Activation of Ras and Formation of GAP Complex During TPA-Induced Monocytic Differentiation of HL-60 Cells

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In this study, it was shown that the proportion of guanosine triphosphate (GTP)-bound active Ras increased in TPA (12-O-tetradecanoylphorbol-13-acetate)-induced monocytic differentiation of HL-60 cells. The increase of active Ras was observed at 24 hours after TPA stimulation and attained to threefold (15%) over the proportion in nontreated HL-60 cells. Herbimycin A, an inhibitor of tyrosine kinase, prevented the activation of Ras, as well as the induction of monocytic differentiation. In parallel with the activation of Ras, the proteins with molecular weights of 52, 56, 62, and 190 kD were tyrosine-phosphorylated and formed a complex with GTPase-activating protein (GAP) for Ras. In addition to the 116-kD GAP (type I GAP), the 100-kD GAP (type II GAP) molecule was markedly induced at 24 hours after TPA stimulation of HL-60 cells. These phenomena sustained for a further 24 hours during monocytic differentiation. However, they were not observed during retinoic acid-induced granulocytic differentiation of the cells. The HL-60 transfectants, which expressed a dominant inhibitory H-ras Asn17, showed a low level of tyrosine-phosphorylated GAP-associated proteins and did not undergo full differentiation in response to TPA. Taken together, these data indicate that the activation of Ras and GAP complex formation mutually correlate and function downstream of protein-tyrosine kinases in the signaling pathway for monocytic differentiation of HL-60 cells.

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I N HEMATOPOIESIS, progenitor cells proliferate and differentiate along multiple cell lineages. This process is highly controlled through successive stages of differentiation and growth arrest. The induction of specific differentiation in vitro allows us to dissect and analyze myelopoiesis. The human myeloblastic cell line HL-60 is known to be led both to the monocytic differentiation and to the granulocytic one. The former is induced by TPA (12-O-tetradecanoylphorbol-13-acetate) and the latter by retinoic acid (RA). These in vitro systems are available for elucidating the molecules involved in the myeloid differentiation.

Protein tyrosine kinases (PTKs) are important regulatory proteins that control cellular growth and differentiation. Recently, we reported that both fyn and lyn tyrosine kinases were expressed abundantly during TPA-induced monocytic differentiation. The inhibitors of PTK activity, such as herbimycin A and genistein, markedly inhibited monocytic differentiation, suggesting that PTK is involved in the differentiation process. The tyrosine-phosphorylated proteins in TPA-treated cells include cytoskeletal proteins, tubulins, which are associated with Fyn and Lyn PTKs.

The signal from tyrosine kinase has been suggested to be transmitted to Ras through specific protein-protein interactions. In addition to the growth of various origins of the cells, Ras plays an important role in the process of neuronal differentiation of PC12 cells that is triggered by the tyrosine kinase receptor. When PC12 cells respond to nerve growth factor, rapid accumulation of the guanosine triphosphate (GTP)-bound form of Ras occurred. The role of Ras in the PC12 cell differentiation can be mimicked by the action of mutationally activated Ras. Nerve growth factor-induced neurite outgrowth was blocked by the microinjection of an anti-Ras monoclonal antibody (MoAb) or a dominant inhibitory ras mutant.

The mammalian Ras proteins bind guanine nucleotides and possess intrinsic GTPase activity. It alternates between active GTP-bound and inactive guanosine diphosphate (GDP)-bound forms. The relative activities of guanine nucleotide-releasing factors (GRFs) and Ras GTPase-activating proteins (GAPs) acting on Ras at any particular time may determine the activation state of Ras. A family of GRFs directly activate Ras, and Sem/Grb2 mediates the interaction of Sos GRF with activated tyrosine kinase receptors. Furthermore, it was recently reported that p95GRF (Vay), which is specifically expressed in the hematopoietic cells, has the GRF activity toward Ras and may be directly regulated by tyrosine kinase.

The GAP stimulates the intrinsic GTP hydrolytic activity of Ras and promotes the return of active GTP-bound Ras to the inactive GDP-bound state. Therefore, GAP might be anticipated to function as a negative regulator of Ras. The GAP contains two src homology 2 (SH2) domains responsible for interaction with tyrosine-phosphorylated proteins. It has been shown that GAP is immunoprecipitated with two tyrosine-phosphorylated proteins with molecular weights of 62 and 190 kD from lysates of cells expressing activated tyrosine kinases or cells stimulated with growth factors. The complex formation of GAP with these proteins inhibits GAP activity, which results in the activation of Ras. Therefore, functional states of both GRFs and GAPs may be controlled by tyrosine kinases in the signaling pathway.

The GAP-associated tyrosine phosphoproteins p62 and p190 were recently cloned from fibroblasts. The p62 has a significant homology to a putative heterogeneous nuclear...
ribonucleoprotein protein and has the ability to bind to single-stranded DNA and to RNA. The p62 is assumed to play a role in some aspect of mRNA processing or utilization. Because a large portion of p190 is identical to a nuclear transcription factor, p190 may transmit a signal to the nucleus for modulation of specific cellular genes. p190 also has GAP activity towards Rho/Rac family, which regulates the reorganization of actin cytoskeleton in cell growth and differentiation. The p62- and p190-mediated signaling pathway operates independently of Ras followed by tyrosine kinase activation.

In this study, we have reported that, during TPA-induced monocytic differentiation of HL-60 cells, the GAP- and pl90-bound active form of Ras increased in a PTK-dependent manner, and that tyrosine-phosphorylated proteins of 52, 56, 62, and 190 kD associated with GAP. These signaling molecules were indicated to function in the induction of monocytic differentiation.

**MATERIALS AND METHODS**

**Cells.** HL-60 cells were suspended with RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% fetal calf serum (Sigma Chemical Co, St Louis, MO). For differentiation experiments, growing cells were subcultured at a density of 2 x 10^6 cells/mL and treated either with TPA (10 ng/mL; Sigma) or RA (1 mM; Sigma) in the presence or absence of herbimycin A (0.4 μg/mL) for the indicated times. Herbimycin A was kindly donated by Yoshimasa Uehara (National Institute of Health, Tokyo, Japan).

**Antibodies.** 125I-labeled monoclonal antiphosphotyrosine PY-20 (ICN Radiocchemicals, Irvine, CA), monoclonal anti-GAP antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), polyclonal anti-GAP antibody (Upstate Biotechnology, Inc, Lake Placid, NY), polyclonal anti-Vav antibody (Santa Cruz Biotechnology), 125I-labeled monoclonal antiphosphotyrosine MoAb PY-20 (New England Nuclear, Boston, MA), and monoclonal anti-Ras antibody (NC-RASW, Santa Cruz, CA) were used in the immunoprecipitation and immunoblotting as described below.

**Immunoprecipitation.** TPA- or RA-treated HL-60 cells (5 x 10^6) were collected by centrifugation and were lysed on ice for 30 minutes with 1 mL of ristocetin-induced platelet aggregation (RIPA) lysis buffer (1% Triton X-100, 10 mM Tris, 150 mM NaCl, 2 mM EDTA, 10 mM aprotinin, 10 mM NaF, 1 mM NaN3, and 25 mM NaVO4; pH 7.4). The supernatant was preincubated with excess amount of protein G-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). The cleared lysate was incubated with various kinds of antibodies and protein G-Sepharose 4B. The immunoprecipitates were washed with the lysis buffer extensively.

**Immunoblotting.** The 10 mL of the cell lysates or the immunoprecipitated proteins were subjected to electrophoresis on 8% or 12% polyacrylamide/sodium dodecyl sulfate gel. The transfer of the proteins to polyvinylidene difluoride membrane and blotting with 125I-labeled PY-20 or monoclonal anti-GAP antibody plus 125I-labeled antiphosphotyrosine Ig antibody were performed as described. Monoclonal anti-Ras antibody, peroxidase-linked antimouse Ig (ICN), monoclonal anti-Ras antibody (NC-RASW), and peroxidase-linked antimouse Ig (ICN) were used in the immunoprecipitation and immunoblotting as described below.

**Analysis of Ras-bound GDP/GTP.** Cells were washed twice with Tris-buffered saline (20 mM Tris-HCl [pH 7.5], 150 mM NaCl) and were labeled for 6 hours in 1 mL of phosphate-free Dulbecco’s modified Eagle’s medium supplemented with 0.05 mCi/mL of 32P. Nucleotides bound to Ras were analyzed as described.

We reported previously that herbimycin A, an inhibitor of PTK, prevented HL-60 cells from acquiring macrophage-like features such as plastic and cellular adherence after stimulation (Fig 2A). In addition, herbimycin A completely inhibited TPA-induced activity of nonspecific esterase, a marker enzyme for monococytes (Fig 2A). As shown in Fig 2B, the increase of GTP-bound Ras in TPA-treated HL-60 cells was abro-
Fig 1. (A) Accumulation of Ras-GTP in monocytic differentiation, but not in granulocytic differentiation of HL-60 cells is shown. HL-60 cells that were treated with or without TPA or RA for 48 hours were labeled with \(^{32}P\). The cell lysates were subjected to immunoprecipitation with or without (NC) monoclonal anti-Ras antibody Y13-269. The nucleotides in the immunoprecipitates were analyzed by thin-layer chromatography. The origin (ori) and the positions of GDP and GTP are indicated. (B) Time course of Ras activation during monocytic differentiation is shown. HL-60 cells were treated for the indicated times with TPA, and guanine nucleotides that were bound to Ras were analyzed. The relative amount of GTP as a percentage of Ras-associated guanine nucleotides (mean ± SEM; n + 3) is shown.

Gated by herbimycin A. These data suggest that Ras plays roles downstream of tyrosine kinase(s) for the induction of monocytic differentiation.

Recently, the GAP-associated tyrosine phosphoproteins, p62 and p190, were supposed to function as the effectors downstream of signals emitted by Ras. Figure 3A showed that in nontreated HL-60 cells, phosphorytoserine-containing proteins were not detected, and TPA and RA induced a tyrosine phosphorylation of various kinds of proteins. The tyrosine phosphorylation of proteins with molecular weights of 62 and 190 kD were observed in TPA-treated HL-60 cells (Fig 3A). To examine whether these phosphorylated proteins associate with GAP during the monocytic differentiation, the lysates from TPA-treated HL-60 cells were immunoprecipitated with several kinds of monoclonal and polyclonal anti-GAP antibodies, and the precipitates were analyzed by immunoblotting using \(^{125}I\)-labeled antiphosphotyrosine MoAb (PY-20). As shown in Fig 3B, anti-GAP immunoprecipitates from TPA-treated HL-60 cells contained several kinds of tyrosine-phosphorylated proteins (p190, p62, p56, and p52). In contrast, tyrosine-phosphorylated proteins were not present in anti-GAP immunoprecipitate from RA-treated cells (Fig 3B). These tyrosine-phosphorylated molecules in anti-GAP immunoprecipitates were clearly visible at 24 hours after the TPA stimulation and increased thereafter until the completion of monocytic differentiation (Fig 3C). By using monoclonal anti-p62 antibody (data not shown), the tyrosine-phosphorylated 62 kD protein in this study was proven to be GAP-associated protein p62.\(^{15}\) p56 and p52 proteins were neither tubulin nor Shc proteins, which have close molecular weights (data not shown) and have yet to be identified.

The immunoblotting using monoclonal anti-GAP antibody showed that TPA treatment significantly increased the level of type I p116 GAP (Fig 4). In the same cells, expression of the type II p100 GAP molecule was induced. In contrast, RA treatment increased the level of p116 GAP but not of p100 GAP (Fig 4). By using the other two kinds of anti-GAP antibodies, the induction of p100 GAP protein was also clearly shown in the TPA-treated HL-60 cells (data not shown).

Because it has been reported that Vav, which is expressed in hematopoietic cells, is tyrosine-phosphorylated and shows GRF activity toward Ras, we examined tyrosine phosphorylation of Vav in TPA-treated HL-60 cells. As shown in Fig 5, the fourfold to fivefold increase in tyrosine phosphorylation of Vav was observed at 24 hours after TPA stimulation. A dominant inhibitory mutation of Ha-ras that changes Ser-17 to Asn-17 in the gene product p21[H(a-ras)]\(^{15}\) was used to investigate the role of Ras in the monocytic differentiation. The cells transfected with pMMTV ras-H(Asn-17) construct yielded G418-resistant transformants. We screened such transformants to select the clones that were unable to undergo monocytic differentiation by TPA even in the absence of the inducer, dexamethasone, because dexamethasone itself had an inhibitory effect on the differentiation. Among such clones, T-13 and T-65 were chosen for further analyses, which showed the quite low level of adherence and morphologic changes as compared with the
Fig 2. (A) Inhibition of cellular and plastic adhesion and nonspecific esterase activity in TPA-treated HL-60 cells by herbimycin A is shown. HL-60 cells (2 × 10^6 cells/mL) were plated on dishes (35 mm diameter; Falcon Labware, Oxnard, CA). TPA (10 ng/mL) or TPA plus herbimycin A (0.4 mg/mL) were then added. (Lane a) After 2 days, pictures were taken using a microscope (Olympus CK2; Olympus, Tokyo, Japan) (original magnification ×100). (Lane b) After 2 days, cells were incubated in the reaction medium containing alpha-naphtyl butyrate and Fast Garnet GBC and were counterstained with Hematoxylin as described in Materials and Methods (original magnification ×600). (B) Inhibition of Ras activation by herbimycin A is shown. HL-60 cells (5 × 10^5) that had been treated with TPA or TPA plus herbimycin A (0.4 mg/mL) were labeled with 32Pi. The amounts of Ras-GTP in the cells were measured as in Fig 1.

DISCUSSION

In our preceding reports, we have proposed an idea that PTKs play a crucial role in TPA-induced monocytic differentiation of HL-60 cells. In the current study, it was found that both the activation of Ras and the increase in the amount of tyrosine-phosphorylated GAP-associated proteins occurred in a PTK-dependent manner during monocytic differentiation. Ras and GAP were suggested to function downstream of the PTK-signaling pathway during the monocytic differentiation.

The dominant negative mutant Ha-ras gene product inhibited monocytic differentiation (Fig 5), indicating that the activation of Ras was an essential event for the monocytic differentiation of HL-60 cells induced by TPA. The role of Ras in cell differentiation was shown in the neuronal differentiation of PC-12 cells induced by NGF. Our findings in this study again strengthened the importance of Ras in cell differentiation as well as in cell proliferation.

The activation of Ras during TPA-induced monocytic differentiation of HL-60 cells was dependent on tyrosine-kinase...
Fig 3. (A) Tyrosine phosphorylation patterns of TPA- or RA-treated HL-60 cells are shown. The RIPA lysates from TPA- or RA-treated HL-60 cells (5 x 10^6 cells) for 0, 24, and 48 hours were immunoblotted with ^125I-labeled PY-20. The exposure time was 24 hours at -80°C with an intensifier screen. (B) Formation of complex of GAP with tyrosine-phosphorylated proteins is shown in monocytic differentiation, but not in granulocytic differentiation, of HL-60 cells. The RIPA lysates from TPA- or RA-treated HL-60 cells (2.5 x 10^6 cells) for 48 hours were immunoprecipitated with polyclonal anti-GAP antibody or control rabbit IgG and protein G-Sepharose. Anti-GAP-immunoprecipitated proteins were subjected to electrophoresis, transferred to a polyvinylidene difluoride filter, and immunoblotted with ^125I-labeled PY-20. The exposure time was 12 hours at -80°C with an intensifier screen. (C) Time course of GAP complex formation during monocytic differentiation is shown. Cell lysates from 2.5 x 10^6 cells of HL-60 cells that had been incubated with TPA for the indicated times were immunoprecipitated with monoclonal anti-GAP and analyzed by immunoblotting with ^125I-labeled PY-20. The exposure time was 12 hours at -80°C with an intensifier screen.
RA~GAP IN MONOCYTIC DIFFERENTIATION

TPA RA
0 24 48 hr 0 24 48 hr

Anti-GAP Blot

Fig 4. One hundred kilodaltons of GAP protein was induced in monocytic differentiation, but not in granulocytic differentiation, of HL-60 cells. The proteins in the lysates from 5 × 10⁶ of HL-60 cells that had been treated with TPA or RA for 24 and 48 hours were separated on 9% polyacrylamide gel, transferred to a polyvinylidene difluoride filter, and analyzed by immunoblotting with monoclonal anti-GAP antibody and ¹²⁵I-labeled antimouse Ig. The exposure time was 8 hours at −80°C with an intensifier screen.

Phosphorylation (Fig 2), p95Vav is suggested to function as a GRF for Ras, and its activity was directly stimulated by tyrosine kinases. The tyrosine phosphorylation of p95Vav was found to be greatly (fivefold) enhanced after TPA stimulation (Fig 5). RA also induced the tyrosine-phosphorylation of Vav without activation of Ras (Katagiri et al, manuscript submitted). Both TPA and RA induced GAP expression; however, formation of GAP complex, which is enzymatically less active, was observed only in TPA-treated cells. Therefore, the activation of Vav might be cancelled by the increase in the level of GAP activity in RA-treated cells.

HL-60 cells harbor a point mutation in the N-ras gene, whose product may preferentially bind GTP. However, contribution of N-Ras to the total Ras level seems to be rather low, because the basal Ras-GTP level is not as high as is observed in other cells expressing activated forms of Ras.

Downward et al reported that treatment of T cells by TPA results in the activation of Ras within several minutes. We could not detect activation of Ras in such short periods of time after TPA addition. Therefore, the mechanisms that lead to the activation of Ras may be quite different between the two systems.

The several kinds of tyrosine-phosphorylated proteins were shown to associate with GAP during the monocytic differentiation of HL-60 cells. These GAP-associated phosphoproteins contained not only p62 and p190, which were already identified as GAP-associated proteins, but also the unknown p56 and p52.

Type II GAP was reported to be generated by alternative splicing of type I GAP and to lack the hydrophobic amino terminus characteristic to type I GAP. So far, the expression of type II GAP has been observed only in the placenta. In this study, type II GAP in addition to type I GAP was induced in the monocytic differentiation. The expression of type II GAP might be related to the association of p56 and p52 with GAP, because both appear to occur characteristically in TPA-treated HL-60 cells. Therefore, our system provides a good model for the analysis of type II GAP function.

Fig 5. Tyrosine phosphorylation of p95Vav in TPA-treated HL-60 cells is shown. The RIPA lysates from TPA-treated HL-60 cells (2.5 × 10⁶ cells) for 0, 24, and 48 hours were immunoprecipitated with polyclonal anti-Vav and protein G-Sepharose. Anti-Vav-immunoprecipitated proteins were subjected to electrophoresis, transferred to a polyvinylidene difluoride filter, and immunoblotted with ¹²⁵I-labeled PY-20. The exposure time was 12 hours at −80°C with an intensifier screen.
Fig 6. (A) Adhesion of a dominant inhibitory Ha-ras Asn-17 gene-transfected HL-60 cells. Ha-ras Asn-17 transfectants were prepared as described in Materials and Methods. The cells were seeded in 6-well dishes at a 10⁶ cells per well and treated with 10 ng/mL of TPA. Clones T-13 and T-65 showed a diminished cellular and plastic adhesion. In contrast, T-42 and T-54 showed a strong adhesion similar to that of parental HL-60 cells. Two days after TPA stimulation, pictures were taken using a microscope (Olympus CK2). (Original magnification ×50.) (B) Expression of Ha-Ras Asn-17 in HL-60 subclones is shown. Cell lysates from 5 × 10⁶ cells of T-13, T-65, T-42, T-54, and HL-60 cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% polyacrylamide gels, were transferred to a polyvinylidene difluoride filter, and were immunostained with monoclonal anti-Ras (NC-004155 peroxidase-linked antimouse Ig F(ab)² fragments and then treated with Amersham ECL reagents. (C) Decreased GAP complex formation in T-13 and T-65 cells is shown. Cell lysates from 10⁶ cells of T-13, T-65, T-42, T-54, and parental HL-60 cells that had been incubated with TPA for 48 hours were immunoprecipitated with monoclonal anti-GAP and analyzed by immunoblotting with ¹²⁵I-labeled PY-20. The exposure time was 12 hours at −80°C with an intensifier screen.
It was reported that GAP-associated proteins p62 and p190 bound tightly to GAP in vitro. This binding is dependent on the tyrosine-phosphorylation of GAP-associated proteins and occurs through the N-terminal SH2 region of GAP that recognizes phosphotyrosine.30,33,34 The N-terminal SH2 domain of GAP conjugated with agarose beads bound tyrosine-phosphorylated GAP-associated proteins, as above, in the lysate of TPA-treated HL-60 cells (data not shown). Therefore, tyrosine-phosphorylation of GAP-associated proteins seems to be a necessary event for a formation of GAP complex through its SH2 domain in TPA-treated HL-60 cells.

The active form of Ras was assumed to affect the tertiary structure of GAP and enables its SH2 domain to interact efficiently with phosphoproteins.4,8 The dominant inhibitory mutant Ha-ras Asn-17 decreased the amount of tyrosine-phosphorylated GAP-associated proteins in the TPA-stimulated HL-60 cells. This result suggests that active Ras might promote the formation of a complex between GAP and tyrosine-phosphorylated GAP-associated proteins in TPA-treated cells.

GAP was shown to serve as an effector of Ras in the K+ channel linked to a muscarinic receptor in atrial membranes.40 Recently, the N-terminal domain of GAP was reported to show the same effect.49 The N-terminal domain of GAP directly interacts with p190, and exerts effector functions for regulating the cytoskeletal organization and the cell adhesion.49 In TPA-treated HL-60 cells, GAP also serves as an effector of Ras and influences the functions of its downstream target molecules, which might be GAP-associated proteins. Accordingly, the GAP complex might play crucial roles for coupling Ras with the downstream signaling molecules in the monocytic differentiation.

We summarized our results in Fig 7, where the Ras and GAP fit into the model for differentiation-inducing signal triggered by activation of protein kinase Cβ (PKCβ) by TPA. The activation of PKCβ results in the expression of early response genes,50 the products of which might lead to the induction of various kinds of PTKs at several hours after TPA stimulation.5 The PTKs phosphorylate Vav and GAP-associated proteins and the activation of Ras is then induced. GTP-bound active Ras may cause conformational change of GAP to promote GAP complex formation. GAP complex may transduce signals from Ras to the nucleus and affect the organization of the cytoskeleton. Mitogen-activated protein kinase functions at the distal end of a ras-dependent signaling pathway, transducing signals to the nucleus.51-54 These signals altogether might elicit the activation of a cascade of genes that code for the complete expression of macrophage-specific phenotypes.

Our findings strongly suggest that Ras and GAP work in the PTK-mediated differentiation of the cells. Future studies aimed at identifying the GAP-associated proteins p56 and p52 and the tyrosine kinases involved in tyrosine phosphorylation of GAP-associated proteins will aid in elucidating the signaling pathway that activates a genetic program for monocytic differentiation.

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