules involved in the Ras pathway in Mo7e cells. Many growth factors and cytokines have been proved to activate the Ras signaling pathway in target cells.[21] Therefore, we have analyzed whether TPO induces phosphorylation of the signaling molecules involved in the Ras pathway in Mo7e cells. As shown in Fig.3-A and -B, TPO induced tyrosine phosphorylation of Shc and its association to Grb-2/Ash adapter protein. Although three types of Shc, p46, p52 and p66, have so far been reported,[22] only one type of Shc (p52) was revealed to be tyrosine-phosphorylated by GM-CSF or TPO in this experiment. Another tyrosine-phosphorylated protein whose molecular weight is approximately 70kDa was also observed. Tyrosine phosphorylation of p70 is prominent in GM-

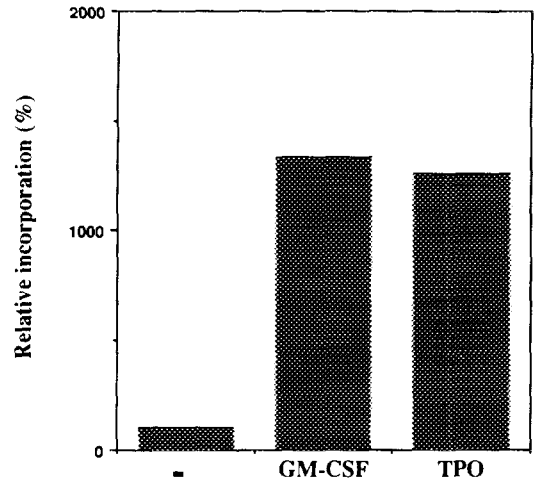

Fig. 1. [^{3}H]Thymidine incorporation in Mo7e cells. [^{3}H]Thymidine incorporation was determined as described in MATERIALS AND METHODS. Data that are the average of 10 independent experiments represent the relative [^{3}H]thymidine incorporation that was normalized to the incorporation without stimulation (-).

CSF-stimulated cells. As for the nature of p70, there are several candidates including p66^{Shc}. As shown in Fig.3-A, tyrosine phosphorylation of p66^{Shc} is not observed in GM-CSF-treated Mo7e cells. Therefore, p70 is not a tyrosine-phosphorylated form of p66^{Shc}. Furthermore, as shown in Fig. 3-C, in the result of immunoblotting using anti-Sos antibody, the bands corresponding to Sos were detected to be shifted only at the lane for the sample that was treated with TPO or GM-CSF. The decreased mobility of Sos in SDS-PAGE was thought to result from their phosphorylation. These evidences show that TPO induces phosphorylation of the signaling molecules involved in the Ras pathway in Mo7e cells.

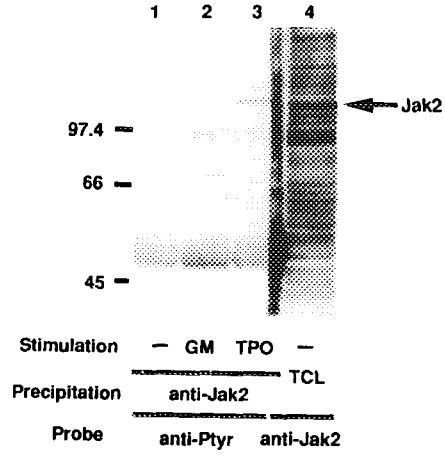

Fig. 2. TPO induces tyrosine phosphorylation of Jak-2. From the lysates of Mo7e cells with no stimulation (lane 1), GM-CSF (lane 2) or TPO (lane 3) stimulation, Jak-2 tyrosine kinase was specifically immunoprecipitated using polyclonal antibody against Jak-2. Tyrosine phosphorylation of Jak-2 was detected by immunoblotting using antiphosphotyrosine monoclonal antibody, 4G10. Total cell lysate from unstimulated Mo7e cells was also included (lane 4) for identification of Jak-2 protein (indicated by the arrow).

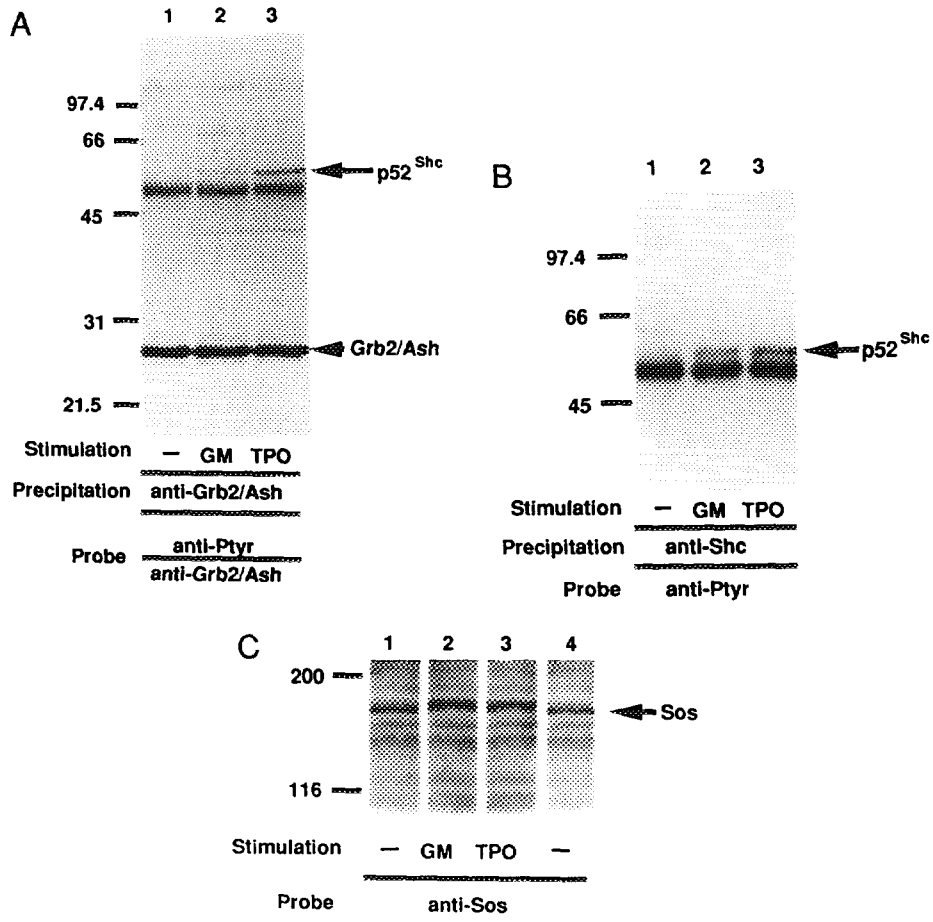

Fig. 3. Analyses of signaling molecules of the Ras pathway in TPO-treated Mo7e cells. (A) Tyrosine phosphorylation of $p52^{Shc}$. From the lysate of untreated (lane 1), GM-CSF-treated (lane 2), or TPO-treated (lane 3) Mo7e cells (1×10^7 cells/lane), Shc was immunoprecipitated by anti-Shc polyclonal antibody, and immunoblotted with antiphosphotyrosine monoclonal antibody. The arrow indicates the position of $p52^{Shc}$. (B) Association of Grb-2/Ash with the tyrosine-phosphorylated form of $p52^{Shc}$. Lysates of untreated (lane 1), GM-CSF-treated (lane 2) or TPO-treated (lane 3) Mo7e cells (1×10^7 cells/lane) were immunoprecipitated with polyclonal anti-Grb-2/Ash antibody and blotted onto a membrane. The membrane was cut into two pieces and separately probed with antiphosphotyrosine antibody (top) or anti-Grb-2/Ash antibody (bottom). The arrow and arrow head indicate the mobilities of $p52^{Shc}$ and Grb-2/Ash, respectively. (C) Phosphorylation of Sos. The total cell lysates were immunoblotted with anti-Sos antibody. Decreased mobilities of Sos protein resulting from phosphorylation were observed in TPO- (lanes 3) or GM-CSF-treated cells (lanes 2). The arrow indicates the position of Sos.

TPO induces tyrosine phosphorylation of the proto-oncogene product Vav. Recent studies have reported that the proto-oncogene product Vav was tyrosine-phosphorylated in response to the stimulation of GM-CSF or IL-3,[23] and that Jak-2 tyrosine kinase potentially tyrosine-phosphorylated Vav in Mo7e cells.[24] Since, in our study, tyrosine phosphorylation of Jak-2 was induced by TPO or GM-CSF, we compared the levels of tyrosine phosphorylation of Vav before and after the stimulation with these factors. As shown in Fig. 4, we found that both TPO and GM-CSF induce increased levels of phosphorylation of Vav on tyrosine residues in Mo7e cells.

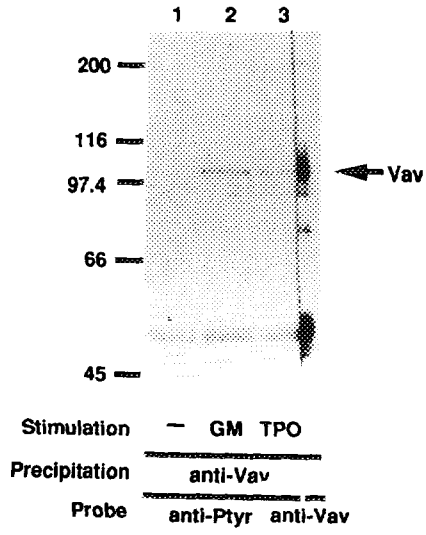

Fig. 4. TPO induces tyrosine phosphorylation of the proto-oncogene product Vav. From the lysates of Mo7e cells with no stimulation (lane 1), GM-CSF (lane 2) or TPO (lane 3) stimulation, the proto-oncogene product Vav was specifically immunoprecipitated with anti-Vav polyclonal antibody and immunoblotted with antiphosphotyrosine monoclonal antibody. A part of the lane 3 (right) was separately probed with anti-Vav antibody for identification of Vav protein. The arrow indicates the position of Vav.

TPO induces tyrosine phosphorylation of proto-oncogene product c-Cbl. We have recently demonstrated that the c-Cbl proto-oncogene product was tyrosine-phosphorylated by the stimulation with GM-CSF or EPO in human hematopoietic cell lines.[25] As shown in Fig. 5-A., we have analyzed tyrosine phosphorylation of c-Cbl in Mo7e cells. Although a low level of tyrosine phosphorylation of c-Cbl was observed in the untreated cells, tyrosine phosphorylation of c-Cbl was revealed to increase by the stimulation with TPO or GM-CSF. This observation indicates that the proto-oncogene product c-Cbl is involved in the signaling pathway downstream of the c-mpl product as well.

The proto-oncogene product, c-Cbl, constitutively associates Grb-2/Ash adapter protein through the proline-rich region. We have recently observed that the proto-oncogene product c-Cbl constitutively associates with Grb-2/Ash adapter protein through the N-terminal SH3 domain of Grb-2/Ash in UT7 cells.[25] Therefore, we have analyzed the association between Grb-2/Ash adapter protein and the proto-oncogene product c-Cbl in Mo7e cells. As shown in Fig. 5-B, anti-c-Cbl polyclonal antibody could precipitate the tyrosine-phosphorylated form of c-Cbl and also coprecipitate Grb-2/Ash adapter protein from the lysate of Mo7e cells, regardless of the stimulation with TPO or GM-CSF. Furthermore, to investigate which domain of c-Cbl associates with the N-terminal SH3 domain of Grb-2/Ash, we have produced several recombinant c-Cbl proteins fused to glutathione S-transferase (GST) protein as described in MATERIALS AND METHODS (Fig. 5-C). As shown in Fig.5-D, some types of GST-Cbl fusion proteins (Types B and D) were proved to precipitate Grb-2/Ash adapter protein regardless of TPO/GM-CSF stimulation. Since both types B and D of GST-Cbl fusion proteins contain a proline rich region, c-Cbl was considered to associate with Grb-2/Ash constitutively through its proline rich region. Furthermore, both types of GST-Cbl fusion proteins could

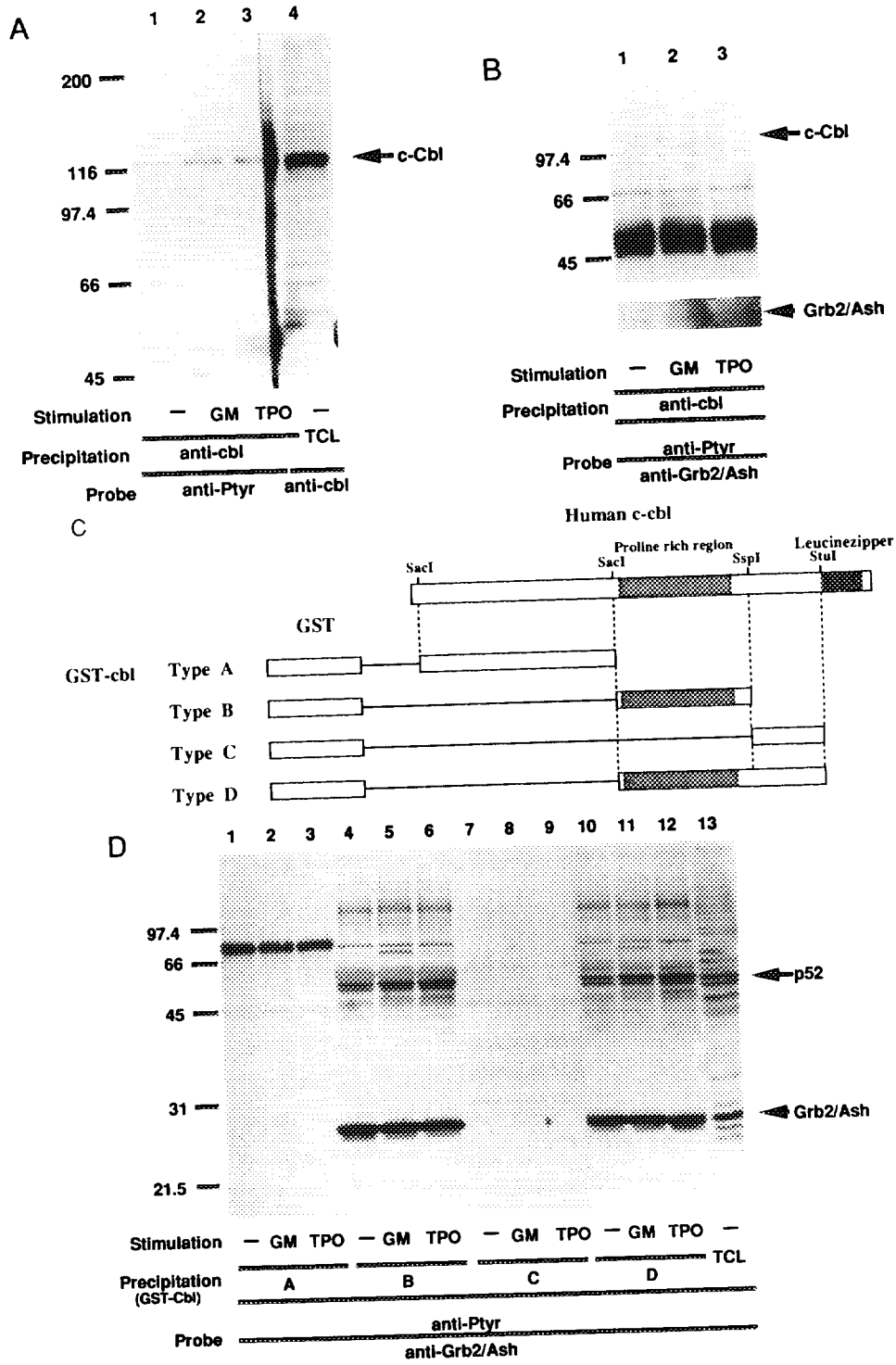

Fig. 5. TPO increases tyrosine phosphorylation of the proto-oncogene product c-Cbl that constitutively associates with Grb-2/Ash adapter protein in Mo7e cells. (A) TPO increases tyrosine phosphorylation of the proto-oncogene product c-Cbl. Lysates of untreated (lane 1), GM-CSF-treated (lane 2) or TPO-treated (lane 3) Mo7e cells (5 x 10^7

precipitate several tyrosine phosphorylated proteins. Among the tyrosine-phosphorylated proteins, a protein (p52, indicated by the arrow in Fig.5-D) whose molecular weight corresponds to that of $p52^{Shc}$ was inducibly precipitated by the stimulation with TPO or GM-CSF.

DISCUSSION

Recent studies have revealed that the c-mpl product is a receptor for TPO.[11-14,26] The c-mpl gene was molecularly cloned as a human homolog of myeloproliferative leukemia virus (MPLV) that caused a broad spectrum of myeloid leukemias in mice, and was revealed to be a member of the cytokine receptor superfamily.[17] Although the intracellular signals triggered by TPO should be transmitted through c-mpl, little is known about the intracellular signaling pathway downstream of c-mpl.

All the members of cytokine receptor superfamily do not have tyrosine kinase activity by itself. However, many cytokines have been proved to induce tyrosine phosphorylation of multiple cellular proteins. This evidence indirectly indicates activation of cytoplasmic tyrosine kinase(s) via binding of a cytokine to its receptor. Among cytoplasmic protein tyrosine kinases, Jak-2 tyrosine kinase was known to be phosphorylated and activated by the stimulation with several hematopoietic growth factors including GM-CSF.[20] As shown in Fig. 2, TPO or GM-CSF induced tyrosine phosphorylation of Jak-2 kinase, suggesting its possible activation. This result indicates that TPO might utilize Jak/Stat pathway in target cells. To further clarity this signaling pathway, it is necessary to identify the functional molecule of Stat family. One candidate of this family is Stat5/MGF, since recent reports have shown that GM-CSF activates Stat5/MGF.[27] As shown in Fig. 3, we also found that treatment of Mo7e cells with TPO could induce tyrosine phosphorylation of Shc and its association to Grb-2/Ash and that Sos was phosphorylated by the stimulation with TPO, as was the case with GM-CSF. Recent study also reported tyrosine phosphorylation of Jak2 and Shc by the stimulation with TPO in c-mpl-transfected BaF/3 cells.[28] These findings might support the hypothesis that TPO-binding to c-mpl could also activate the Ras signaling pathway.

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cells/lane) were immunoprecipitated with polyclonal anti-c-Cbl antibody and immunoblotted with antiphosphotyrosine antibody. A part of the lane 3 (right) and total cell lysate of untreated cells (lane 4) were separately immunoblotted with anti-c-Cbl antibody for identification of c-Cbl protein. (B) The proto-oncogene product c-Cbl constitutively associates with Grb-2/Ash adapter protein in Mo7e cells. Lysates of untreated (lane 1), GM-CSF-treated (lane 2) or TPO-treated (lane 3) Mo7e cells (5×10^7 cells/lane) were immunoprecipitated with polyclonal anti-c-Cbl antibody. The immunoprecipitates and total cell lysate of untreated cells (lane 4) were blotted onto a membrane. The membrane was cut into two parts and separately probed with antiphosphotyrosine antibody (top) or anti-Grb-2/Ash antibody (bottom). The arrow and arrow head indicate the positions of tyrosine-phosphorylated c-Cbl and Grb-2/Ash, respectively. (C) Construction of glutathione S-transferase (GST) fusion proteins that contain various domains of c-Cbl. (D) Grb-2/Ash adapter protein associates constitutively with the proto-oncogene product, c-Cbl, through its proline-rich region and it inducibly associates tyrosine phosphorylated proteins. Lysates of untreated (lanes 1, 4, 7 and 10), GM-CSF-treated (lanes 2, 5, 8 and 11) or TPO-treated (lanes 3, 6, 9 and 12) Mo7e cells (5×10^7 cells/lane) were independently mixed with the series of GST-Cbl fusion proteins noncovalently coupled to glutathione-agarose beads and precipitated as described in MATERIALS AND METHODS. The immunoblotted membrane was cut into two parts and probed separately with antiphosphotyrosine antibody (top) or anti-Grb-2/Ash antibody (bottom). Molecular weight markers, indicated at the left, are given in kDa. The arrow and arrow head indicate tyrosine phosphorylated protein (p52) and Grb-2/Ash, respectively.

Grb-2/Ash adaptor protein is proved to associate with phosphorylated tyrosine residues of EGF receptor,[29] Shc,[30] IRS-1,[31] and Syp[32] through its SH2 domain, and with Sos,[33] C3G,[34] Dynamin,[35] Vav,[36] and Cbl[25] through its SH3 domain(s). Recent studies have reported that Vav confers the activity of guanine nucleotide exchange factor (GEF) for Ras[37] and is tyrosine phosphorylated by the stimulation with GM-CSF, IL-3, or EPO. In this paper, we demonstrated that TPO or GM-CSF could trigger tyrosine phosphorylation of Vav in Mo7e cells. This evidence supports the hypothesis that Vav should be involved in the signaling pathway downstream of c-mpl.

The c-cbl gene was molecularly cloned as the cellular homolog of the v-Cbl oncogene carried by Cas NS-1 retrovirus that induces pre-B cell lymphomas and myeloid leukemias in mice. We have recently reported that proto-oncogene product c-Cbl was constitutively associate with Grb-2/Ash adaptor protein through the N-terminal SH3 domain of Grb-2/Ash, and that c-Cbl was tyrosine phosphorylated by the stimulation with GM-CSF or EPO in a human leukemia cell line, UT-7.[25] From these results, we suggested the possibility that c-Cbl was involved in the signaling pathway downstream of the receptor for GM-CSF or EPO. In this work, we observed that tyrosine phosphorylation of c-Cbl was increased by the stimulation with TPO in Mo7e cells as was seen by the treatment with GM-CSF. Moreover, using GST-Cbl fusion proteins, we found that c-Cbl is constitutively associated with Grb-2/Ash through the proline rich region of c-Cbl. This is the first report that identifies the domain of c-Cbl through which c-Cbl is constitutively associated with the N-terminal SH3 domain of Grb-2/Ash. Although guanine nucleotide exchange factor (GEF), Sos, that is involved in the Ras pathway, was also associated constitutively with the SH3 domain of Grb-2/Ash, c-Cbl does not harbour any structural homology to the GEF domain of Sos or the other GEFs.[38] From this result, c-Cbl might be involved in another signaling pathway different from the Ras pathway. From these lines of evidence, we conclude that TPO could activate the Jak/Stat pathway, the Ras pathway and another pathway involving c-Cbl.

REFERENCES

1. Gordon MS, and Hoffman R Blood 80: 302, 1992
2. Williams N, Jackson H, Walker F, and Oon SH Blood Cells 15: 123, 1989
3. March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, Gillis S, Henney CS, Kronheim SR, Grabstein K, Conlon PJ, Hopp TP, and Cosman D Nature 315: 641, 1985
4. Ikebuchi K, Wong GG, Clark SC, Ihle JN, Hirai Y, and Ogawa M Proc Natl Acad Sci U S A 84: 9035, 1987
5. Ishibashi T, Kimura H, Uchida T, Kariyone S, Friese P, and Burstein SA Proc Natl Acad Sci U S A 86: 5953, 1989
6. Teramura M, Kobayashi S, Hoshino S, Oshimi K, and Mizoguchi H Blood 79: 327, 1992
7. Wong GG, Witek JS, Temple PA, Wilkens KM, Leary AC, Luxenberg DP, Jones SS, Brown EL, Kay RM, Orr EC, Shoemaker C, Golde DW, Kaufman RJ, Hewick RM, Wang EA, and Clark SC Science 228: 810, 1985
8. Jacobs K, Shoemaker C, Rudersdorf R, Neill SD, Kaufman RJ, Mufson A, Seehra J, Jones SS, Hewick R, Fritsch EF, Kawahakita M, Shimizu T, and Miyake T Nature 313: 806, 1985
9. Hendrie PC, Miyazawa K, Yang YC, Langefeld CD, and Broxmeyer HE Exp Hematol 19: 1031, 1991
10. Metcalf D, Hilton D, and Nicola NA Blood 77: 2150, 1991
11. Bartley TD, Bogenberger J, Hunt, P, Li YS, Lu HS, Martin F, Chang MS, Samal B, Nichol JL, Swift S, Johnson MJ, Hsu RY, Parker VP, Suggs S, Skrine JD, Merewether LA, Clogston C, Hsu E, Hokom MM, Hornkohl A, Choi E, Pangelinan M, Sun Y, Mar V, McNinch J, Simonet L, Jacobsen F, Xie C, Shutter J, Chute H, Basu R, Selander L, Trollinger D, Sieu L, Padilla D,

Trail G, Elliott G, Izumi R, Covey T, Crouse J, Garcia A, Xu W, Del Castillo J, Brion J, Cole S, Hu MCT, Pacifici R, Ponting I, Saris C, Wen D, Yung YP, Lin H, and Bosselman RA Cell 77: 1117, 1994
12. de Sauvage Fj, Hass PE, Spencer SD, Malloy BE, Gurney AL, Spencer SA, Darbonne WC, Henzel WJ, Wong SC, Kuang WJ, Oles KJ, Hultgren B, Solberg LA Jr, Goeddel DV, and Eaton DL Nature 369: 533, 1994
13. Lok S, Kaushansky K, Holly RD, Kuijper JL, Lofton Day CE, Oort PJ, Grant FJ, Heipel MD, Burkhead SK, Kramer JM, Bell LA, Sprecher CA, Blumberg H, Johnson R, Prunkard D, Ching AFT, Mathewes SL, Bailey MC, Forstrom JW, Buddle MM, Osborn SG, Evans SJ Sheppard PO, Presnell SR, O'Hara PJ, Hagen FS, Roth GJ, and Foster DC Nature 369: 565, 1994
14. Kaushansky K, Lok S, Holly RD, Broudy VC, Lin N, Bailey MC, Forstrom JW, Buddle MM, Oort PJ, Hagen FS, Roth GJ, Papayannopoulou T, and Foster DC Nature 369: 568, 1994
15. Kato T, Iwamatsu A, Shimada Y, Horie K, Kokubo A, Maeda E, Ogami K, Sohma Y, Akahori H, Kudo Y, Kubomura N, Matsumoto A, Tahara T, Ohashi H, Tsumura H, Inoue H, Kawamura K, and Miyazaki H Blood 84: 329a, 1994
16. Ogami K, Shimada Y, Sohma Y, Akahori H, Seki N, Hori T, Kobayashi K, Kawagishi M, Yamada Y, Horie K, Maeda E, Kudo Y, Kato T, Miyazaki H, and Kawamura K Blood 84: 326a, 1994
17. Vigon I, Mornon JP, Cocault L, Mitjavila MT, Tambourin P, Gisselbrecht S, and Souyri M Proc Natl Acad Sci U S A 89: 5640, 1992
18. Skoda RC, Seldin DC, Chiang MK, Peichel CL, Vogt TF, and Leder P Embo J 12: 2645, 1993
19. Avanzi GC, Lista P, Giovinazzo B, Miniero R, Saglio G, Benetton G, Coda R, Cattoretti G, and Pegoraro L Br J Haematol 69: 359, 1988
20. Quelle FW, Sato N, Witthuhn BA, Inhorn RC, Eder M, Miyajima A, Griffin JD, and Ihle JN Mol Cell Biol 14: 4335, 1994
21. Feig LA: Science 260: 767, 1993
22. Pelicci G, Lanfrancone L, Grignani F, McGlade J, Cavallo F, Forni G, Nicoletti I, Grignani F, Pawson T, and Pelicci PG Cell 70: 93, 1992
23. Hanazono Y, Sasaki K, Odai H, Mimura T, Mitani K, Yazaki Y, and Hirai H Cancer Res 86: 336, 1995
24. Matsuguchi T, Inhorn RC, Carlesso N, Xu G, Druker B, and Griffin JD Embo J 14: 257, 1995
25. Odai H, Sasaki K, Iwamatsu A, Hanazono Y, Tanaka T, Mitani K, Yazaki Y, and Hirai H J Biol Chem 270: 10800-10805 1995
26. Wendling F, Maraskovsky E, Debili N, Florindo C, Teepe M, Titeux M, Methia N Breton, Gorius J, Cosman D, and Vainchenker W Nature 369: 571, 1994
27. Mui AL, Wakao H, O'Farrell AM, Harada N, and Miyajima A Embo J 14: 1166, 1995
28. Drachman JG, Griffin JD, and Kaushansky K J Biol Chem 270: 4979, 1995
29. Matuoka K, Shibasaki F, Shibata M, and Takenawa T Embo J 12: 3467, 1993
30. Batzer AG, Rotin D, Urena JM, Skolnik EY, and Schlessinger J Mol Cell Biol 14: 5192, 1994
31. Myers Mg Jr, Wang LM, Sun XJ, Zhang Y, Yenush L, Schlessinger J, Pierce JH, and White MF Mol Cell Biol 14: 3577, 1994
32. Bennett AM, Tang TL, Sugimoto S, Walsh CT, and Neel BG Proc Natl Acad Sci U S A 91: 7335, 1994
33. Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, and Weinberg RA Nature 363: 45, 1993
34. Tanaka S, Morishita T, Hashimoto Y, Hattori S, Nakamura S, Shibuya M, Matuoka K, Takenawa T, Kurata T, Nagashima K, and Matsuda M Proc Natl Acad Sci U S A 91: 3443, 1994
35. Seedorf K, Kostka G, Lammers R, Bashkin P, Daly R, Burgess WH, van der Bliek Am, Schlessinger J, and Ullrich A J Biol Chem 269: 16009, 1994
36. Ramos Morales F, Druker BJ, and Fischer S Oncogene 9: 1917, 1994
37. Gulbins E, Coggeshall KM, Langlet C, Baier G, Bonnefoy Berard N, Burn P, Wittinghofer A, Katzav S, and Altman A Mol Cell Biol 14: 906, 1994
38. Blake TJ, Shapiro M, Morse Hc 3d, and Langdon WY Oncogene 6: 653, 1991