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Functional analysis of the cytoplasmic domain of the human Mpl receptor for tyrosine-phosphorylation of the signaling molecules, proliferation and differentiation

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Abstract To investigate the functional domains for signal transduction of human Mpl, we constructed a series of human c-mpl cDNAs with various deletions in the cytoplasmic domain, and then introduced each cDNA into murine IL-3-dependent myeloid leukemia FDC/P2 cells to establish stable transformants. We examined the growth and differentiation responses and tyrosine phosphorylation of the intracellular signaling proteins including Jak2, Tyk2, Stat3, Stat5, Vav, SHPTP2, Cbl, Shc and Shc-associated p145 when receptor stimulation occurred after thrombopoietin (TPO) binding. TPO stimulated cell proliferation and induced the expression of megakaryocyte lineage-specific AP-51 and CD61 cell surface antigens and tyrosine phosphorylation of the signaling proteins in transformants expressing full length human Mpl. These results suggested that Mpl not only induced proliferation but also transduced megakaryocyte-specific differentiation signals into FDC/P2 cells. Mutational analysis of human Mpl indicated that the N-terminal region of its cytoplasmic domain is necessary and sufficient to transduce proliferation and differentiation signals into cells, while the C-terminal region may also play important roles in transducing the differentiation signals.

Key words: Mpl; Thrombopoietin; Proliferation; Differentiation; Signal transduction

1. Introduction

The proto-oncogene c-mpl [1,2] is a member of the cytokine receptor superfamily, including the receptors for other hematopoietic growth factors such as interleukins (ILs), colony-stimulation factors (CSFs) and growth hormone (GH). We and other groups identified c-mpl ligand, thrombopoietin (TPO), which regulates megakaryopoiesis and thrombopoiesis [3–6]. Human Mpl consists of 635 amino acids, containing the extracellular domain with two repeats of a characteristic domain structure that includes four conserved cysteine residues and a WSXWS motif, a single transmembrane domain and a cytoplasmic domain with two structural motifs called box1 and box2. Like other members of the cytokine receptor superfamily, Mpl does not contain kinase-related or nucleotide-binding consensus sequences in its cytoplasmic domain, and its intracellular signaling pathways have not yet been fully elucidated.

Recent studies by several groups, including us, indicated that the activated Mpl induces cellular proliferation and differentiation [7–16]. The cytoplasmic domain of the Mpl can transmit proliferative signals in wild or chimeric receptor constructs. Moreover, Mpl mediates the differentiation signals, including those for the induction of megakaryocyte lineage-specific transcriptional factors and cell surface antigens in several hematopoietic cell lines. These results indicated that the cytoplasmic domain of the Mpl plays important roles in mitogenic and differentiation signal transduction. It is thought that the Mpl mediates proliferation and differentiation signals through the phosphorylation of intracellular signaling proteins. We demonstrated that TPO induces the tyrosine phosphorylation of several intracellular proteins including Janus kinases (Jaks), Stats (signal transducers and activators of transcription) [17,18], and others [19–21]. To investigate the functional domains for signal transduction of the human Mpl, we have established FDC/P2 expressing a series of truncated human Mpl receptors in murine IL-3-dependent myeloid leukemic FDC/P2 cells and examined the tyrosine phosphorylation of signaling proteins and the proliferative and differentiation responses by TPO stimulation in each transformant. In this report, we discuss the relationship between the tyrosine phosphorylation of signaling proteins and cellular responses, including proliferation and differentiation.

2. Materials and methods

2.1. Cytokines and antibodies

Recombinant murine IL-3 from *Escherichia coli* and recombinant human TPO from Chinese hamster ovary (CHO) cells were provided by the Production and Technology group of Kirin Brewery Co., Ltd. Rabbit anti-human Mpl polyclonal antibody was generated by immunizing rabbits with purified CHO-derived soluble human Mpl [6]. Mouse anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit polyclonal antibodies including anti-Jak2, anti-Tyk2, anti-Stat3, anti-Stat5, anti-Vav, anti-SHPTP2, anti-Cbl and anti-Shc were from Santa Cruz Laboratories Inc. (Santa Cruz, CA). Goat anti-mouse or rabbit Ig $F(ab')_2$ conjugated horseradish peroxidase were from Amersham (Arlington Heights, IL). Anti-murine CD61 antibody was obtained from Pharmingen (San Diego, CA). Anti-murine megakaryocyte/platelet antigen antibody AP-51 [15] was provided by Dr. T. Nagasawa of Tsukuba University. Anti-rat or hamster antibody conjugated with fluorescence isothiocyanate (FITC) was from Biosource International Inc. (Camarrilo, CA).

2.2. Plasmid construction

The humpl-Pasl2 plasmid [6] carrying a full length of the human p-form c-mpl cDNA[2] was provided by Amgen Inc. (Thousand Oaks, CA). Truncated human c-mpl cDNAs were constructed as follows. The 1.5 kb DNA fragment for the N-terminal region of human mpl was isolated by digestion of the humpl-Pasl2 with *EcoRI* and *SacI* restriction enzymes. The DNA fragments for various lengths of the C-terminal region of human mpl was generated by PCR, using a sense strand primer (5'-GGGGAGCTCGTGGTCGGACCCAACTAGG-

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G-3′) encoding the inner *Sac*I site of c-mpl and each reverse primer (5′-GGGACTAGTCCTGTAGTGTGCAGGAAACTG-3′, Mpl522; 5′-GGGACTAGTGCCTAGGACCCGGTGCAGGTC-3′, Mpl540; 5′-GGGACTAGTATCTGAGACTGTGGCCTTGGG-3′, Mpl558; 5′-GGGACTAGTCTCTGAGGACTTGGGGAGGAT-3′, Mpl576; 5′-GGGACTAGTCAATCTTCGGTAGTCCATCTG-3′, Mpl594; 5′-GGGACTAGTCTCAGCCATGGGTGGGCACAC-3′, Mpl612; 5′-GGGACTAGTAGGCTGCTGCCAATAGCTTAG-3′, Mpl635) containing an *Spe*I site. These were digested with the restriction enzymes *Sac*I and *Spe*I. The N- and C-terminal DNA fragments were ligated and inserted into the *Eco*RI and *Spe*I sites downstream of the promoter/enhancer sequence in the mammalian expression vector pSMT201 (Ohashi et al., unpublished) containing the influenza virus hemagglutinin (HA) tag [22] and hexahistidine tag sequences and stop codon downstream of the *Spe*I site.

2.3. Cells and transfection

IL-3-dependent mouse myeloid leukemia cell line FDC/P2 cells [23] were maintained in Iscove's modified Dulbecco's medium (IMDM) (Gibco/BRL, Gaithersburg, MD) containing 10% fetal calf serum (FCS) and 1 ng/ml IL-3. The plasmid was transformed into cells as described [24,25]. Briefly, pSMT201 plasmids carrying various lengths of truncated c-mpl cDNA were transfected into FDC/P2 cells with the pMCneo plasmid [26] by electroporation, then selected in IMDM containing 10% FCS, 1 ng/ml IL-3 and 0.5 mg/ml G418. Selected transformants were cloned by limiting dilution.

2.4. Cell proliferation assay

Cell proliferation was measured by means of an MTS colorimetric assay (Promega, Madison, WI) [27] based on the activity of dehydrogenase enzyme in metabolically active cells. Exponentially growing cells were washed three times with IMDM containing 10% FCS, then resuspended with IMDM containing 10% FCS at a density of 2.5×10^4 cells/ml. Cell suspension (100 μl) was seeded into each well of 96-well tissue culture plates. Various concentrations of TPO (100 μl) diluted with IMDM medium containing 10% FCS were added into each well. The plates were then incubated in a CO_2 incubator for 72 h at 37°C. Thereafter, 20 μl of MTS/PMS was added into each well and the plates were further incubated for 4 h at 37°C. Cell growth was determined by measuring the absorbance at 492 nm.

2.5. Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting analysis were performed as described [25]. Briefly, 2×10^7 cells starved for 10 h in IMDM containing 10% FCS were incubated in the absence or presence of 5 ng/ml TPO for 15 min at 37°C, then solubilized with lysis buffer: 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% NP40, 150 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, and complete protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). Clear cell lysates were immunoprecipitated with the desired polyclonal antibody and protein G Sepharose FF gels (Pharmacia Biotech, Uppsala, Sweden) for 2 h at 4°C. After centrifugation, the precipitates were subjected to 7.5% SDS-PAGE and then immunoblotted with anti-HA antibody 12CA5 or anti-phosphotyrosine monoclonal antibody (4G10). After extensive washing, the blots were visualized with an ECL immunoblotting detection system (Amersham) according to the manufacturer's instructions.

2.6. Flow cytometry

The cell surface expression of the hematopoietic cell surface antigens in IL-3- or TPO-stimulated cells was analyzed using flow cytometry. Cells were cultured with IL-3 or TPO (1×10^6 cells) were washed, then resuspended in phosphate buffered saline (PBS) containing 5% FCS and 0.1% NaN_3. They were incubated for 30 min at 4°C with first antibody in a final volume of 0.1 ml, followed by FITC-conjugated second antibody for 30 min at 4°C. All measurements were performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

3. Results

3.1. Generation of FDC/P2 transformants expressing truncated human Mpl

To determine the functional domains of the human Mpl for signal transduction, we constructed a series of human c-mpl cDNAs with various deletions in their cytoplasmic domains. Fig. 1 shows a schematic representation of the human Mpl mutants. The cDNAs included the full length human c-mpl cDNA (mpl635) and six mutants (mpl612, mpl594, mpl576, mpl558, mpl540, and mpl522) in which the cytoplasmic domain was deleted from the C-terminal end up to amino acid positions 613, 595, 559, 577, 541 and 523, respectively. These cDNAs also contained the sequence encoding the viral HA epitope and hexahistidine tags in their C-termini recognized by the mouse monoclonal antibody 12CA5 [22] or nickel chelate gels. FDC/P2 cells were transformed with expression plasmids carrying the mutant cDNA as described above. Stable transformants expressing each mutant were isolated and designated FDCP-Mpl635, FDCP-Mpl612, FDCP-Mpl594, FDCP-Mpl576, FDCP-Mpl558, FDCP-Mpl540 and FDCP-Mpl522. Expression of the mutant Mpl in these transformants was first analyzed by immunoprecipitation and immunoblotting using the 12CA5 monoclonal antibody. As shown in Fig. 2, each transformant expressed mature Mpl receptor with molecular weights predicted from the size of the deletion. The bands with a lower molecular weight seen in all transformants are likely to be immature Mpl, because they were not tyrosine phosphorylated after TPO stimulation (Fig. 3A). Parental FDC/P2 cells have no c-mpl transcript and did not express the murine Mpl receptor on the cell surface. We confirmed the endogenous expression of the murine Mpl on established transformants by means of reverse-transcriptional PCR using murine c-mpl-specific primers. The murine c-mpl transcript was undetectable in all transformants (data not shown). These results indicated that all mutants of the human Mpl were expressed in the transformants.

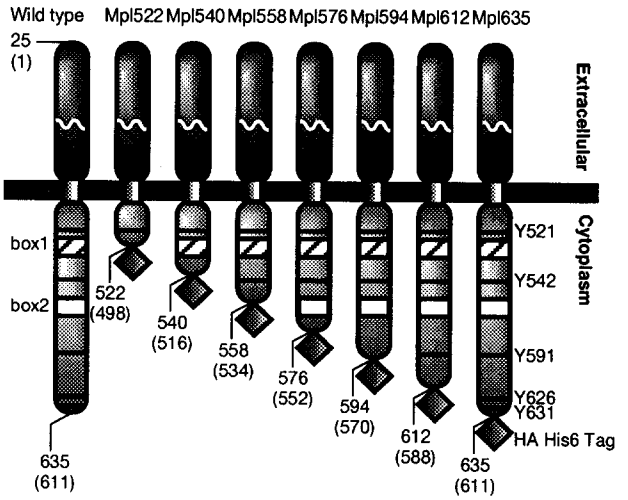

Fig. 1. Schematic representation of truncated c-Mpl. A viral HA epitope and hexahistidine tags were introduced into the C-teminal region of the receptors (indicated by diamonds). Boxes 1 and 2 are indicated by hatched and open boxes, respectively. Tyrosine residues in the cytoplasmic domain (Y521, Y542, Y591, Y626, Y631) are indicated by the thick line.

3.2. Tyrosine phosphorylation of cellular signaling proteins mediated by the truncated human Mpl receptors

In the cytokine receptor system, several cellular proteins are tyrosine-phosphorylated after ligand binding to the receptor and involved in intracellular signal transduction. We demon-

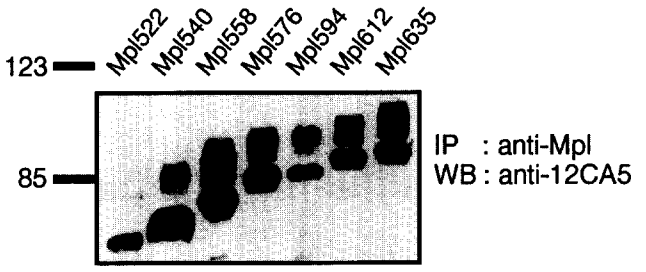

Fig. 2. Mpl receptor expression in the FDC/P2 transformants. Cell lysates of each transformant were prepared as described in Section 2. Mpl proteins were immunoprecipitated (IP) with anti-human Mpl polyclonal antibody, separated on 7.5% SDS-PAGE, and then analyzed by immunoblotting with 12CA5 mouse monoclonal antibody (WB). The mobilities of prestained molecular markers are shown on the left of the panel. Lanes 1–7 indicates FDCP-Mpl522, FDCP-Mpl540, FDCP-Mpl558, FDCP-Mpl576, FDCP-Mpl594, FDCP-Mpl612, and FDCP-Mpl635, respectively.

strated that TPO induced the tyrosine phosphorylation of Jak2 and Tyk2, Stat3 and Stat5 in FDCP-hMpl5 cells engineered to express full-length human Mpl [14]. Other proteins, including Cbl, Vav, SHPTP2, Shc, Shc-associated p145 and Mpl itself are also tyrosine-phosphorylated by TPO in our and other cell systems [8,9,11–14,28]. To investigate the Mpl functional domains for phosphorylation of the cellular proteins, we examined the TPO-induced tyrosine phosphorylation of these proteins by TPO stimulation in transformants. Starved cells were stimulated or not with TPO, lysed, immunoprecipitated with the indicated antibody, resolved by SDS-PAGE and immunoblotted using an anti-phosphotyrosine 4G10 antibody. Fig. 3 shows the tyrosine phosphorylation of signaling proteins in Mpl deletion mutants. Mpl itself, Jak2 and Tyk2 were tyrosine-phosphorylated by TPO stimulation in FDCP-Mpl635, FDCP-Mpl612, FDCP-Mpl594, FDCP-Mpl576 and FDCP-Mpl558 cells, but not in unstimulated cells (Fig. 3A–C). The phosphorylation level of these proteins in FDCP-Mpl558 cells was relatively low. These proteins were not tyrosine-phosphorylated in FDCP-Mpl540 and FDCP-Mpl522 cells. Although Vav was weakly tyrosine-phosphorylated before TPO stimulation, it was obviously phosphorylated thereafter. The phosphorylation spectrum of Vav in transformants was the same as that of Mpl, Jak2 or Tyk2 (Fig. 3D). These results demonstrated that the phosphorylation of Mpl, Jak2, Tyk2 and Vav after TPO stimulation required the membrane proximal region of the human Mpl, which contains the box1 region conserved in other members of the cytokine receptor superfamily. On the contrary, tyrosine phosphorylation of the Stat3, Stat5, Cbl and SHPTP2 required the membrane proximal domain containing 18 more distal amino acids (amino acid positions 512–576). TPO induced the tyrosine-phosphorylation of these proteins in FDCP-Mpl635, FDCP-Mpl612, FDCP-Mpl594 and FDCP-Mpl576 cells, but not in FDCP-Mpl558, FDCP-Mpl540 and FDCP-Mpl522 cells (Fig. 3E–H). On the other hand, phosphorylation of the Shc required the most distal region of the Mpl receptor (Fig. 3I). Only full-length Mpl635 (FDCP-Mpl635) mediated full tyrosine phosphorylation of the Shc. Shc was very weakly tyrosine-phosphorylated in FDCP-Mpl612 and FDCP-Mpl594 cells. However, tyrosine-phosphorylated Shc-associated p145 co-immunoprecipitated with Shc in FDCP-Mpl635, FDCP-Mpl612, FDCP-Mpl594 and FDCP-Mpl576 cells (Fig. 3J).

3.3. *Mpl-mediated cell proliferation requires the 45 membrane-proximal amino acids of human Mpl*

To determine the Mpl cytoplasmic region required for cell proliferation, we examined the TPO-dependent growth of the transformants. As shown in Fig. 4, FDCP-Mpl635, FDCP-Mpl612, FDCP-Mpl594, FDCP-Mpl576 and FDCP-Mpl558 cells proliferated in response to TPO in a dose-dependent manner. FDCP-Mpl558 cells had a lower growth response than the other transformants. However, FDCP-Mpl540, FDCP-Mpl522 and parental FDC/P2 cells did not proliferate in response to TPO even at very high concentrations. These results indicated that the membrane proximal region in Mpl cytoplasmic domain is required for cell proliferation and that it is involved in Mpl-mediated growth signal transduction. As described above, tyrosine-phosphorylation of Jak2, Tyk2 and Vav required the 45 amino acids of Mpl proximal to the plasma membrane (Fig. 3B–D). Moreover, the tyrosine phosphorylation levels of these proteins in FDCP-Mpl558 cells were low. These results indicate that the cytoplasmic domain of the Mpl required for cell proliferation corresponds to that for the tyrosine-phosphorylation of these signaling proteins.

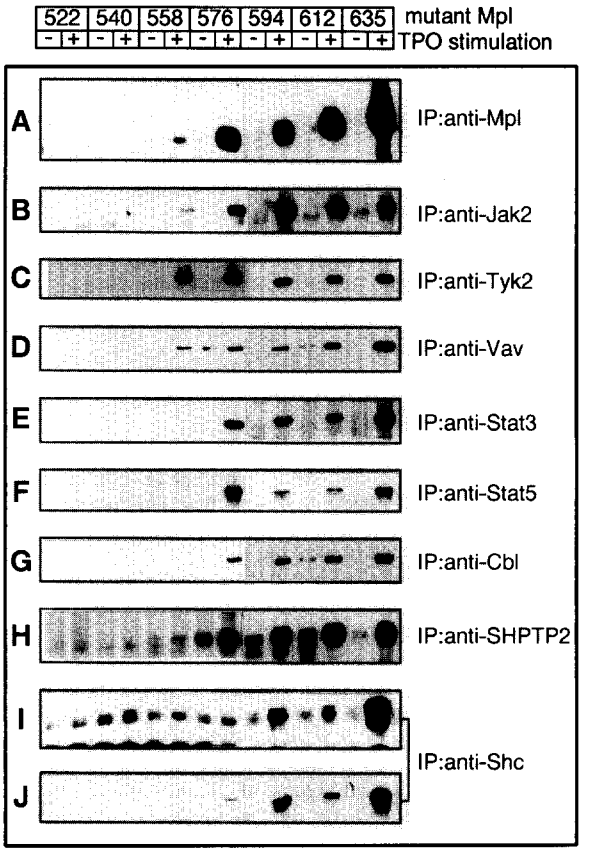

Fig. 3. Tyrosine phosphorylation of Mpl (A), Jak2 (B), Tyk2 (C), Vav (D), STAT3 (E), STAT5 (F), Cbl (G), SH-PTP2 (H), Shc (I) and Shc-associated p145 (J) in FDC/P2 cells expressing various Mpl mutants. Starved cells were incubated in the absence (−) or presence (+) of TPO for 15 min. The cells were lysed and then immunoprecipitated with an indicated polyclonal antibody. Immunoprecipitates were separated on 7.5% SDS-PAGE and then analyzed by immunoblotting with anti-phosphotyrosine antibody 4G10.

Moreover, these results suggested that these signaling proteins are involved in the Mpl-mediated signal transduction pathways for cell proliferation.

3.4. Megakaryocyte lineage-specific differentiation signals mediated by the truncated human Mpls

TPO induces expression of megakaryocyte-specific transcriptional factors and cell surface antigens on some hematopoietic cell lines including CMK [29], UT7 [30] and FD-TPO [15], which endogenously or exogenously express Mpl receptor. In FDCP-hMpl5 cells [14], TPO increased the expression of megakaryocyte/platelet-specific cell surface antigens AP-51 and CD61 (data not shown). To determine the Mpl cytoplasmic regions functional for cellular maturation signals, we examined the capacity of mutant Mpl receptors to increase the expression of megakaryocyte-specific antigens AP-51 and CD61 upon TPO stimulation. All transformants except FDCP-Mpl522 and FDCP-Mpl540 were cultured in the presence of IL-3 or TPO and the expression of cell surface antigens was analyzed by means of flow cytometry. FDCP-Mpl522 and FDCP-Mpl540 cells were grown in medium containing IL3, or IL3 and TPO because these cells lack a proliferative response to TPO. As shown in Fig. 5, the expression of both AP-51 and CD61 antigens on FDCP-Mpl635, FDCP-Mpl576, FDCP-Mpl558 cells was enhanced by TPO. In contrast, there were no changes in expression levels of AP-51 and CD61 in FDCP-Mpl612, FDCP-Mpl594, FDCP-Mpl540 and FDCP-Mpl522 cells in the presence of TPO. No changes in expression of these antigens were induced even after 1 month of culture in medium containing TPO. Furthermore, in other FDC/P2 transformants transfected with mpl612 or mpl594 cDNA, the expression of cell surface antigens was not enhanced in response to TPO. These results indicated that the C-terminal (aa 595–634) and membrane-proximal regions (aa 512–558) of the Mpl cytoplasmic domain play important roles for cellular differentiation signaling, including the inducible expression of megakaryocyte-specific cell surface antigens.

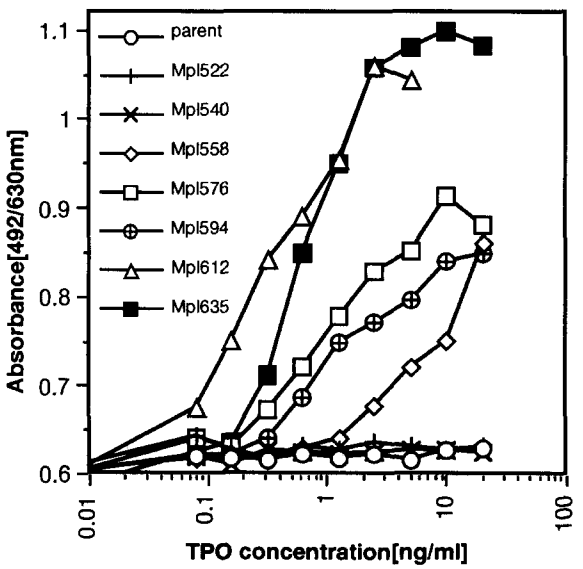

Fig. 4. The TPO dose-response curves of FDC/P2 transformants expressing Mpl mutants and parental FDC/P2 cells. Cells were cultured in the presence of various concentrations of TPO for 3 days. The number of viable cells was then measured by means of an MTS colorimetric assay.

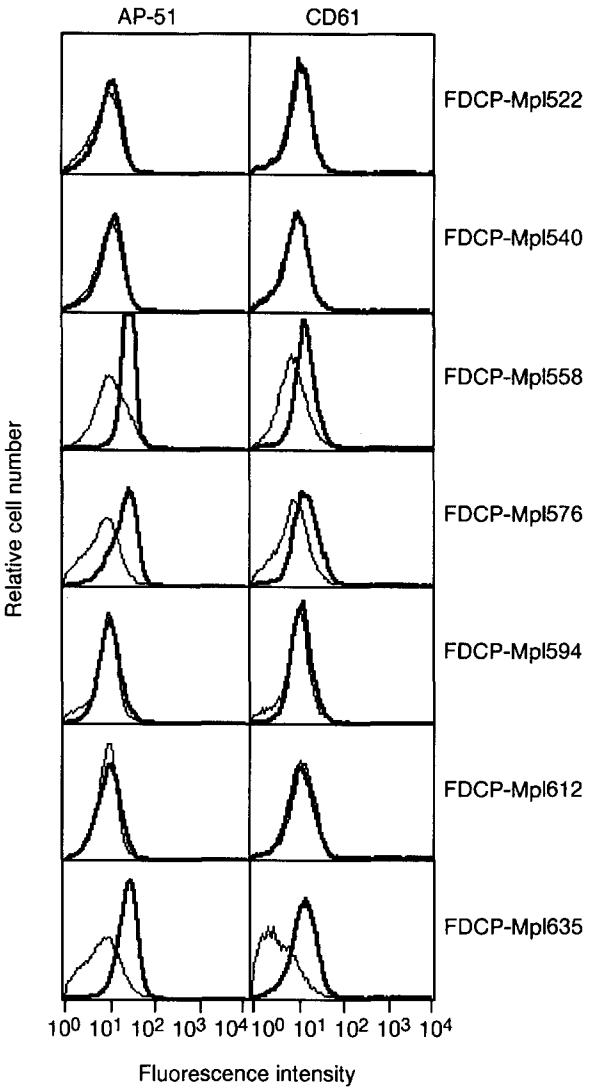

Fig. 5. Flow cytometry of megakaryocyte/platelet-specific cell surface antigens, AP-51 (left panels) and CD61 (right panels) in transformants stimulated by IL-3 or TPO. Cells cultured with IL-3 (thin line) or TPO (thick line) were incubated for 30 min at 4°C with anti-AP-51 or anti-CD61 antibody. After washing, the cells were then stained for 30 min with FITC-conjugated second antibody. Fluorescence intensity was determined using a FACScan flow cytometer.

The results also indicated that the cytoplasmic domain of human Mpl receptor for cell differentiation signals is identical to that for cell proliferative signals. As described above, intracellular signaling proteins were tyrosine-phosphorylated by Mpl activation after TPO binding. However, there was no correlation between the tyrosine phosphorylation of signaling proteins and the induction of megakaryocyte-specific cell surface antigen expression in each transformant. These findings suggested that these differentiation signals are mediated by other signal transduction pathways through unknown signaling molecules.

4. Discussion

We demonstrated the functional domains of human Mpl for the tyrosine phosphorylation of cellular proteins, as well as proliferation and differentiation, using mouse IL-3-dependent

myeloid leukemia FDC/P2 transformants engineered to express a series of truncated Mpl receptors. In the FDCP-Mpl635 transformant expressing a full-length Mpl, TPO induce the tyrosine phosphorylation of several cellular signaling proteins, including Mpl itself and stimulated the proliferation and expression of megakaryocyte/platelet-specific cell surface antigens. These results indicated that the Mpl expressed in FDC/P2 cells mediates signals for TPO-triggered proliferation and differentiation. Mutational analysis of Mpl further showed that two distinct regions of its cytoplasmic domain are involved in proliferation and differentiation signal transduction.

The Mpl558 receptor, including a region proximal to the plasma membrane, mediated proliferative and differentiation signals and induced the tyrosine phosphorylation of Jak2, Tyk2, Vav and Mpl itself in the presence of TPO, indicating that 45 membrane proximal amino acids are required for producing both the proliferation and differentiation signals. This region contained the box1, but not box2 motif conserved in other cytokine receptors such as the erythropoietin and G-CSF receptors and gp130. This suggested that the region including the box2 motif in the Mpl cytoplasmic domain is not essential for receptor-mediated cellular behavior, particularly proliferation. Several investigators have examined the signal transduction for cell proliferation mediated by mutants of several cytokine receptors (reviewed in [17,18]). These studies revealed that the membrane proximal region of the cytokine receptors is necessary and sufficient for cytokine-induced mitogenic signaling, and that box1 alone and in combination with box2 is required for binding of Jaks and for activation of the Jak-Stat pathway. Our findings that the box2 region is not necessary for producing Mpl-mediated signals is not in agreement with the data reported by Gurney et al. [8]. They showed that the Mpl cytoplasmic region within 63 amino acids, including the box2 motif, transmits proliferative signals and activates Jak2, Stat1 and Stat3 whereas that within 42 amino acids did not. This means that the 3 extra amino acids (aa 556–558) in Mpl are critical for producing proliferative signals. These differences may be due to the cell line used. In this study, however, we found that the membrane-proximal 45 amino acid sufficient for producing proliferative signals could not induce the tyrosine-phosphorylation of Stat3 and Stat5, and that tyrosine phosphorylation of these Stats required the membrane proximal region containing 18 more distal amino acids. We also showed that the tyrosine phosphorylation of Stats induced by Mpl activation is not essential for proliferative signaling in our FDC/P2 system. FDCP-Mpl558 cells exhibited lower proliferation rates and tyrosine phosphorylation levels of Jak2, Tyk2, Vav and Mpl, although the expression level of the receptor was the same as or higher than those of other transformants. This suggested that the degree of phosphorylation of these signaling proteins directly contributes to the proliferative response to TPO. Thus our data suggested that Jak2, Tyk2 and Vav are involved in Mpl-mediated signal transduction for both proliferation and differentiation, whereas Stat3 and Stat5 are not. Moreover, the results also suggested that there are proliferative signal transduction pathways other than those of Jak-Stat involved in Mpl-mediated signaling.

The Mpl receptor activates Stat1, 3 and 5 in various types of cells [8,10,11,14]. However the role of Stats in signal transduction of the Mpl receptor remains unclear. They were apparently unnecessary for proliferation and differentiation in our in vitro cell system. Stats do not appear to be involved in mitogenic responses to cytokines, as IFNs do not induced cell proliferation [31]. IL-6 [32], IL-2 [33] and IL-4 [34] mutant receptors can trigger cell proliferation without the activation of Stat3, 5 and 6, respectively. Although biological functions of Stat3 and 5 are not fully elucidated, our data indicated that Stat3 and 5 are not directly involved in cell proliferation or the inducible expression of megakaryocyte specific cell surface antigens including AP-51 and CD61. However, Stats regulate the expression of a wide range of genes induced by the activation of the receptors for cytokines such as IFNs, IL-4 and IL-6 [18]. For example, Stat3 [35] is required for the IL-6-induced expression of acute-phase response genes, whereas Stat5 mediates the gene transcription of several milk proteins induced by prolactin [36]. Thus Stats play important roles for transcription of many genes induced by cytokines. Therefore, Stat3 and 5 may mediate the TPO-induced transcription of several proteins that are specifically expressed in megakaryocyte-lineage cell type except AP-51 and CD61.

As described above, Vav is also tyrosine-phosphorylated by TPO stimulation in FDC/P2 transformants which Jak2 and Tyk2 were tyrosine-phosphorylated and proliferative responses were seen. Vav are selectively expressed in hematopoietic cells, and transiently tyrosine-phosphorylated in response to stimulation of the cytokine, c-Kit tyrosine kinase and T-cell antigen receptors [37–41]. The Vav exogenously expressed in fibroblast cells displays constitutive activation of the Ras-MAPK pathway [42]. Moreover, an activated erythropoietin receptor mutant constitutively associated with Jak2 and induced tyrosine phosphorylation of Vav [40], suggesting that Vav protein functions as a downstream signaling molecule for Jaks. Thus Vav protein may be positioned between Jaks and Ras. Our experiments showed that the tyrosine phosphorylation of Vav in Mpl mutants positively correlates with proliferative responses. In this study, however, we did not examine the phosphorylation and activation of proteins directly involved in the Ras-MAPK signal transduction pathway. Further investigation is under way to elucidate the role of Vav in Mpl-mediated signal transduction pathways.

Reports have described that the C-terminal region of several cytokine receptors plays an important role in transducing differentiation signals with no effect upon cell growth [43–48]. Deleting this region from the G-CSF receptor [43–45] caused phenotypic changes in the cell surface antigens, a loss of gene expression of neutrophil-specific enzymes, and the inhibition of morphological differentiation into neutrophils. In this study, we found that the C-terminal region (aa 595–635) of the Mpl, which is dispensable for proliferation, is also required for producing differentiation signals that enhance the expression of megakaryocyte-specific cell surface antigens. On the other hand, this C-terminal region was necessary for inducing tyrosine phosphorylation of Shc. Thus the results suggested that the phosphorylation and activation of Shc is involved in the differentiation activity of the human Mpl receptor. Little is known regarding the role of this Shc in the differentiation of hematopoietic cells. The adaptor protein Shc is tyrosine-phosphorylated during cytokine receptor activation, then it forms a complex with Grb2 and Sos1 resulting in activation of the Ras-MAPK signal transduction pathway [49,50]. In fact, we confirmed that phosphorylated Shc binds to the Grb2/Sos1 complex in our cell system (data not shown).

Moreover, Shc activation is involved in both proliferative and differentiation responses in non-hematopoietic cells [51]. However, the inhibition of $p21^{Ras}$ activation blocks proliferation, but not the differentiation of 32Dcl13 cell into neutrophils [52]. These findings suggested that the signal transduction for cellular differentiation mediated by the cytokine receptor is involved in activating Shc protein but not the Ras-MAPK pathway. Shc also associates with p145 protein to form a complex in several cytokine receptor systems [53,54]. The formation of an Shc-p145 complex has been implicated in the cytokine-induced activation of the Ras-MAPK signal transduction pathway in hematopoietic cells. Tyrosine phosphorylation of Shc-associated p145 protein by TPO stimulation occurs in FDCP-Mpl612, FDCP-Mpl594 and FDCP576 cells in which no phosphorylation of Shc was apparent. Thus p145 phosphorylation did not correspond with that of Shc. The results indicated that the elements required for the phosphorylation of p145 and of Shc are different. Deletion of over 18 amino acids (aa 575–594) restored the differentiation activities of mutant Mpl, although Shc was not phosphorylated. However, no signaling proteins phosphorylated in a manner similar to differentiation of mutant Mpls were found. These results suggested that unknown signal transduction pathways through a signaling molecule other than Shc are involved in cellular differentiation. Alternatively, there may be signaling molecules that suppress differentiation signals and deletion of the C-terminal region may allow these molecules to bind the middle region (aa 577–612) of the Mpl. Thus it is likely that differentiation signals from the Mpl receptor are mediated by several signal transduction pathways.

Here we demonstrated that Grb2-associated proteins, including SHPHTP2 and Cbl, are involved in signal transduction of the Mpl. SHPTP2, which is a tyrosine-specific protein phosphatase, is thought to stimulate the signaling mediated by cytokine receptor [55]. SHPTP-2 contains two SH2 domains that allow it to bind receptors for cytokines. It have been reported that SHPTP2 directly binds to tyrosine-phosphorylated erythropoietin receptor and to Grb2 upon erythropoietin stimulation in Mo7e cells [56]. SHPTP2 also associated with the PDGF and c-Kit receptors [57,58]. On the other hand, Cbl is involved in signal transduction in various cells. This protein is tyrosine-phosphorylated by stimulating the receptors for granulocyte macrophage-CSF, erythropoietin, epidermal growth factor, fibroblast growth factor and nerve growth factor [59,60]. Cbl is associated with bcr-abl and v-abl, suggesting that it is involved in the pathogenesis of chronic myelogenous leukemia [61]. The roles of these proteins in Mpl-mediated signal transduction are still unclear. As described above, we demonstrated that Cbl was tyrosine-phosphorylated in FDCP-Mpl576, FDCP-Mpl594, FDCP-Mpl612 and FDCP-Mpl635 cells, but not in FDCP-Mpl558 cells, indicating that the region required for the phosphorylation of these proteins is not related to that for proliferation. By studying TPO-Mpl signal transduction using platelets, we have recently reported that Cbl is involved in signaling during platelet aggregation by redistribution to the cytoskeleton [28]. Thus, these Grb2-associated signaling proteins may function as potential mediators of other types of Mpl-mediated signaling.

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References

[1] Skoda, R.C., Seldin, D.C., Chiang, M., Peichel, C.L., Vogt, T.F. and Leder, P. (1993) EMBO J. 12, 2645–2653.
[2] Vigon, I., Mornon, J.P., Cocault, L., Mitjavila, M., Tambourin, P., Gisselbrecht, S. and Souyri, M. (1992) Proc. Natl. Acad. Sci. USA 89, 5640–5646.
[3] Kato, T., Ogami, K., Shimada, Y., Iwamatsu, A., Sohma, Y., Akahori, H., Horie, K., Kokubo, A., Kudo, Y., Maeda, E., Kobayashi, K., Ohashi, H., Ozawa, T., Inoue, H., Kawamura, K. and Miyazaki, H. (1995). J. Biochem. 118, 229–236.
[4] De Sauvage, F.J., Hass, P.E., Spencer, S.D., Malloy, B.E., Gurney, A.L., Spencer, S.A., Darbonne, W.C., Henzel, W.J., Wong, S.C., Kaung, W.J., Oles, K.J., Hultgren, B., Solberg Jr, L.A., Goeddel, D.V. and Eaton, D.L. (1994) Nature 369, 533–538.
[5] Lok, S., Kaushansky, K., Holly, R.D., Kuijper, J.L., Lofton-Day, C.E., Oort, P.J., Grant, F.J., Heipel, M.D., Burkhead, S.K., Kramer, J.M., Bell, L.A., Sprecher, C.A., Blumberg, H., Johnson, R., Prunkard, D., Cing, A.F.T., Mathewes, S.J., Bailey, M.C., Forstom, J.W., Buddle, M.M., Osborn, S.G., Evans, S.J., Sheppard, P.O., Preanell, S.R., O'Hara, P.J., Hagen, F.S., Roth, G.J. and Foster, D.C. (1994). Nature 369, 565–568.
[6] Bartley, T.D., Bogenberger, J., Hunt, P., Li, Y.-S., Lu, H.S., Martin, F., Chang, M-S., Samal, B., Nichol, J.L., Swift, S., Johnson, M.J., Hsu, R-Y., Parker, V.P., Suggs, S., Skrine, J.D., Merewether, L.A., Clogston, C., Hsu, E., Hokom, M.M, Hornkohl, A., Choi, E., Pangelinan, M., Sun, Y., Mar, V., McNinch, J., Siminet, L., Jacobsen, F., Xie, C., Shutter, J., Chutte, H., Basu, R., Selander, L., Trollinger, D., Sieu, L., Padilla, D., Trail, G., Eliott, G., Izumi, R., Covey, T., Crouse, J., Garcia, A., Xu, W., Del Castillo, J., Biron, J., Cole, S., Hu, M.C-T., Pacifici, R., Ponting, I., Wen, D., Yung, Y.P., Lin, H. and Bosselman, R.A. (1994) Cell 77, 1117–1124.
[7] Drachman, J.G., Griffin, J.D. and Kaushansky, K. (1995) J. Biol. Chem. 270, 4979–4982.
[8] Gurney, A.L., Wong, S.C., Henzel, W.J. and de Sauvage, F.J. (1995) Proc. Natl. Acad. Sci. USA 92, 5292–5296.
[9] Miyakawa, Y., Oda, A., Drucker, B.J., Kato, T., Miyazaki, H., Handa, M. and Ikeda, Y. (1995) Blood 86, 23–27.
[10] Pallard, C., Gouilleux, F., Benit, L., Cocault, L., Souyri, M., Levy, D., Groner B., Gisslbrecht, S. and Dusanter-Fourt, I. (1995) EMBO J. 14, 2847–2856.
[11] Sattler, M., Durstein, M.A., Frank, D.A., Okuda, K., Kaushansky, K., Salgia, R. and Griffin, J.D. (1995) Exp. Hematol. 23, 1040–1048.
[12] Bacon, C.M., Tortolani, P.J., Shimosaka, A., Rees, R.C., Longo, D.L. and O'Shea, J.J. (1995) FEBS Lett. 370, 63–68.
[13] Sasaki, K., Odai, H., Hanazono, Y., Ogawa, S., Langdon, W.Y., Tanaka, T., Miyagawa, K., Mitani, K., Yazaki, Y. and Hirai, H. (1995) Biochem. Biophys. Res. Commun. 216, 338–347.
[14] Miyakawa, Y., Oda, A., Brucker, B.J., Miyazaki, H., Handa, M., Ohashi, H. and Ikeda, Y. (1996) Blood 87, 439–446.
[15] Nagata, Y., Nagahisa, H., Aida, Y., Okutomi, K., Nagasawa, T. and Todokoro, K. (1995) J. Biol. Chem. 270, 19673–19675.
[16] Debili, N., Wendling, F., Katz, A., Grichard, J., Breton-Gorius, J., Hunt, P. and Vainchenker, W. (1995) Blood 86, 2516–2525.
[17] Heldin, C.-H. (1995) Cell 80, 213–223.
[18] Ihle, J.N. (1995) Nature 377, 591–594.
[19] Yamada, M., Komatsu, N., Okada, K., Kato, T., Miyazaki, H. and Miura, Y. (1995) Biochem. Biophys. Res. Commun. 217, 230–237.
[20] Mu, S.X., Xia, M., Elliott, G., Bogenberger, J., Swift, S., Bennett, L., Lappinga, D.L., Hecht, R., Lee, R. and Saris, C.J.M. (1995) Blood 86, 4532–4543.
[21] Nagata, Y. and Todokoro, K. (1995) FEBS Lett. 377, 497–501.
[22] Kolodziej, P.A. and Young, R.A. (1991) Methods Enzymol. 194, 277–285.
[23] Dexter, T.M., Garland, J., Scott, D., Scolnick, E. and Metcalff, D. (1980) J. Exp. Med. 152, 1036–1047.
[24] Ohashi, H., Kameda, R., Nishikawa, M., Kawagishi, M. and Liu, Y.-C. (1994) Cytotechnology 16, 27–35.

[25] Ohashi, H., Maruyama, K., Liu, Y-C. and Yoshimura, A. (1994) Proc. Natl. Acad. Sci. USA 91, 158–162.
[26] Thomas, K.R. and Capecchi, M.R. (1987) Cell 51, 503–512.
[27] Buttke, T.M., McCurbrey, J.A. and Owen, T.C. (1993) J. Immunol. Methods 157, 233–240.
[28] Oda, A., Ozaki, K., Brucker, B.J., Miyakawa, Y., Miyazaki, H., Handa, M., Morita, H., Ohashi, H. and Ikeda, Y. (1996) Blood (in press).
[29] Kauchansky, K., Lok, S., Holly, R.D., Broudy, V.C., Lin, N., Bailey, M.C., Forstrom, J.W., Buddie, M.D., Oort, P.J., Hagen, F.S., Roth, G.J., Papayannopoulou, T. and Foster, D.C. (1994) Nature 369, 568–571.
[30] Porteu, F., Rouyez, M-C., Cocault, L., Benit, L., Charon, M., Picard, F., Gisselbrecht, S., Souyri, M. and Dusanter-Fourt, I. (1996) Mol. Cell. Biol. 16, 2473–2482.
[31] Darnell, J.E. Jr., Kerr, I.M. and Stark, G.R. (1994) Nature 264, 1415–1421.
[32] Stahl, N., Farruggella, T.J., Boulton, T.G., Zhong, Z., Darnell, J.E. Jr. and Yancopoulos, G.D. (1995) Science 267, 1349–1353.
[33] Fujii, H., Nakagawa, Y., Schindler, U., Kawahara, A., Mori, H., Gouilleux, F., Groner, B., Ihle, J.N., Minami, Y., Miyazaki, T. and Taniguchi, T. (1995) Proc. Natl. Acad. Sci. USA 92, 5482–5486.
[34] Quelle, F.W., Shimoda, K., Thierfelder, W., Fischer, C., Kim, A., Ruben, S.M., Cleveland, J.L., Pierce, J.H., Keegan, A.D., Nelms, K., Paul, W.E. and Ihle, J.N. (1995) Mol. Cell. Biol. 15, 3336–3343.
[35] Kishimoto, T., Taga, T. and Akira, S. (1994) Cell 76, 253–262.
[36] Wakao, H., Gouilleux, F. and Groner, B. (1994) EMBO J. 13, 2182–2191.
[37] Gulbins, E., Coggeshall, K.M., Baier, G., Katzav, S., Burn, P. and Altman, A. (1993) Science 260, 822–825.
[38] Alai, M., Mui, A.L., Cutler, R.L., Bustelo, X.R., Barbacid, M. and Krystal, G. (1992) J. Biol. Chem. 267, 18021–18025.
[39] Miura, O., Miura, Y., Nakamura, N., Quelle, F.W., Wittuhn, B.A., Ihle, J.N. and Aoki, N. (1994) Blood 84, 4135–4141.
[40] Matsuguchi, T., Inhorn, R.C., Carlesso, N., Xu, G., Drucker, B. and Griffin, J.D. (1995) EMBO. J. 14, 257–265.
[41] Fischer, K.D., Zmuidzinas, A., Gardner, S., Barbacid, M., Bernstein, A. and Guidos, C. (1995) Nature 374, 474–477.
[42] Bustelo, X.R., Suen, K., Leftheris, K., Meyer, C.A. and Marbacid, M. (1994) Oncogen 9, 2405–2413.
[43] Fukunaga, R., Ishizaka-Ikeda, E. and Nagata, S. (1993) Cell 74, 1079–1087.
[44] Dong, F., van Buitenen, C., Pouwels, K., Hoefsloot, L.H., Lowenberg, B. and Touw, I.P. (1993) Mol. Cell. Biol. 13, 7774–7781.
[45] Ziegler, S.F., Bird, T.A., Morella, K.K., Mosley, B., Gearing, D.P. and Baumann, H. (1993) Mol. Cell. Biol. 13, 2384–2390.
[46] Baumann, H., Symes, A.J., Comeau, M.R., Morella, K.K., Wang, Y., Friend, D., Ziegler, S.F., Fink, J.S. and Gearing, D.P. (1994) Mol. Cell. Biol. 14, 138–146.
[47] Ho, A.S.-Y., Wei, S.H.-Y., Mui, A.L.-F., Miyajima, A. and Moore, K.W. (1992) Mol. Cell. Biol. 15, 5043–5053.
[48] Sotiropoulos, A., Perrot-Applanat, M., Dinerstein, H., Palliar, A., Postel-Vinay, M.C., Finidori, J. and Kelley, P.A. (1994) Endocrinology 135, 1292–1298.
[49] Cutler, R.L., Liu, L., Damen, J.E. and Krystal, G. (1993) J. Biol. Chem. 268, 21463–21465.
[50] Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pellici, P.G., Schlessinger, J. and Pawson, T. (1992) Nature 360, 689–692.
[51] Obermeier, A., Bradshaw, R.A., Seedorf, K., Choidas, A., Schlessinger, J. and Ullrich, A. (1994) EMBO. J. 13, 1585–1590.
[52] Okuda, K., Ernst, T.J. and Griffin, J.D. (1994) J. Biol. Chem. 269, 24602–24607.
[53] Damen, J.E., Liu, L., Cutler, R.L. and Krystal, G. (1993) Blood 82, 2296–2303.
[54] Damen, J.E., Liu, L., Rosten, P., Humphries, R.K., Jefferson, A.B., Majerus, P.W. and Krystal, G. (1996) Proc. Natl. Acad. Sci. USA. 93, 1689–1693.
[55] Feng, G.S., Hui, C.C. and Pawson, T. (1993) Science 259, 1607–1610.
[56] Tauchi, Feng, G-S, Shen, R., Hoatlin, M., Bagby, G.C., Kabat, D. and Broxmeyer, H.E. (1995) J. Biol. Chem. 270, 5631–5635.
[57] Bennet, A.M., Tang, T.L., Sugimoto, S., Walsh, C.T. and Neel, B. (1994) Proc. Natl. Acad. Sci. USA. 91, 7335–7339.
[58] Tauchi, T., Feng, G.-S., Marshall, M.S., Shen, R., Mantel, C., Pawson, T. and Broxmeyer, H.E. (1994) J. Biol. Chem. 269, 25206–25211.
[59] Odai, H., Sasaki, K., Iwamatsu, A., Hanazono, Y., Tanaka, T., Mitani, K., Yazaki, Y. and Hirai, H. (1995) J. Biol. Chem. 270, 10800–10805.
[60] Tanaka, S., Neff, L., Baron, R. and Levy, J.B. (1995) J. Biol. Chem. 270, 14347–14351.
[61] Andoniou, C.E., Thien, C.B. and Langdon, W.Y. (1994) EMBO J. 13, 4515–4523.