

G-CSF dependent neutrophil differentiation requires down-regulation of MAPK activities through Gab2 signaling pathway.

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Abbreviations: G-CSF: granulocyte colony-stimulating factor, MAPK: mitogen-activated protein kinase, Erk1/2: extracellular signal-regulated protein kinase 1 and 2, Gab: Grb2-associated binding protein, SHP2: Src homology region 2-containing protein tyrosine phosphatase 2, PI3-K: phosphatidylinositol 3-kinase, MEK: Mitogen-activated protein kinase kinase, Rsk: ribosomal S6 kinase, Akt: protein kinase B, GST: glutathione S-transferase

Abstract

Granulocyte colony-stimulating factor (G-CSF) stimulation of myeloid cells induced tyrosine-phosphorylation of cellular proteins. One of the tyrosine-phosphorylated proteins was found to be a scaffold protein, Grb2-associated binding protein 2 (Gab2). Another member of Gab family protein, Gab3, was exogenously overexpressed in neutrophil progenitor cells to make the Gab3 protein to compete with the endogenous Gab2 for the G-CSF dependent signaling. In Gab3-overexpressed cells, the level of tyrosine phosphorylation of endogenous Gab2 by G-CSF stimulation was markedly down-regulated, while the phosphorylation of Gab3 was significantly enhanced. The Gab3-overexpressed cells continuously proliferated in the medium containing G-CSF and lost the ability to differentiate to the mature neutrophil, characterized by the lobulated nucleus. The G-CSF stimulation-dependent tyrosine phosphorylation of Gab3, the association of SHP2 to Gab3 and the following MAPK activation were prolonged in the Gab3-overexpressed cells, compared to the parental cells, where the binding of SHP2 to Gab2 protein and thereby the activation of MAPK were not sustained after G-CSF stimulation. Inhibition of MAPK by pharmaceutical inhibitor restored the Gab3-overexpressed cells to the ability to differentiate to mature neutrophil. Therefore, G-CSF dependent Gab2 phosphorylation and following its down-regulation led the short-term MAPK activation. The down-regulation of MAPK after transient Gab2 phosphorylation was necessary for the consequent neutrophil differentiation induced by G-CSF stimulation.

1. Introduction

Granulocyte colony-stimulating factor (G-CSF) is a critical regulator of neutrophilic granulocyte production and stimulates the proliferation, survival, maturation, and functional activation of the cells of the granulocytic lineage (Demetri and Griffin, 1991; Murakami and Nagata, 1998). G-CSF binding to its receptor leads to receptor homodimerization (Ishizaka-Ikeda et al., 1993) and the activation of receptor-associated Janus kinase (Jak) family of protein kinases, which in turn leads to the phosphorylation of four tyrosine residues (Tyr703, 728, 743, and 763 of the murine G-CSF receptor) within the cytoplasmic domain of the receptor. (Ward et al., 1999; Yoshikawa et al., 1995). The signaling molecules, which were reported to be activated through the G-CSF receptor, include the Janus kinases, Jak1, Jak2, and Tyk2 (Avalos et al., 1997; Nicholson et al., 1995; Nicholson et al., 1994; Shimoda et al., 1997; Tian et al., 1996), the signal transducer and activator of transcription (STAT) proteins, STAT1, STAT3, and STAT5 (Chakraborty et al., 1996; Nicholson et al., 1996; Tian et al., 1996; Tweardy et al., 1995), the Src kinases Lyn and Hck (Corey et al., 1994; Corey et al., 1998; Ward et al., 1998a), and components of the Ras, Raf, mitogen-activated protein kinase (MAPK), and MAPK-related pathways (Barge et al., 1996; Bashey et al., 1994; de Koning et al., 1996; de Koning et al., 1998; Nicholson et al., 1995; Ward et al., 1998b) as well as the phosphatidylinositol-3 kinase (PI3-K)-Akt pathway (Dong and Lerner, 2000).

When neutrophil precursor cells, GM-I62-1, are stimulated with G-CSF, a number of cellular proteins are tyrosine-phosphorylated, and cells first proliferate, then stop proliferating and eventually differentiate to mature neutrophil characterized by lobulated nuclei (Oka et al., 2006; Omura et al., 2002; Yoshikawa et al., 1995). Grb2 associated binder 2 (Gab2) protein was among the phosphorylated proteins by G-CSF stimulation (Zhu et al., 2004). Gab2 is a scaffold protein, which belongs to the Gab family proteins, including Gab1, Gab2 and Gab3 in mammals. They are involved in various signaling pathways, including the PI3-K-Akt and Ras-MAPK pathways downstream of receptor tyrosine kinases and cytokine receptors (Arnaud et al., 2004; Crouin et al., 2001; Nishida and Hirano, 2003; Nishida et al., 1999; Seiffert et al., 2003). They have conserved motifs, that is, amino-terminal pleckstrin homology (PH) domain, two proline-rich Src-homology (SH)3 docking sites and multiple tyrosine residues, which provide the binding sites for SH2 domain containing proteins when they are phosphorylated (Liu and Rohrschneider, 2002).

Among the Gab family proteins, Gab2 is predominantly expressed in hematopoietic cells and is tyrosine-phosphorylated upon cytokine stimulations. As expected, Gab2 was one

of the major tyrosine phosphorylated proteins in neutrophil precursor cell, GM-I62-1, by G-CSF stimulation as shown previously (Zhu et al., 2004) and also in this report. Therefore, it is likely that Gab2 scaffold protein is involved in the G-CSF-dependent signaling.

In order to elucidate the role of Gab2 in neutrophil differentiation induced by G-CSF, we took advantage of the overall structural similarities as well as their confined differences between Gab2 and Gab3 proteins. We expressed Gab3 in neutrophil precursor cell, GM-I62-1, to make it compete with the endogenous Gab2 for G-CSF-stimulated signaling. In Gab3-overexpressed cells, GM-Gab3, exogenously expressed Gab3 was tyrosine-phosphorylated by G-CSF stimulation, along with the decreased phosphorylation of endogenous Gab2. The Gab3-overexpressed cells responded to G-CSF for continuous proliferation, and nuclear morphological changes, i.e. nuclear lobulation, were not observed. Therefore, G-CSF-dependent neutrophil differentiation was blocked in the Gab3-overexpressed cells. Furthermore, we found that the activation of MAPK downstream of Gab2 was down-regulated due to the negative feedback regulation of Gab2 phosphorylation and that the MAPK activity downstream of Gab3 was kept higher after G-CSF stimulation. The down-regulation of MAPK activity appeared to be necessary for the G-CSF-induced neutrophil differentiation.

2. Materials and Methods

2.1. Growth Factors and cell lines

Mouse recombinant interleukin-3 (IL-3) and G-CSF were used as described previously (Fukunaga et al., 1990; Omura et al., 2002). Mouse myeloid cell line, GM-I62-1 is a clonal cell line of GM-I62M described previously (Yoshikawa et al., 1995) and shows the same properties as GM-I62M. These cells express mouse G-CSF receptor and respond to G-CSF with neutrophil differentiation. Another mouse neutrophil precursor cell line, L-G, have been described previously (Lee et al., 1991). Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco Life Technologies, USA) and 45 units/ml IL-3.

2.2. Plasmid construction

Murine cDNA was synthesized by Superscript III (Invitrogen, USA) with total RNA isolated from GM-I62M cells (Yoshikawa et al., 1995) which were grown in the medium supplemented with IL-3 according to the manufacturer's instructions. The Gab3-Flag expression plasmid was constructed as follows. PCR was carried out using Gab3_Eco-F (CGAATTCAGGATGAGCACTGGTGACACTA) and Gab3-1100-R (CCACATGGTCTAACCCAGA) for Gab3-N-terminal, and Gab3-1050-F (CAAGAAGCCAGGGTATACT) and Gab3-XbaI-R (CGTCTAGACACTTTGGATTGCCTCTCATC) for Gab3-C-terminal, with murine Gab3 cDNA as a template. Both products were isolated by agarose gel electrophoresis. The Gab3-N-terminal PCR product was digested with EcoRI and KpnI, and resultant 1030-bp DNA fragment was inserted into pBluescript II SK (+) (Stratagene, La Jolla, CA, USA) which had been digested with EcoRI and KpnI. The Gab3-C-terminal PCR product was digested with KpnI and XbaI, and resultant 760-bp DNA fragment was inserted into pBluescript II SK (+), which had been digested with KpnI and XbaI. The authenticity of the resulting plasmids (pBS II SK (+) Gab3-N and pBS II SK (+) Gab3-C) were confirmed by DNA sequencing. The EcoRI-KpnI fragment of pBS II SK (+) Gab3-N terminal and the KpnI-XbaI fragment of pBS II SK (+) Gab3-C terminal were isolated and inserted into the pEF-BOS-EX-C-Flag (procedure to construct pEF-BOS-EX-C-Flag will be described elsewhere) which had been

digested with EcoRI and XbaI. The plasmid was designated pBOS-Gab3-Flag and used as an expression plasmid for Flag-tagged Gab3.

N-terminal HA-tag expression vector pEF-BOS-EX-HA was constructed as follows: HA-tag oligonucleotides, HA-for (catggccTACCCCTACGACGTGCCCCGACTACGCCg) and HA-rev (aattcGGCGTAGTCGGGCACGTCTAGGGGTAaggc) which have NcoI site and EcoRI site, were annealed and inserted into pEF-BOS-EX-Flag plasmid (Omura et al., 2002) which has been digested with EcoRI and partially with NcoI to remove DNA fragment for Flag-tag. The obtained plasmid was designated as pEF-BOS-EX-HA.

The HA-tagged Gab2 expression plasmid was constructed as follows. N-terminal and C-terminal Gab2 cDNA fragments were amplified by PCR using primers, either Gab2-N-f (GCGGTACCGACATGAGCGGCGGCGGC) and Gab2-N-r (CTGTGAGGCTGCTCTTGGTG) or Gab2-C-f (GCCAAGCCGACACAATACAG) and Gab2-C-r (CGTCTAGATTACAGCTTGGCACCCCTTGG) with cDNA prepared with total RNA prepared from GM-I62M cells. Amplified N-terminal and C-terminal Gab2 cDNA fragment were digested with KpnI and EcoRI or EcoRI and XbaI, respectively and were inserted into the corresponding restriction sites of pBluescript II SK (+). The resulting plasmids were pBSIISK(+)Gab2-N and pBSIISK(+)Gab2-C, respectively. The authenticity of these plasmids was confirmed by DNA sequencing. The Gab2 N-terminal and C-terminal fragments were again isolated from these plasmids and were inserted together into the KpnI and XbaI sites of pEF-BOS-EX -HA. The resulting plasmid was designated pBOS-HA-Gab2.

Flag-tagged Gab2 expression plasmid, pBOS-Flag-Gab2, was constructed as follows. The SphI-KpnI fragment harboring promoter region and HA-sequence just upstream of Gab2 cDNA in pBOS-HA-Gab2 was replaced by the corresponding fragment from pEF-BOS-EX-Flag (Omura et al., 2002).

To construct the Gab3FF-Flag expression plasmid, where two tyrosine residues Tyr542 and Tyr569, to which SHP2 binds, were replaced with Phe, site-directed mutagenesis was carried out. The primers used for the PCR were Gab3-Y395F-F (GAAGACAGCTATGTGCCCTGA) and Gab3-Y542F-R-1 (TCCAGGGCCAAAAAATCCAAGCTGAATTTC) for one reaction, and Gab3-Y542F-F-1 (TTCAGCTTGGATTTTTTGGCCCTGGACTTC) and BOS-A2 (GGAGACAAGAAATCCCTGTT) for another, with pBOS-Gab3-Flag plasmid DNA as a template, to replace Y542. Both products were isolated by agarose gel electrophoresis, then mixed 1:1 and used as templates for secondary PCR with Gab3-Y395F-F and BOS-A2 as

primers. The resulting PCR product, Gab3-Y542, was isolated by agarose gel electrophoresis. The second mutation was introduced by the following PCR. The primers used for the reactions were Gab3-Y416F-F (AGGATGACTTCATTCCAATGAG) and Gab3-Y569F-R (TCCACTTGAACAAAGTCTACTCTCTGCTCT) for one reaction, and Gab3-Y569F-F (CAGAGAGTAGACTTTGTTCAAGTGGATGAA) and BOS-A2 for another, with the above DNA fragment, Gab3-Y542 as a template. Both products were isolated by agarose gel electrophoresis, then mixed 1:1 and used as templates for secondary PCR with Gab3-Y416F-F and BOS-A2 as primers. The resulting PCR product was digested with PstI and XbaI, and the obtained 2933-bp DNA fragment was inserted into pBluescript II KS (+), which had been digested with PstI and XbaI. The authenticity of the plasmid obtained (pBS-Gab3FF) was confirmed by DNA sequencing. The 430-bp XhoI–XbaI fragment of pBS-Gab3FF was isolated again and ligated with the 1364 bp EcoRI–XhoI fragment and the 5264-bp EcoRI–XbaI fragment of pBOS-Gab3-Flag. The plasmid obtained was designated pBOS-Gab3FF-Flag and used as an expression plasmid for Flag-tagged Gab3FF.

GST-Gab3-C protein expression plasmid was constructed as follows. The pBS II SK (+) Gab3-N was digested with NcoI, and the NcoI end was filled in by Klenow polymerase. Then, the obtained fragment was again digested with KpnI. The blunt-ended NcoI-KpnI Gab3-N fragment and the KpnI-NotI fragment isolated from pBS II SK (+) Gab3-C plasmid were ligated with the 4900 bp SmaI-NotI fragment of pGEX4T-2 (GE Healthcare Life Sciences). The obtained plasmid was designated pGEX-Gab3-C and used as an E. coli expression plasmid for GST-tagged Gab3-C.

GST-Gab2-C protein expression plasmid was constructed as follows. The EcoRI-NotI fragment of pBS II SK (+) Gab2-C terminal were isolated and ligated with the 4900-bp EcoRI-NotI fragment of pGEX 4T-1 (GE Healthcare Life Sciences). The plasmid was designated pGEX-Gab2-C and used as an expression plasmid for GST-tagged Gab2-C.

2.3. Preparation of antibodies against murine Gab2 and Gab3

GST-Gab2-C and GST-Gab3-C fusion proteins were expressed in E. coli and purified using Glutathione-Sepharose CL-4B affinity resin (GE Healthcare Life Sciences). Briefly, E. coli BL21(DE3) harboring either pGEX-Gab2-C or pGEX-Gab3-C was cultured in the M9 medium containing 0.2% glucose, 0.1% tryptone and 0.1 mg/ml ampicillin until the turbidity

at 600 nm reached to 0.6. The expression of the plasmid-harboring genes was induced by adding IPTG at 0.1mM and the cells were further cultured for another 3 hrs at 30 °C.

The *E. coli* cells were harvested, washed with buffer A (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA and 1 mM PMSF) and suspended with the same buffer. After the treatment with lysozyme at a concentration of 0.1mg/ml, DTT and Sarkosyl were supplemented to the solution at 5mM and 0.5%, respectively, and cells were disrupted by sonication and cytosolic proteins were recovered by centrifugation at 12,000 x g for 15min at 4 °C. Tritone X-100 was added to the obtained supernatant at the concentration of 0.5% and GST fusion proteins were absorbed to the Glutathione-Sepharose 4B (GE Healthcare Life Sciences, Marlborough, MA) for 12 hr at 4°C. After the resin was packed into a column and was extensively washed with the buffer A containing 0.5% Triton X-100, the GST fusion proteins were eluted from the column with buffer B (50mM Tris HCl pH8.8, 150mMNaCl, 1mM EDTA and 1mM PMSF) containing 10mM glutathione. The obtained proteins were used to immunize rabbits (female New Zealand White) with either complete or incomplete Freund's adjuvants (BD Difco Laboratories, Detroit, MI). Blood was collected from ear vein and serum was prepared (Harlow and Lane, 1988).

2.4. Transfection

Mouse GM-I62-1 or L-G cells were transfected with pBOS-Gab3-Flag or pBOS-Gab3FF-Flag with pBSpacΔp (de la Luna et al., 1988), which carries the puromycin-resistance gene, as described (Omura et al., 2002).

Clonal puromycin-resistant cells were analyzed for their expression of the Flag-tagged Gab3 or Gab3FF proteins by immunoblot analysis with an anti-Flag M2 IgG (Sigma, St Louis, MO, USA) and anti-Gab3 antiserum prepared as above. Transformants were grown in RPMI 1640 medium containing 10% fetal bovine serum and mouse IL-3 (45 units/mL).

2.5. Assay of long-term cell growth and morphological examination

To determine the long-term growth potential of Gab3- or Gab3FF-expressing transfectants, cells were incubated at an initial density of 2×10^5 cells/mL in medium containing no factor, 150 units/mL mouse G-CSF, or 45 units/mL mouse IL-3. The medium was replenished every 2-4 days to maintain the cell density at $(1-5) \times 10^5$ cells/mL. Viable cells were stained with

trypan blue and counted under the light microscope. To analyze the morphological changes, cells were collected on glass slides by centrifugation (850 x g for 8 min) and stained with Wright–Giemsa solutions (Merck KGaA, Darmstadt, Germany).

2.6. Assay for the effects of MAPK inhibition on the neutrophil differentiation

MEK inhibitor U0126 (EMD Millipore Corporation, MA USA) was dissolved in DMSO at 20mM. The reagent was diluted with DMSO to the concentration 200 times more than that used in each assay. To examine the effects on the phosphorylation of other signaling proteins such as MAPK upon G-CSF stimulation, U0126 was added in the medium 30 minutes before the cytokine stimulation. To determine the effects on the growth and morphological changes, the inhibitor was added to the medium 24hr after the cells were cultured in the indicated medium and the inhibitor was supplemented in every replenished media thereafter.

2.6. Cytokine stimulation and preparation of cell lysate

Cells were grown in the presence of IL-3 to a density of up to 1×10^6 cells/mL, washed three times with factor-free medium containing 5% fetal bovine serum, and starved in the factor-free medium with 10% fetal bovine serum at 1.5×10^6 cells/mL for 4-6 hours in 5%CO₂ incubator at 37 °C. After being stimulated with 150 U/mL G-CSF for the period indicated for each experiment, cells were immediately chilled on ice/water, washed twice with ice-cold PBS containing 0.2mM Na₃VO₄, and lysed with lysis buffer [50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na₃VO₄, 10 mM sodium pyrophosphate, 0.5% CHAPS, and protease inhibitors (1 mM phenylmethanesulfonyl fluoride (PMSF), 1 µg/mL each leupeptin and pepstatin A; Sigma, St. Louis, MO)] for 15 min on ice at a cell density of 1×10^8 cells/mL. Insoluble materials were removed by centrifugation at 17,400×g for 15 min at 4 °C.

2.7. Immunoprecipitation

Immunoprecipitation of each target protein was performed as below. Cell lysates prepared from $3-5 \times 10^6$ cells which had been stimulated with G-CSF as described above were diluted 20-30 times with the lysis buffer containing 1% TritonX-100 instead of 0.5% CHAPS,

followed by being pre-cleared by centrifugation at 17,400×g for 10 min at 4 °C. Subsequently, antiserum against Gab2, or Gab3 was added to the pre-cleared cell lysates followed by incubation for 1 hr at 4°C. The immune complexes were captured by adding 5 mg of Protein-A Sepharose CL-4B (GE Healthcare Bio-Sciences AB, Sweden) and further incubated for 12 hr at 4°C. The immune complexes with Protein A-Sepharose were centrifuged at 5,000 x g for 30 sec at 4°C, followed by washing 4 times with the lysis buffer containing 1% TritonX-100 and once with lysis buffer without any detergent. Proteins were solubilized with SDS sample buffer and subjected to SDS-PAGE and immunoblotting, as described previously (Oka et al., 2006).

2.8. Immunoblotting

Cellular proteins were subjected to SDS/PAGE and blotted on to GVHP membranes (Millipore Corp., Bedford, MA, USA) as described previously (Omura et al., 2002). The membranes were treated as described previously (Oka et al., 2006). Primary antibodies used are as follows: anti-phosphotyrosine IgG (4G10) (Millipore (Cat. # 05-321)), anti-Flag M2 IgG (Sigma, St. Louis, MO), β -actin (#4970, Cell Signaling Technology), SHP2 (SH-PTP2 (N-16): sc-424, Santa Cruz Biotechnology, Inc.), p-MAPK (#9101, Cell Signaling Technology), MAPK (Millipore (Cat. # 06-128)). Anti-sera against Gab2 and Gab3 were prepared as above. Secondary antibodies and reagent conjugated with HRP were rabbit anti-mouse IgG peroxidase (HRP)-conjugated antibodies (#P0260, DakoCytomation Denmark A/S, Denmark), polyclonal goat anti-rabbit immunoglobulins/HRP (#P0448 DakoCytomation Denmark A/S, Denmark), and Protein A-peroxidase-linked (NA9120, GE Healthcare, UK). Reacted proteins were visualized by enhanced chemiluminescence (Haan and Behrmann, 2007) and recorded with Fujifilm Lumino-Image analyzer LAS-4000mini (FUJIFILM, Tokyo).

2.9. Statistical analysis

Values are presented as the mean $\pm$ standard deviation or $\pm$ standard error where indicated. Statistical significance was assessed using a two-sided Student t-test.

3. Results

3.1. Scaffold protein Gab2 is one of the major phosphorylated proteins in GM-I62-1 by G-CSF stimulation

GM-I62-1, one of the clonal cell lines of GM-I62M (Yoshikawa et al., 1995), which was established by introducing G-CSF receptor cDNA into neutrophil/monocyte progenitor cell L-GM (Lee et al., 1991). GM-I62-1 showed the properties of neutrophil progenitor cell, that is, it responds to G-CSF with differentiating to the mature neutrophil, characterized by the growth suppression and nuclear lobulation as GM-I62M does (Dong et al., 1993; Yoshikawa et al., 1995). When GM-I62-1 cells were stimulated by G-CSF, a number of cellular proteins were tyrosine-phosphorylated (Yoshikawa et al., 1995) (Figure 1A), among which one of the strongly phosphorylated proteins with relative molecular weight of 75kDa was identified as Gab2, as it was immunoprecipitated with anti-Gab2 antibodies (Figure 1A, 1B and 1D). Therefore, Gab2 protein appeared to be one of the major tyrosine-phosphorylated proteins in GM-I62-1 cells by G-CSF stimulation.

3.2. Overexpression of Gab3 protein in GM-I62-1 cells suppressed phosphorylation of Gab2 and inhibited the neutrophil differentiation induced by G-CSF

Among the Gab family proteins, Gab1, Gab2 and Gab3, GM-I62-1 cell has mainly Gab2, a much lower amount of Gab3 (Figure 1A, 1B and 1C) and an undetectable amount of Gab1 examined by immunoblotting (data not shown). As Gab2 is phosphorylated heavily by G-CSF stimulation (Figure 1A and 1D), it likely has some roles in the G-CSF dependent signaling pathways. In order to study the functional differences of Gab family proteins and to shed light on the essential properties of Gab2 in G-CSF induced neutrophil differentiation processes, Gab3 protein, another member of Gab family protein, was over-expressed in the neutrophil precursor cell line GM-I62-1 to compete with endogenous Gab2 protein for the G-CSF induced signal transduction reactions and the effects of Gab3 over-expression on the G-CSF dependent neutrophil differentiation were analyzed. Gab3 expression plasmid pBOS-Gab3-Flag was constructed with carboxyl-terminal Flag-tag as shown in Figure 2A. The plasmid was stably transfected to GM-I62-1 cell and Gab3-expressing clones were isolated. Relative amounts of the exogenously expressed Gab3 protein as well as the endogenous Gab2

protein in one of the clone GM-Gab3-Flag (clone1-A-4) were analyzed by immunoblotting (Figure 2B). The amounts of Gab3-Flag protein in GM-Gab3 cells were about 12 times more than endogenous Gab3 and were comparable with the endogenous Gab2, estimated by the immunoblot analysis with increasing amounts of Flag-Gab2 protein expressed in Cos-7 cells as a control (data not shown).

The exogenously expressed Gab3 seems to compete with the endogenous Gab2 for the G-CSF stimulated signaling in Gab3-overexpressing GM-Gab3 cells, as the tyrosine phosphorylation of Gab2 was largely suppressed along with the elevated phosphorylation of Gab3 by G-CSF stimulation (Figure 1A, 1C and 1D). Next, the effects of Gab3 overexpression on the G-CSF-induced neutrophil differentiation were examined in GM-Gab3 cells. When the neutrophil progenitor cell, GM-I62-1, is cultured in the presence of G-CSF, the cell proliferates for a few days, followed by the growth arrest which leads to the mature neutrophil with lobulated nucleus (Figure 3A and 3B) (Omura et al., 2002; Yoshikawa et al., 1995). In contrast to GM-I62-1, Gab3-overexpressing GM-Gab3 cells kept proliferating in the G-CSF containing medium as the cells cultured in the medium containing IL-3 (Figure 3A) and the neutrophil differentiation characterized by the nuclear morphological changes was largely blocked, as shown in Figure 3B and 3C. The inhibition of neutrophil differentiation in the medium with G-CSF by Gab3 overexpression was also observed in a couple of other clones stably transfected with Gab3 expression plasmid (data not shown). These observations suggested that overexpressed Gab3 in the neutrophil progenitor cell, GM-I62-1, competed the endogenous Gab2 for the G-CSF stimulated differentiation signals, thereby inhibited the neutrophil differentiation.

These inhibitory effects against neutrophil differentiation were not due to the overexpression of any Gab family protein but to the overexpression of Gab3 isoform, as the overexpression of Gab2 in GM-I62-1 at the equivalent level of the exogenously expressed Gab3 in GM-Gab3 cells had no detectable effect on the G-CSF-stimulated neutrophil differentiation (data not shown).

3.3. Overexpression of a mutant form of Gab3 which lacks the SHP2-binding tyrosine residues had no inhibitory effect on the G-CSF-induced neutrophil differentiation.

When cells are stimulated by growth factors or cytokines, the members of Gab family proteins are phosphorylated on their tyrosine residues. Among these residues, three tyrosine

(Tyr395, Tyr416, Tyr515 in mouse Gab3) residues are suggested to be the binding sites for p85 subunit of PI3-kinase (PI3-K) upon phosphorylation, resulting in the activation of PI3-K-Akt signal transduction pathway. Other two tyrosine residues (Tyr542, Tyr569) located near the carboxyl terminus of the proteins are proposed to be the binding sites for SHP2, leading to the activation of the Ras-MAPK signaling pathway (Liu and Rohrschneider, 2002). In order to determine the signaling pathways involved in the inhibitory effects on the G-CSF stimulated neutrophil differentiation in GM-I62-1 cells by the Gab3 overexpression, the expression plasmids for Gab3 were constructed, of which either the tyrosine residues for the binding of p85 subunit of PI3-K or the residues for the binding of SHP2 were replaced by phenylalanines (Gab3-F3 and Gab3FF, respectively), and the plasmids were transfected to the neutrophil progenitor cell, GM-I62-1. The clones of the cells overexpressing these mutant Gab3 proteins were isolated and the overexpression was confirmed by immunoblot analysis (Figure 2B for GM-Gab3FF, no data was shown for Gab3-F3). The effects of the overexpression of these mutant Gab3 proteins on the G-CSF stimulated neutrophil differentiation were again examined.

GM-Gab3-F3 cells overexpressing Gab3-F3, in which the three tyrosine residues for the binding of PI3-K were mutated to phenylalanines, continuously proliferated in the media containing G-CSF, that is, G-CSF-dependent growth suppression was still inhibited in GM-Gab3-F3 cells (data not shown), although the proliferation rate decreased compared to that of GM-Gab3. Furthermore, G-CSF-induced nuclear morphological changes, were largely suppressed in GM-Gab3-F3 cells as in GM-Gab3 cells, compared to GM-I62-1 cells (data not shown), although nuclei of small number of GM-Gab3-F3 cells became lobulated in some degree. These nuclear lobulation observed in some GM-Gab3-F3 cells might be due to the detectable level of activation of PI3-K-Akt signaling pathway in GM-Gab3-F3 cells stimulated by G-CSF. Therefore, the overexpression of Gab3-F3 appeared to still have the inhibitory effects on the G-CSF dependent neutrophil differentiation as the Gab3-overexpression did.

In contrast to GM-Gab3-F3, GM-Gab3FF cells overexpressing Gab3FF-Flag protein, in which two tyrosine residues (Tyr542, Tyr569) for the SHP2 binding were mutated, differentiated to mature neutrophils in the medium containing G-CSF, that is, the proliferation of the GM-Gab3FF cells was suppressed (Figure 3A) and the nuclear morphology turned to be lobulated as GM-I62-1 cells which were cultured in the presence of G-CSF (Figure 3B and 3C). Therefore, the inhibition of neutrophil differentiation caused by

the overexpression of Gab3 appeared to be due to the SHP2-Ras-MAPK signaling through the phosphorylated tyrosine residues of SHP2 binding sites (Tyr542 and Tyr569) of the Gab3 protein.

3.4. Overexpression of Gab3 caused the sustained MAPK activation by G-CSF stimulation.

It has been reported that the binding of SHP2 to the phosphorylated tyrosine residues of Gab2 was negatively regulated by the phosphorylation of the particular Ser/Thr residues of Gab2, which was caused by the activation of Rsk or other kinases downstream of Ras-MAPK signaling pathway (Zhang et al., 2013). Therefore, the activation of MAPK resulting from the binding of SHP2 to the phosphorylated Gab2 is expected to be transient in cells harboring predominantly Gab2. However, there is no corresponding Rsk phosphorylation site in Gab3 protein, suggesting that, unlike Gab2, Gab3 might not have the negative feedback regulation of tyrosine phosphorylation by Rsk downstream of Ras-MAPK signaling pathway, which would end up with the prolonged MAPK activation. In order to confirm the lack of negative feedback regulation of Ras-MAPK signaling in the Gab3-overexpressed cells, GM-Gab3, the G-CSF-dependent MAPK activation and the SHP2 binding to Gab2 or Gab3 proteins after G-CSF stimulation were examined by immunoblot analysis of cell lysates or immunoprecipitates with either anti-Gab2 or anti-Gab3 antibodies (Figure 4A). In GM-I62-1 cells (Figure 4A lanes 1-5), where Gab2 is the major isoform among Gab family proteins, SHP2 bound to Gab2 as early as 5 minutes after G-CSF stimulation, followed by its dissociation from Gab2 protein 20 minutes after the stimulation. On the other hand, in GM-Gab3 cells (Figure 4A lanes 6-10), where Gab3 was overexpressed, SHP2 bound to Gab3, within five minutes after G-CSF stimulation. However, the SHP2 kept binding to Gab3 more than 35 minutes after the stimulation. Once SHP2 binds to the Gab proteins, Ras-MAPK signaling is activated. The activation, that is, phosphorylation of the activation loop of MAPK (Erk1/2), shown as p-MAPK in Figure 4A, was observed as early as 5 minutes after G-CSF stimulation. As in the case of the SHP2 binding to Gab2, the MAPK activation was down-regulated 20 minutes after G-CSF stimulation in GM-I62-1 cells. By contrast, the MAPK activation was prolonged in GM-Gab3 cells. The level of tyrosine-phosphorylated MAPK 35 min after G-CSF stimulation was higher in GM-Gab3 than in GM-I62-1 as shown in Figure 4B. The prolonged MAPK activation in GM-Gab3 cells likely caused the GM-Gab3

cells to continuously proliferate and suppressed the neutrophil differentiation in the medium containing G-CSF (Figure 3).

On the other hand, no binding of SHP2 to Gab3 upon G-CSF stimulation was observed in GM-Gab3FF cells, where two tyrosine residues for binding of SHP2 of exogenously expressed Gab3 protein (Tyr542, Tyr569) were replaced by phenylalanine (Figure 4A, lanes 11-15). Furthermore, very little amount of SHP2 binding to Gab2 was detected in GM-Gab3FF cells, because G-CSF dependent tyrosine-phosphorylation of Gab2 was suppressed in GM-Gab3FF cells (Figure 4A, lanes 11-15) as in GM-Gab3 cells (Figure 1D) because of the competition for G-CSF-dependent signaling between endogenous Gab2 and exogenously overexpressed Gab3FF. Even in the GM-Gab3FF cells, some degree of time-dependent MAPK activation, i.e. MAPK phosphorylation, was observed, which was suppressed 35 minutes after G-CSF stimulation (Figure 4A) as in GM-I62-1 cells. This MAPK activation was probably due to the residual phosphorylation of endogenous Gab2 protein, to which SHP2 bound, resulting in the lower degree of activation of Ras-MAPK signaling pathway. Therefore, as supposed from the lower and transient MAPK activation (MAPK phosphorylation) in GM-Gab3FF (Figure 4B), overexpression of Gab3FF had negligible inhibitory effects on the G-CSF dependent neutrophil differentiation characterized by the growth suppression and the nuclear lobulation in GM-Gab3FF cells (Figure 3).

3.5. Pharmaceutical inhibitor against MEK can rescue GM-Gab3 cells from the Gab3-overexpression-causing inhibition of G-CSF dependent neutrophil differentiation.

In order to confirm the assumption that the sustained activation of MAPK caused by the Gab3 overexpression inhibited G-CSF dependent neutrophil differentiation, effects of pharmaceutical inhibitor against MEK, U0126, which inhibits MEK resulting in the suppression of MAPK (Erk1/2) phosphorylation was examined on the G-CSF induced neutrophil differentiation in GM-Gab3 cells. In the presence of 10 μ M U0126 in the medium, the phosphorylation of MAPK (Erk1/2) was approximately 50% suppressed, while MAPK was inhibited more than 95% in the presence of 100 μ M U0126, examined by the immunoblot with anti p-MAPK antibodies (data not shown). Then, effects of the MEK inhibitor on the G-CSF dependent neutrophil differentiation was examined. In the presence of 10 μ M U0126, GM-Gab3 cells ceased to proliferate 6 to 8 days after the cells were cultured in the medium containing G-CSF (Figure 5A), like GM-I62-1 cells shown in Figure 3A. The

nuclear morphological changes, that is, nuclear lobulation characteristic to the differentiated neutrophils, were also observed in GM-Gab3 cells cultured in the medium containing G-CSF in the presence of the MEK inhibitor U0126 (Figure 5B and 5C). These observations again demonstrated that the sustained MAPK activation due to the overexpression of Gab3 inhibited the G-CSF induced neutrophil differentiation characterized by the G-CSF dependent proliferation-arrest as well as the induction of nuclear lobulation. Furthermore, the partial inhibition of MAPK by 10 μ M of the MEK inhibitor, U0126, restored the G-CSF dependent neutrophil differentiation, at least partially, in GM-Gab3 cells.

3.6. Gab3 overexpression in another neutrophil progenitor cell L-G also suppressed the G-CSF dependent neutrophil differentiation.

Gab3-Flag and Gab3FF-Flag proteins were also exogenously expressed in another neutrophil progenitor cell, L-G. The amount of Gab3 proteins in LG-Gab3 and LG-Gab3FF were equivalent and about 2 times more than those in GM-Gab3 and GM-Gab3FF cells, respectively (Figure 6A).

Cells were cultured either in the absence or in the presence of IL-3 or G-CSF. L-G cells underwent neutrophil differentiation in the medium containing G-CSF. LG-Gab3 cells, where Gab3 protein was overexpressed, continuously proliferated in the medium with G-CSF as in the medium with IL-3, showing that the overexpression of Gab3 inhibited G-CSF dependent neutrophil differentiation also in L-G cells. On the other hand, LG-Gab3FF cells still show the growth suppression in the medium containing G-CSF (Figure 6B) even though equivalent amount of Gab3 or Gab3FF proteins were exogenously expressed in LG-Gab3 and LG-Gab3FF cells (Figure 6A).

L-G cells underwent differentiation with lobulated nucleus after 10 days culture in G-CSF containing medium as GM-I62-1 cells did (Figure 6C and 6D). Overexpression of Gab3 in L-G cells (LG-Gab3) suppressed the morphological changes of nucleus even after more than 10 days culture in the presence of G-CSF. In contrast, LG-Gab3FF cells showed the nuclear lobulation after 10 days culture in the medium containing G-CSF (Figure 6C and 6D), that is, overexpression of Gab3FF had negligible inhibitory effects on the morphological changes typical to the neutrophil differentiation induced in the medium containing G-CSF. LG-Gab3FF cells went through apoptotic cell death, starting on day 8 after culturing in the

G-CSF-containing medium and very few cells survived on day 13, which made the examination of cell morphology very difficult.

These observations again suggested that the sustained activation of Gab3-SHP2-Ras-MEK-MAPK signaling in the Gab3-overexpressed cells inhibited G-CSF-induced neutrophil differentiation. In other words, transient activation and following down-regulation of MAPK activities through Gab2, rather than Gab3 is necessary for normal neutrophil differentiation induced by G-CSF stimulation.

4. Discussion

In GM-I62-1 cells, G-CSF stimulation activates Jak family of kinases and in turn they tyrosine-phosphorylate other cellular proteins, resulting in the complex formation of signaling proteins, including G-CSF-receptor-Shc-Grb2-Gab2-SHP2, which activates Ras-MAPK signaling. When Gab3 was overexpressed, Gab3 appeared to compete endogenous Gab2 protein for the binding to Grb2 adaptor protein and Grb2-Gab3-SHP2 complex was formed, instead of Grb2-Gab2-SHP2 complex.

We have shown in this report that the overexpression of Gab3 in the neutrophil precursor cell, GM-I62-1, kept the cells to continuously proliferate in the G-CSF-containing medium and suppressed the G-CSF-dependent neutrophil differentiation, characterized by the nuclear lobulation. The inhibitory effects of the Gab3-overexpression on the G-CSF dependent neutrophil differentiation is apparently due to the sustained tyrosine phosphorylation of Gab3 and its binding to SHP2, followed by the prolonged MAPK activation by G-CSF stimulation.

The down-regulations of tyrosine phosphorylation of Gab2 following stimulation of growth factors or cytokines have been reported previously (Lynch and Daly, 2002; Zhang et al., 2013; Zhang et al., 2017). In human adenocarcinoma cell line MCF-7, heregulin-activated ErbB receptor induced tyrosine phosphorylation of Gab2 leading to the activation of Ras-MAPK signaling as well as the PI-3K-Akt signaling. The activated Akt phosphorylated Ser159 of Gab2, resulting in the dephosphorylation of the tyrosine residues of Gab2. This negative feedback regulation of the signalling pathway in the epithelial cells restrains the further mitogenic responses of cells by heregulin stimulation (Lynch and Daly, 2002). In another report (Zhang et al., 2013), using human embryonic kidney cell line, HEK293, EGF stimulated tyrosine phosphorylation of Gab2 led the activation of Ras-MAPK signalling, resulting in the phosphorylation and activation of Rsk (p90 ribosomal S6 kinase) downstream of MAPK. The activated Rsk, in turn, phosphorylates three Ser residues of Gab2 (Ser 160, Ser211 and Ser620 in human Gab2) and downregulated the recruitment of SHP2 to Gab2 resulting in the termination of MAPK activation. This feedback effects were reported to be specific to the SHP2 recruitment to Gab2, that is, binding of p85 subunit of PI3-K to Gab2 was not affected by the Ser phosphorylation caused by Rsk (Zhang et al., 2013). Furthermore, same group of researchers reported that, using HEK293 and MCF-10A cell lines, EGF-stimulated activation of Ras-MAPK signaling caused the phosphorylation of four Ser

residues, Ser469, Ser591, Ser612, and Ser614 of mouse Gab2, (Ser612 of mouse Gab2 corresponds to Ser623 in human Gab2), which subsequently attenuated the phosphorylation and activation of Akt (PKB) downstream of PI3-K (Zhang et al., 2017). Differences of the down-regulatory mechanisms of Gab2 phosphorylation might be due to the differences of the cells and/or the growth factors to stimulate the cells. These reports demonstrated that the phosphorylation of certain Ser or Thr residues of Gab2 caused to downregulate either Ras-MAPK or PI3-K-Akt signaling, or both signaling pathways. However, the negative feedback mechanisms of tyrosine phosphorylation of Gab2 protein and the participating phosphatases have not yet been reported.

In the neutrophil precursor cell, GM-I62-1, where Gab2 protein is mainly expressed among the three members of Gab family proteins, G-CSF dependent tyrosine phosphorylation of Gab2 was transient and was down-regulated at 35 min after G-CSF stimulation (Figure 4A). This down-regulation was probably due to the same negative feedback mechanisms reported as described above. The downregulation of Gab2 tyrosine phosphorylation caused the suppression of SHP2-binding to Gab2 as well as of MAPK activation in GM-I62-1 cells (Figure 4). The resulting low MAPK activity allows GM-I62-1 cell to differentiate to mature neutrophil in the medium containing G-CSF.

Alignment of amino acid sequences of Gab2 and another member of the Gab family protein, Gab3, revealed that the most Ser or Thr residues in Gab2 which reported to be phosphorylated by either Akt, Rsk or MAPK as described above (Lynch and Daly, 2002; Zhang et al., 2013; Zhang et al., 2017) are not conserved in Gab3, except Ser612 which reported to be phosphorylated by MAPK (Zhang et al., 2017). Lack of these Ser/Thr phosphorylation sites in Gab3 suggested that the signals transduced through Gab3 protein would not undergo negative feedback regulation. Indeed, tyrosine phosphorylation of Gab3 by G-CSF stimulation was stronger and prolonged for longer periods of time in GM-Gab3, where Gab3 was over-expressed (Figure 4A). The resulting prolonged higher MAPK activation appeared to support the sustained proliferation and to inhibit neutrophil differentiation characterized by nuclear lobulation in GM-Gab3 cells. The sustained proliferation and inhibition of neutrophil differentiation in the medium containing G-CSF was not observed in Gab2-overexpressed cells (data not shown). Therefore, the suppression of neutrophil differentiation observed in this report was exclusively due to Gab3 and was not observed in cells where Gab2 was overexpressed in the equivalent amount.

It has been reported the Gab2^{-/-} knockout mouse is generally healthy, lacks allergic response (Gu et al., 2001) and defects in the mast cell development (Nishida et al., 2002). There is no difference observed in red blood cell, lymphocyte, neutrophil and monocyte counts in the steady state peripheral blood between wild-type and Gab2^{-/-} mice (Zhang et al., 2007). However, Gab2-deficient hematopoietic cells are deficient in cytokine responsiveness, that is, they showed the reduction of total CFU-C as well as CFUs of individual cell types including CFU-G in methylcellulose progenitor assay in the presence of several cytokines (Zhang et al., 2007). Therefore, Gab2 is necessary for the efficient hematopoietic cell responses to these cytokines. Nonetheless, it has not been clear which functional properties of Gab2 are essential and cannot be replaced by other member of Gab family proteins, even though they share the structural similarities. In this report, we found that the tyrosine phosphorylation of Gab2 is kept low by its negative feedback system and thereby activation of MAPK downstream of Gab2 is kept low, which is necessary for the G-CSF-induced neutrophil differentiation.

Enhancement of proliferation activity caused by overexpression of Gab3 protein was reported in some tumor cells (Jia et al., 2017; Xiang et al., 2017). Overexpression of Gab2 has also been reported in ovarian cancer and hepatocellular carcinoma (Cheng et al., 2017; Duckworth et al., 2016). However, in the neutrophil precursor cell, GM-I62-1, Gab2 overexpression did not affect the G-CSF dependent neutrophil differentiation, probably due to the difference between myeloid cells and epithelial cells.

It has been reported that sustained and enhanced activation of MAPK (Erk1/2) inhibits granulocyte differentiation and augments the commitment to monocyte in myeloid precursor cells (Carras et al., 2016; Hu et al., 2015; Jack et al., 2009). In this report, overexpression of Gab3 in GM-I62-1 cells resulted in the sustained activation of MAPK in the medium containing G-CSF. Therefore, in Gab3-overexpressing GM-Gab3 cells, G-CSF stimulation might confer the cells to monocytic development and inhibit neutrophilic differentiation with continuous proliferation and suppressed nuclear lobulation. Whether the GM-Gab3 cells cultured in the G-CSF-containing medium acquire monocytic properties remains to be elucidated.

Previously, S. J. Corey and his colleagues reported that the Src family kinase Lyn forms a complex with Gab2 and that G-CSF stimulation induced Gab2 phosphorylation and its association with a phosphatase, SHP2, which in turn activated Lyn (Futami et al., 2011). They suggested that the activation of Lyn through the interaction of Gab2 may be involved in

the G-CSF induced Ba/F3 cell proliferation and/or differentiation. In order to examine the activation of Lyn, first, we analyzed the association of Lyn with Gab2, Gab3 or Gab3FF in GM-I62-1, GM-Gab3 and GM-Gab3FF cells by immunoprecipitation with anti-Lyn antibodies followed by the immunoblotting with either anti-Gab2 or anti-Gab3 antibodies. The binding of Lyn to Gab2 was observed in GM-I62-1 cells. However, the association of Lyn with Gab3 or Gab3FF was hardly detected in GM-Gab3 or GM-Gab3FF, even though the amounts of the overexpressed Gab3 and Gab3FF were more than the endogenous Gab2 in these cells. Furthermore, the activation of Lyn, that is, the phosphorylation of the tyrosine residue (Tyr397) of the activation-loop of Lyn kinase in GM-Gab3 and GM-Gab3FF was significantly low compared to that of GM-I62-1 cell upon G-CSF stimulation. These observations suggested that the properties of proliferation and differentiation observed in GM-I62-1, GM-Gab3 and GM-Gab3FF cells in G-CSF containing media were not related to the Lyn kinase activities.

Same group of researchers reported that G-CSF induced Gab2 phosphorylation is Lyn kinase dependent using pharmacological Src kinase inhibitors (Zhu et al., 2004). We examined the G-CSF dependent Gab2 tyrosine phosphorylation in the presence of 10 μ M PP1 or 3 μ M PP2. Both inhibitors inhibited the phosphorylation of the tyrosine residue in the activation-loop of Lyn. However, no inhibition of G-CSF induced Gab2 tyrosine phosphorylation was observed in the presence of these inhibitors in GM-I62-1 cells. Therefore, Lyn kinase activity did not appear to be involved in the G-CSF dependent phosphorylation of Gab family proteins and the induction of the neutrophil differentiation in GM-I62-1 cells.

We reported here that the activation of initial Ras-MAPK signaling, which should be followed by its downregulation through the dephosphorylation of Gab2 and dissociation of SHP2 from it, is necessary for the G-CSF dependent neutrophil differentiation. Furthermore, the sustained activation of MAPK through the overexpressed Gab3 rather than the endogenous Gab2 inhibited the G-CSF-dependent neutrophil differentiation.

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7. Footnote

The authors declare that they have no conflicts of interest with the contents of this article.

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9. Figure Legends

Figure 1. Tyrosine phosphorylation of Gab2 and Gab3 in GM-I62-1 and GM-Gab3 cells by G-CSF stimulation. (A) Cells were either unstimulated (lanes 1, 3) or stimulated (lanes 2, 4) with G-CSF and proteins were separated with SDS-PAGE, followed by immunoblot with antibodies against phosphotyrosine (4G10). (B), (C) Antibodies were stripped off from the filter and re-probed with antibodies against Gab2 or Gab3 protein. Migration positions of Gab2 and Gab3 proteins in (A) are indicated with arrows on the right. GM-I62-1 (lanes 1, 2), GM-Gab3 (lanes 3, 4). Migration positions of molecular weight marker proteins were indicated on the left in (A). (D) Gab2 and Gab3 proteins were immunoprecipitated with anti-Gab2 or anti-Gab3 antibodies from cell lysates in (A) and were subjected to the immunoblot with antibodies against phosphotyrosine (4G10). Antibodies were stripped off and each filter was reprobed with antibodies against either Gab2 or Gab3. GM-I62-1 (lanes 1, 2), GM-Gab3 (lanes 3, 4).

Figure 2. Schematic structures of Gab proteins and their expressions in Gab3-overexpressed cell lines. (A) Structures of Gab2, Gab3-Flag and its mutant, Gab3FF-Flag, which lacks SHP2 binding sites (Y542F, Y569F). Positions of phosphatidylinositol-3 kinase (PI3-K) and SHP2 binding sites are also shown on the top. Flag-tag is attached to the C-terminus of Gab3 proteins. PH-domains and proline-rich regions which were proposed to be the SH3-binding sites are shown by underscores. Amino acid number is shown under each protein. (B) Expression of Gab2, Gab3-Flag and Gab3FF-Flag in GM-I62-1 and its Gab3-Flag-transfected cell lines, GM-Gab3 and GM-Gab3FF. Equivalent amounts of proteins in cell lysates were separated by SDS-PAGE, followed by sequential immunoblotting with antibodies against Flag-tag, Gab3, Gab2 or beta-actin. Migrate positions of endogenous Gab2 and Gab3 proteins are indicated by arrows on the left. Asterisk indicates other cellular protein than Gab proteins which reacted with the antibodies. Immunoblot with anti beta-actin antibody indicated the equal loading of cell lysate proteins. Lane 1: GM-I62-1, lane 2: GM-Gab3, lane 3: GM-Gab3FF.

Figure 3. Growth properties and morphological changes of GM-I62-1 and its transfectant cell lines overexpressing Gab3 or Gab3FF. (A) Cells, which had been maintained in the medium containing IL-3, were washed to remove IL-3 and were cultured in the media

either without (factor free (open triangle) or with IL-3 (open circle) or G-CSF (closed circle). Cell numbers were counted after trypan blue staining. Standard errors are shown with error bars which were calculated from more than 4 experiments. (B) Cells were cultured in the medium containing IL-3 or G-CSF for 10 or 13 days and were stained with Wright-Giemsa solution and cellular morphologies were observed under microscope. One set of results among three experiments are shown. Bar shows 20 micro meters. (C) Quantitative analysis of the morphological changes shown in (B). More than 100 cells in each preparation from more than 3 independent experiments were inspected under a microscope and classified into 4 categories (a-d) indicated at the bottom.

Figure 4. G-CSF-stimulation dependent binding of SHP2 to the Gab proteins and the subsequent phosphorylation of MAPK in GM-I62-1, GM-Gab3 and GM-Gab3FF. (A) Cells were starved for cytokine for 5 hours, then either unstimulated or stimulated with G-CSF for the indicated time shown on the top. Gab2 and Gab3 proteins were immunoprecipitated with the corresponding antibodies from the cell lysates and the precipitated proteins were separated by SDS-PAGE and analyzed by immunoblot with anti-SHP2 antibodies for the binding of SHP2 to Gab proteins or with anti-phosphotyrosine antibody (4G10) for the tyrosine phosphorylation of Gab proteins. The amounts of precipitated Gab proteins were analyzed with anti-Gab2 and anti-Gab3 antibodies shown on the bottom of the figure. G-CSF dependent phosphorylation of MAPK (Erk1/2) and MAPK protein contents in the cell lysates were also examined by immunoblots with anti-phospho MAPK and anti-MAPK antibodies. Equivalent amount of SHP2 in each cell lysate was confirmed with the immunoblot with anti-SHP2 antibodies. (B) Quantitative analysis of phosphorylation of MAPK by G-CSF stimulation in (A). The intensities of phospho-MAPK bands (p-Erk1 and p-Erk2) in the immunoblots were normalized by the intensities of the MAPK bands (Erk1 and Erk2). Relative phosphorylation was calculated compared to the one in the GM-I62-1 cells stimulated by G-CSF for 5 min and shown in the graph. Standard deviations were calculated with three independent experiments and data are presented as mean $\pm$ S.D. p-values were calculated by Student's paired t-test and the values of < 0.05 and < 0.01 were shown as * and ** and were considered significant.

Figure 5. Effects of U0126 on the growth properties and the morphological changes of GM-Gab3 cells. (A) GM-Gab3 cells were cultured without cytokine (factor free (open

triangle)), or with IL-3 (open circle) or G-CSF (closed circle). Twenty-four hours after starting culture, either no inhibitor, MEK inhibitor U0126, or the vehicle solution, DMSO, was added to the medium at 1/200th of the volume at the concentration of 10 micro molar indicated at the bottom by arrows. Cells were stained with trypan blue and cell number was counted under microscope. Standard errors were calculated from more than 3 experiments and shown as error bars. (B) Cells which were cultured in the presence of IL-3 or G-CSF for 10 days and 13 days were collected on the glass slides and stained with Wright-Giemsa solution in (A). Scale bar indicates 20 micro meters. (C) Quantitative analysis of the morphological changes shown in (B). More than 100 cells in each preparation from more than 3 independent experiments were inspected under a microscope and classified into 4 categories (a-d) indicated at the bottom.

Figure 6. Growth properties and G-CSF dependent morphological changes of another granulocyte progenitor cell line, L-G and its Gab3-Flag or Gab3FF-Flag overexpressing cell lines, LG-Gab3 and LG-Gab3FF. (A) Proteins of equivalent amount of cell lysates prepared from GM-I62-1 and L-G cells over-expressing Gab3 were separated by SDS-PAGE, followed by immunoblot analysis with antibodies against either Flag-tag, Gab2, Gab3 or beta-actin. Arrows on the left indicate the migrating position of endogenous Gab2 and Gab3 proteins. Asterisk shows a cellular protein with which anti-Gab3 antibodies cross-reacted. Immunoblot with anti beta-actin antibody showed the equal loading of proteins. (B) Growth properties of L-G, LG-Gab3 and LG-Gab3FF cells in the medium without cytokine (factor free (open triangle)), with IL-3 (open circle) or G-CSF (closed circle) as Figure 3A. Standard errors were calculated from more than 3 experiments and shown as error bars. (C) Morphological changes of cells in (B) either cultured in the medium with IL-3 or G-CSF for 10 or 13 days were analyzed under a microscope as in Figure 3B. LG-Gab3FF cells underwent apoptosis when they were cultured in the medium containing G-CSF for more than 10 days. At day 13, too few cells left survived to analyze under microscope. Scale bar indicates 20 micro meters. (D) Quantitative analysis of cell morphology in (C). More than 100 cells from 2 independent experiments were analyzed and classified as in Figure 3.