p56^{dok-2} as a cytokine-inducible inhibitor of cell proliferation and signal transduction

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p56^{dok-2} acts as a multiple docking protein downstream of receptor or non-receptor tyrosine kinases. However, the role of p56^{dok-2} in biological functions of cells is not clear. We found that transcription of the p56^{dok-2} gene in macrophages was increased markedly in response to cytokines such as macrophage colony-stimulating factor (M-CSF), granulocyte/macrophage-CSF and interleukin-3 (IL-3). Forced expression of p56^{dok-2} inhibited M-CSF-, granulocyte-CSF-, IL-3- and stem cell factor-induced proliferation of myeloid leukemia cells, M-NFS-60. The p56^{dok-2}-overexpressing cells showed an impaired induction of *c-myc* but not of *c-jun*, *junB* or *c-fos* when stimulated with M-CSF. Consistent with these results, the peritoneal cavity of the hairless (*hr/hr*) strain of mutant mice, whose cells expressed less p56^{dok-2} than wild-type mice, contained more macrophages than that of *+/hr* mice. Moreover, the inhibition of endogenous p56^{dok-2} expression in macrophage-like tumor cells, J774A.1, by stable expression of antisense p56^{dok-2} mRNA accelerated cell proliferation. The study identifies a novel role for p56^{dok-2} as a molecule that negatively regulates signal transduction and cell proliferation mediated by cytokines in a feedback loop.

Keywords: cell proliferation/cytokine/Dok-R/FRIP/p56^{dok-2}

Introduction

The proliferation and differentiation of cells of many lineages are tightly regulated by proteins known as cytokines. The binding of cytokines to their receptors activates multiple tyrosine kinases including receptor tyrosine kinases, JAK family kinases and Src family kinases (reviewed in Ihle, 1995). The receptors for cytokines such as macrophage colony-stimulating factor (M-CSF) and stem cell factor (SCF) are transmembrane tyrosine kinases and the cytoplasmic domain of these receptors encodes a tyrosine kinase (Sherr *et al.*, 1985; Huang *et al.*, 1990; Williams *et al.*, 1990; Zsebo *et al.*, 1990). In contrast, the receptors for the majority of cytokines do not encode a tyrosine kinase catalytic

domain. Studies conducted with a variety of cytokine receptors have provided evidence that, regardless of receptor type, the activation of tyrosine kinases and the phosphorylation of substrates drive cell proliferation or differentiation (reviewed in Ihle, 1995).

More recent studies have also elucidated the mechanisms responsible for negatively regulating the cytokine signals. SHP-1 is a tyrosine phosphatase that binds to the erythropoietin (Epo) receptor and the β chain of interleukin-3 (IL-3) receptor (Yi *et al.*, 1993, 1995). A mutation in the SHP-1 gene results in hyper tyrosine phosphorylation of cellular proteins and increased levels of macrophages and erythrocytes (Shultz *et al.*, 1993; Tsui *et al.*, 1993). SHIP, SH2-containing inositol phosphatase, has also been shown to function as a growth inhibitory molecule and its targeted disruption results in hematopoietic perturbations as a consequence of hyper-responsiveness to stimulation by IL-3, M-CSF and granulocyte/macrophage-CSF (GM-CSF) (Liu *et al.*, 1997; Helgason *et al.*, 1998).

The CIS/JAB/SOCS/SSI family consists of relatively small proteins with a centrally located SH2 domain and a unique carboxyl motif termed the SOCS box (Yoshimura *et al.*, 1995; Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997). Studies have also implicated these molecules in the negative regulation of cytokine signal transduction. For example, CIS protein binds tyrosine phosphorylated Epo receptor and can block Epo signaling (Yoshimura *et al.*, 1995). Importantly, stimulation with a broad array of cytokines is capable of upregulating mRNA for one or more members of the family (Yoshimura *et al.*, 1995; Starr *et al.*, 1997), suggesting that these molecules act in a classic negative feedback loop to regulate cytokine signal transduction.

Here, we provide evidence that p56^{dok-2} is also a cytokine-inducible inhibitor of cytokine signal transduction. p56^{dok-2} (also known as Dok-R or FRIP) is a member of a newly identified subfamily of docking proteins (Di Cristofano *et al.*, 1998; Jones and Dumont, 1998; Nelms *et al.*, 1998), of which p62^{dok} is the prototype (Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997). More recently, the third member of this *dok* family, Dok-L/Dok-3, has been cloned (Cong *et al.*, 1999; Lemay *et al.*, 2000). Analysis of the expressed sequence tag database reveals the presence of additional members of the family (Di Cristofano *et al.*, 1998). The N-terminal part of dok proteins contains a pleckstrin homology domain thought to be involved in the membrane localization of proteins (Noguchi *et al.*, 1999). There is a potential phosphotyrosine binding domain near the central region (Jones and Dumont, 1998). The C-terminal region contains a number of potential tyrosine phosphorylation and SH2 domain interaction sites (Bhat *et al.*, 1998; Jones and Dumont, 1999; Lock *et al.*, 1999; Noguchi *et al.*, 1999),

leading to the suggestion that dok proteins act as multiple docking proteins downstream of tyrosine kinases. In fact, in reconstitution experiments using transiently transfected cells, epitope-tagged p56^{dok-2} was phosphorylated on tyrosine residues when co-expressed with Bcr-Abl tyrosine kinase (Di Cristofano *et al.*, 1998), an activated form of Tek/Tie2 receptor tyrosine kinase (Jones and Dumont, 1998) or Src family kinases, Lyn, Hck or Src (Lock *et al.*, 1999). Moreover, stimulation of cell lines that stably expressed p56^{dok-2} with growth factors or cytokines, including epidermal growth factor, insulin, IL-2, IL-3 or IL-4, also resulted in rapid phosphorylation of p56^{dok-2} (Nelms *et al.*, 1998; Lock *et al.*, 1999). Once phosphorylated, p56^{dok-2} was capable of interacting via specific binding sites with SH2 domains of rasGTPase-activating protein (rasGAP) (Di Cristofano *et al.*, 1998; Jones and Dumont, 1998, 1999; Nelms *et al.*, 1998; Lock *et al.*, 1999) and with an adapter protein, Nck (Jones and Dumont, 1998; Lock *et al.*, 1999).

Although it was found that ectopic overexpression of p62^{dok} upregulated motility of chinese hamster ovary cells (Noguchi *et al.*, 1999), more recent studies clearly show that p62^{dok}, at least in B cells, is a negative regulator of MAP kinase and cell proliferation (Tamir *et al.*, 2000; Yamanashi *et al.*, 2000). Inactivation of the p62^{dok} gene by homologous recombination has shown that upon B cell receptor cross-linking, p62^{dok} suppresses MAP kinase and is dispensable for FcγRIIB-mediated negative regulation of cell proliferation (Yamanashi *et al.*, 2000). Furthermore, transfection experiments in a B cell line, A20, suggest that Dok-L/Dok-3 is also a negative regulator of immunoreceptor signaling (Lemay *et al.*, 2000). In contrast to the p62^{dok} and Dok-L/Dok-3 genes, p56^{dok-2} gene expression is not normally detectable in B cells but is high in T cells and macrophages (Nelms *et al.*, 1998; Lemay *et al.*, 2000). Therefore, p56^{dok-2} is thought to have a role in T cells and macrophages. In fact, the results of experiments done with the hairless (*hr*) strain of mutant mice raise the possibility that p56^{dok-2} negatively regulates the proliferation of T cells but not of B cells: axillary lymph node T cells of *hr/hr* mice expressed 3- to 5-fold less p56^{dok-2} than wild-type mice and were hyper-responsive to stimulation with IL-2 and T cell receptor activation (Nelms *et al.*, 1998). However, this result is not necessarily evidence that p56^{dok-2} functions as a growth inhibitory molecule since the hairless phenotype is thought to result from the disruption of a gene termed *hairless*, a putative zinc-finger gene, not the p56^{dok-2} gene (Cachon-Gonzalez *et al.*, 1994). Based on an overexpression study and antisense inhibition of gene expression, we show here that p56^{dok-2} functions as a negative regulator of cell proliferation induced by a range of cytokines. Importantly, we show that the expression of such a negative regulator is induced by cytokines themselves.

Results

Identification of p56^{dok-2} as a cytokine-inducible gene

To obtain genes whose induction is mediated by cytokines, we employed the cDNA library subtraction technique (Diatchenko *et al.*, 1996). We prepared a cDNA library of

Fig. 1. Northern blot (A and B) and immunoblot analyses (C) of p56^{dok-2}. (A) Bone marrow macrophages, peritoneal macrophages or M-NFS-60 cells were factor depleted and then stimulated with M-CSF for the periods indicated. J774A.1 cells were serum depleted for 24 h. Total RNA (5 µg/lane) was blotted to membrane and hybridized with probe to p56^{dok-2} or p62^{dok}. To verify the amount of RNA loaded, the same blots were hybridized with GAPDH probe. Exposure times: top and middle panels, 14 h; bottom panel, 1 h. (B) Bone marrow macrophages were factor depleted for 12 h and then stimulated with either M-CSF, GM-CSF or IL-3 for the periods indicated. Total RNA (5 µg/lane) was blotted to membrane and hybridized with probes to p56^{dok-2} or GAPDH. Exposure times: top panel, 12 h; bottom panel, 2 h. (C) M-NFS-60 cells were factor depleted for 4 h and then stimulated with M-CSF for the periods indicated. The cleared cell lysates containing equal amounts of protein (5 µg/lane) were blotted to membrane and probed with anti-p56^{dok-2} antibody. To verify the amount of protein loaded, the same blot was probed with anti-rasGAP antibody. (A, B and C) Data shown are representative of two independent experiments with similar results.

murine bone marrow-derived macrophages cultured in cytokine-free medium and subtracted this cDNA library from that of cells stimulated with a macrophage-specific growth factor, M-CSF, for 3 h. To identify genes induced by M-CSF, clones isolated randomly from the subtracted library were screened by northern blot analysis. The study identifies a number of novel or known genes (S.Suzu, K.Nomaguchi, M.Yamada, H.Hayasawa, F.Kimura and K.Motoyoshi, manuscript in preparation). One of the genes frequently isolated by these procedures was that encoding p56^{dok-2}.

The induction of the p56^{dok-2} gene by M-CSF was observed in murine bone marrow-derived macrophages, murine peritoneal macrophages and factor-dependent murine myeloid leukemia M-NFS-60 cells (Figure 1A, top panel). In contrast to these primary macrophages and M-NFS-60 cells, the immortalized murine macrophage-like tumor cells, J774A.1, expressed p56^{dok-2} transcript at a high level even when the cells were serum depleted for 24 h (Figure 1A, top panel). The relatively high expression of

the $p56^{dok-2}$ gene in J774A.1 cells was also noted by others (Lemay *et al.*, 2000). No obvious change in $p62^{dok}$ expression was observed in these cells (Figure 1A, middle panel). A major $p56^{dok-2}$ transcript of 2 kb was induced within 1.5 h after stimulation of bone marrow macrophages with M-CSF and the expression was maintained thereafter (Figure 1B). A minor transcript of 3 kb, which was noted earlier by others (Jones and Dumont, 1998), was also detected (Figure 1B, upper band). $p56^{dok-2}$ could be induced not only by M-CSF but also by other growth factors for macrophages: GM-CSF and IL-3 induced $p56^{dok-2}$ expression in bone marrow macrophages (Figure 1B). The induction of $p56^{dok-2}$ protein was confirmed by an immunoblotting analysis. The induced expression in M-NFS-60 cells was detected within 3 h of stimulation with M-CSF and the level of expression increased until 18 h (Figure 1C). Collectively, these results clearly indicate that $p56^{dok-2}$ is a cytokine-inducible gene.

Suppression of cell proliferation by forced expression of $p56^{dok-2}$

To prove the proposed hypothesis that $p56^{dok-2}$ negatively regulates cell proliferation, we attempted to express $p56^{dok-2}$ cDNA using a strong constitutive promoter (Mizushima and Nagata, 1990; Kimura *et al.*, 1994; Suzu *et al.*, 1998) in factor-dependent murine myeloid leukemia M-NFS-60 cells (Nakoinz *et al.*, 1990; Suzu *et al.*, 1997). The cells were shown to express more $p56^{dok-2}$ gene in response to M-CSF stimulation (see Figure 1A). The cells were also known to proliferate well in response to M-CSF and weakly in response to SCF, IL-3 or granulocyte-CSF (G-CSF) (Nakoinz *et al.*, 1990). The transfectants were screened for their expression of $p56^{dok-2}$ protein by immunoblotting. Few of the G418-resistant clones obtained showed an increase in the expression of $p56^{dok-2}$ but four independent clones that expressed more $p56^{dok-2}$ than those obtained by the transfection of empty vector were isolated (Figure 2A). As shown in Figure 2B, all clones of M-NFS-60 cells expressing $p56^{dok-2}$ (NFS-dok2, clones 7, 17, 25 and 26) showed a reduced proliferation rate in response to M-CSF when compared with parental cells (NFS-wt) or cells transfected with empty vector (NFS-vector, clones 1 and 2). Among $p56^{dok-2}$ -expressing cells, clones 25 and 26, which expressed more $p56^{dok-2}$ than clone 7 or 17 (see Figure 2A), showed a markedly reduced proliferation rate (Figure 2B). If clone 26 was maintained in the antibiotic G418-free medium, the expression of $p56^{dok-2}$ was lost with time and the cells regained their responsiveness to M-CSF (data not shown), excluding the possibility of a change that affected the intrinsic ability of the cells to proliferate. The inhibitory effect of the constitutive expression of $p56^{dok-2}$ was not specific for M-CSF-dependent cell proliferation. Even if the $p56^{dok-2}$ -overexpressing M-NFS-60 cells (clone 26, see Figure 2A and B) were cultured in the presence of SCF, IL-3 or G-CSF, they showed a reduced proliferative response to these cytokines (Figure 2C). These results suggest that $p56^{dok-2}$ is a negative regulator of cell proliferation mediated by a range of cytokines in M-NFS-60 cells.

Fig. 2. Establishment of transfectants of M-NFS-60 cells expressing $p56^{dok-2}$ and their proliferation rates. (A) M-NFS-60 cells were transfected with empty vector (NFS-vector) or with $p56^{dok-2}$ cDNA (NFS-dok2). The transfectants were analyzed for their expression of $p56^{dok-2}$ protein by immunoblotting. The M-NFS-60 cell lines were factor depleted for 4 h in RPMI 1640 medium containing 1% BSA and the cell lysates were prepared from the cells. The cleared cell lysates containing equal amounts of protein (5 μ g/lane) were blotted to membrane and probed with anti- $p56^{dok-2}$ antibody. To verify the amount of protein loaded, the same blot was probed with anti-rasGAP antibody. Data shown are representative of two independent experiments with similar results. (B) The proliferation of parental M-NFS-60 cells (NFS-wt), cells transfected with empty vector (NFS-vector) or cells transfected with $p56^{dok-2}$ cDNA (NFS-dok2) in response to M-CSF. The cells were seeded at a density of 1×10^4 cells/ml and cultured for 2 or 3 days in medium containing 10% FCS and M-CSF. Error bars from triplicate assays are shown. These results are representative of three independent experiments. (C) The proliferation of parental M-NFS-60 cells (open bars) or cells expressing $p56^{dok-2}$ (clone 26, see A and B) (solid bars) cultured in the absence of factor (None), or the presence of SCF, IL-3 or G-CSF. Cells were seeded at a density of 2×10^3 cells/well and cultured for 72 h. The cells were then pulsed with [³H]thymidine and the incorporated radioactivity was measured. Error bars from triplicate assays are shown. These results are representative of three independent experiments.

Analyses of $p56^{dok-2}$ expression and peritoneal cells in *hr/hr* mice

The $p56^{dok-2}$ gene was shown to be localized to mouse chromosome 14 at a previously characterized locus termed *hairless* (Nelms *et al.*, 1998), but the *hairless* phenotype, lymphadenopathy and splenomegaly, is thought to result from the disruption of a putative zinc-finger gene termed *hairless*, not the $p56^{dok-2}$ gene (Cachon-Gonzalez *et al.*, 1994). Nevertheless, lymph node T cells from mice homozygous for the *hairless* allele (*hr/hr*) have a significantly lower level of $p56^{dok-2}$ transcript than those from

Fig. 3. Analyses of p56^{dok-2} expression and peritoneal cells in *hr/hr* mice. (A) Total RNA from bone marrow cells or spleen cells of 10- or 13-week-old *hr/hr* mice and heterozygous control (+/*hr*) mice was prepared, blotted to membrane (5 µg/lane for bone marrow cells and 2.5 µg/lane for spleen cells) and then hybridized with probe to p56^{dok-2} or GAPDH. Exposure times: top panel, 3 days; bottom panel, 1 h. (B) FACS profiles of peritoneal cells from *hr/hr* and +/*hr* mice. Cells were stained with anti-Mac-1 antibody and analyzed by flow cytometry. (C) Wright-Giemsa-stained cytopsin preparations of peritoneal cells collected from *hr/hr* and +/*hr* mice. Arrows indicate typical cells with macrophage morphology. (B and C) Data shown are representative of an analysis with 8 *hr/hr* mice and 8 +/*hr* mice 13 weeks old. The experiments were repeated using 10-week-old mice.

heterozygous (+/*hr*) mice, which may be due to the defect of the putative zinc-finger gene (Nelms *et al.*, 1998). We also found that both bone marrow cells and spleen cells from *hr/hr* mice expressed less p56^{dok-2} transcript than those from +/*hr* mice (Figure 3A). Thus, the mutant mice are thought to be useful for analyzing the physiological role of p56^{dok-2}.

Because we identified p56^{dok-2} as a cytokine-inducible gene in macrophages (see Figure 1), we attempted to analyze the change in the mononuclear phagocyte system in *hr/hr* mice. For this purpose, we compared the phenotype of peritoneal cells of *hr/hr* mice with that of +/*hr* control mice. Although there was no change in the total number of peritoneal cells between *hr/hr* mice and +/*hr* mice ($3.9 \pm 0.4 \times 10^5$ cells for *hr/hr* and $4.1 \pm 1.2 \times 10^5$ cells for +/*hr*), the percentage of cells expressing a surface marker for macrophages, Mac-1, of *hr/hr* mice was higher than that of +/*hr* mice ($30 \pm 6\%$ for *hr/hr* mice and $14 \pm 2\%$ for +/*hr* mice) (Figure 3B). There was no difference in the median log fluorescence on Mac-1-positive cells between *hr/hr* mice and +/*hr* mice (Figure 3B). The fact that the peritoneal cavity of *hr/hr* mice contained more macrophages than that of +/*hr* mice was confirmed by enumerating cells with macrophage morphology in cytocentrifuge preparations (Figure 3C). Our data and the result reported previously with *hr/hr* mice

Fig. 4. Establishment of transfectants of J774A.1 cells expressing antisense p56^{dok-2} mRNA and its proliferation rate. (A) J774A.1 cells were transfected with empty vector (J774-vector) or with vector containing the full-length p56^{dok-2} cDNA in the antisense orientation (J774-AS-dok2). The transfectants were analyzed for their expression of p56^{dok-2} protein by immunoblotting. At the exponential growth stage, cells were harvested and lysates were prepared from the cells. The cleared cell lysates containing equal amounts of protein (2.5 µg/lane) were blotted to membrane and probed with anti-p56^{dok-2} antibody. To verify the amount of protein loaded, the same blot was probed with anti-rasGAP antibody. Data shown are representative of two independent experiments with similar results. (B) The proliferation of J774A.1 cells transfected with empty vector (J774-vector) or cells transfected with antisense p56^{dok-2} cDNA (J774-AS-dok2). The cells were seeded at a density of 1×10^4 cells/ml and cultured for 3 (open bars) or 4 days (solid bars) in medium containing 10% FCS. Error bars from triplicate assays are shown. These results are representative of three independent experiments.

(Nelms *et al.*, 1998) raise the possibility that p56^{dok-2} is critical in regulating the proliferation of at least macrophages and T cells.

Acceleration of cell proliferation by antisense inhibition of p56^{dok-2} expression

To prove more convincingly that p56^{dok-2} is a negative regulator of cell proliferation of macrophages, we attempted to express antisense p56^{dok-2} mRNA in macrophage-like J774A.1 cells. The cells were shown to constitutively express p56^{dok-2} transcript at a high level (see Figure 1A). The cells were transfected with a selectable expression plasmid containing full-length p56^{dok-2} cDNA in the antisense orientation. As a control, the cells were transfected with empty vector. Transfected clones were selected in the presence of G418. A total of four antisense-transfected G418-resistant clones was isolated and a clone (J774-AS-dok2) that showed a significant decrease in the level of p56^{dok-2} protein compared with two control clones (J774-vector, clones 1 and 2) was obtained (Figure 4A). Among the control clones, clone 1 contained slightly less p56^{dok-2} protein than clone 2 (Figure 2A), and the level of p56^{dok-2} protein of clone 2 was comparable to that of parental cells (data not shown). If the antisense clone was maintained in G418-free medium, the level of p56^{dok-2} protein returned to that of parental cells (data not shown). Thus, the proliferation rate of the antisense clone

Fig. 5. Expression of immediate-early response genes in M-NFS-60 cells. Parental M-NFS-60 cells (NFS-wt) or p56^{dok-2}-overexpressing M-NFS-60 cells (NFS-dok2) (clone 26, see Figure 2) were factor depleted for 4 h in RPMI 1640 medium containing 1% BSA and then stimulated with M-CSF for the periods indicated. Total RNA (5 µg/lane) was blotted to membrane and hybridized with probe to *c-myc*, *c-jun*, *junB*, *c-fos* or GAPDH. Exposure times: *c-myc* and GAPDH, 4 h; *c-jun*, *junB* and *c-fos*, 8 h. Data shown are representative of two independent experiments with similar results.

was compared with that of the control clones maintained in G418-containing medium. Although J774A.1 cells are immortalized tumor cells and proliferate in the absence of exogenously added growth factor (Fan *et al.*, 1993), the antisense clone (J774-AS-dok2) showed an accelerated proliferation compared with two control clones (J774-vector, clones 1 and 2) (Figure 4B). Among clones obtained by transfection with empty vector, clone 1, which contained slightly less p56^{dok-2} protein than clone 2 (Figure 4A), proliferated slightly more quickly than clone 2 (Figure 4B). These results strongly suggest that p56^{dok-2} functions as a negative regulator of cell proliferation.

Effects of overexpression of p56^{dok-2} on M-CSF-induced activation of the signaling pathway

We next attempted to clarify the mechanism by which the constitutive overexpression of p56^{dok-2} inhibited the proliferation of M-NFS-60 cells in response to M-CSF. M-CSF receptor, encoded by the *c-fms* proto-oncogene, is a member of the receptor tyrosine kinase family (Sherr *et al.*, 1985). Ligand binding leads to the autophosphorylation of M-CSF receptor and the assembly of phosphotyrosine-dependent signaling complexes (reviewed in Rohrschneider, 1995). Proteins interacting directly with M-CSF receptor have been described. Of these, Src family kinases are shown to associate with phosphotyrosine 559 in the juxtamembrane region of M-CSF receptor (Alonso *et al.*, 1995). Furthermore, the activation of Src kinases and the subsequent induction of an immediately early response gene, *c-myc*, have been shown to be critical for M-CSF-dependent cell proliferation (Roussel *et al.*, 1991; Roche *et al.*, 1995). We therefore assumed that the constitutive overexpression of p56^{dok-2} affected the Src-*c-myc* pathway.

M-CSF stimulation induced the expression of the immediate-early response genes, *c-myc*, *c-jun*, *junB* and *c-fos* in parental M-NFS-60 cells (Figure 5). However, induction of the *c-myc* gene by M-CSF in p56^{dok-2}-overexpressing M-NFS-60 cells was severely impaired (Figure 5). Induction of *junB*- or *c-fos* mRNA in the two

Fig. 6. Tyrosine phosphorylation of p56^{dok-2} in M-NFS-60 cells. (A) Parental M-NFS-60 cells were factor depleted for 4 h in RPMI 1640 medium containing 1% BSA, treated with PP2 for the last 30 min or left untreated, and then stimulated with M-CSF for the periods indicated. (B) Parental M-NFS-60 cells (NFS-wt) or p56^{dok-2}-overexpressing M-NFS-60 cells (NFS-dok2) (clone 26, see Figure 2) were factor depleted for 4 h in RPMI 1640 medium containing 1% BSA and then treated with M-CSF for the periods indicated. (A and B) Immunoprecipitates (IP) were obtained with anti-p56^{dok-2} antibody (goat polyclonal antibody) and analyzed by immunoblotting (Blot) with anti-phosphotyrosine antibody (pTyr). The blots were reprobed with anti-p56^{dok-2} antibody (rabbit antiserum). Data shown are representative of two independent experiments with similar results.

cell lines was comparable and induction of *c-jun* in p56^{dok-2}-overexpressing cells was slightly higher than in parental cells (Figure 5). In any case, the finding that induction of *c-myc* is severely impaired in p56^{dok-2}-overexpressing cells, which show a reduced proliferation rate (see Figure 2), is consistent with the notion that *c-myc* is a key regulator of M-CSF-dependent cell proliferation.

We next examined whether overexpression of p56^{dok-2} affected the activation of Src family kinases. While p56^{dok-2} was originally identified as a substrate of Bcr-Abl tyrosine kinase (Di Cristofano *et al.*, 1998) and a protein capable of interacting with the intracellular domains of IL-4 receptor (Nelms *et al.*, 1998) and with Tek/Tie2 receptor tyrosine kinase (Jones and Dumont, 1998), it was recently identified as a substrate of Src family kinases (Lock *et al.*, 1999). We found that M-CSF stimulation was able to induce the tyrosine phosphorylation of p56^{dok-2} in M-NFS-60 cells and that the phosphorylation was severely reduced when the cells were pretreated with Src family kinase inhibitor, PP2 (Figure 6A), confirming that p56^{dok-2} was a substrate of Src family kinases in the cells. Furthermore, we found that p56^{dok-2} could be phosphorylated in p56^{dok-2}-overexpressing cells upon M-CSF stimulation and that the level of the phosphorylation in the cells was equivalent to that in parental cells (Figure 6B). It therefore seems likely that constitutive overexpression of p56^{dok-2} affected the cascade downstream of Src family kinases but not the activation of the kinases itself.

The Src family kinase-mediated signaling pathway that regulates the expression of *c-myc* gene has remained largely unresolved. However, a recent study raises the possibility that the induction of *c-myc* by Src family kinases is in part dependent on activation of the ras/MAP kinase pathway (Cheng *et al.*, 1999). An adapter molecule, Shc, is a good candidate that links Src family kinases to

Fig. 7. Effect of constitutive overexpression of p56^{dok-2} on tyrosine phosphorylation of Shc and its association with Grb2 (A) and on activation of ERK2 (B). (A and B) Parental M-NFS-60 cells (NFS-wt) or p56^{dok-2}-overexpressing M-NFS-60 cells (NFS-dok2) (clone 26, see Figure 2) were factor depleted for 4 h in RPMI 1640 medium containing 1% BSA and then treated with M-CSF for the periods indicated. (A) Immunoprecipitates (IP) were obtained with anti-Shc antibody (rabbit polyclonal) and analyzed by immunoblotting (Blot) with antibodies to phosphotyrosine (pTyr), Grb2 and Shc (mouse monoclonal IgG). Alternatively, immunoprecipitates were obtained with anti-Grb2 antibody (rabbit polyclonal IgG) and analyzed by immunoblotting with antibodies to Shc and Grb2 (mouse monoclonal IgG). Data shown are representative of two independent experiments with similar results. (B) The cleared cell lysates containing equal amounts of protein (5 µg/lane) were blotted to membrane and probed with antibodies to phosphorylated ERK (pERK) and total ERK (ERK). Data shown are representative of four independent experiments with similar results.

the ras/MAP kinase pathway (reviewed in Corey and Anderson, 1999). We found that Shc was tyrosine phosphorylated rapidly in response to M-CSF stimulation in parental cells but the phosphorylation was severely reduced in p56^{dok-2}-overexpressing cells (Figure 7A). Consistent with the reduction in Shc phosphorylation, the association between Shc and Grb2 in p56^{dok-2}-overexpressing cells was reduced (Figure 7A). Similarly, there was a diminution in the activation of ERK2, one of the MAP kinases, in p56^{dok-2}-overexpressing cells (Figure 7B). However, the diminution in the activation of ERK2 was modest when compared with that in Shc phosphorylation (Figure 7A) and in *c-myc* induction (see Figure 5). Therefore, although the inhibitory effect of p56^{dok-2} on the induction of the *c-myc* gene might be in part dependent on the ras/MAP kinase pathway, it is obvious that other Src kinase-mediated signaling pathways that regulate the induction of *c-myc* are affected by p56^{dok-2}.

Discussion

In this study, we have provided evidence that p56^{dok-2} functions as a negative regulator of cell proliferation.

Forced expression of p56^{dok-2} suppressed M-CSF-, SCF-, IL-3- and G-CSF-dependent proliferation of M-NFS-60 cells (Figure 2). Moreover, the inhibition of endogenous p56^{dok-2} expression in J774A.1 cells by stable expression of antisense p56^{dok-2} mRNA accelerated proliferation of the cells (Figure 4). Recently, Yamanashi *et al.* (2000) generated mice that lack p62^{dok}, which is closely related to p56^{dok-2}, and clearly demonstrated that p62^{dok}, at least in B cells, is a negative regulator of cell proliferation. In contrast to the p62^{dok} gene, the p56^{dok-2} gene is not normally detectable in B cells but is present in T cells and macrophages (Nelms *et al.*, 1998; Lemay *et al.*, 2000). Therefore, p56^{dok-2} might function as a negative regulator of cell proliferation in T cells or macrophages. Studies with *hr/hr* mice support this hypothesis. Axillary lymph node T cells, bone marrow cells and spleen cells of *hr/hr* mice expressed less p56^{dok-2} transcript than those of *+/hr* mice (Nelms *et al.*, 1998; Figure 3A). The mice have lymphadenopathy of axillary lymph nodes (Nelms *et al.*, 1998) and the number of macrophages in the peritoneal cavity is significantly higher in *hr/hr* mice than in *+/hr* mice (Figure 3), in both of which the increase might result from cellular hyperproliferation due to the impaired negative regulation of p56^{dok-2}. However, once again, we must mention that there is no direct evidence that the immunologic/hematologic phenotype of *hr/hr* mice is caused by the altered expression of p56^{dok-2} since the hairless phenotype is thought to result from the disruption of a putative zinc-finger gene, *hairless* (Cachon-Gonzalez *et al.*, 1994). The precise physiological role of p56^{dok-2} will be clarified further by generating mice that lack this gene.

Another noteworthy finding of our study is that p56^{dok-2} is a cytokine-inducible gene. Expression of p56^{dok-2} in macrophages is induced by M-CSF, GM-CSF and IL-3 (Figure 1B). Similarly, p56^{dok-2} is induced in M-NFS-60 cells by M-CSF (Figure 1A). None of the *dok* family genes has been shown to be transcriptionally regulated by extracellular stimuli including cytokine stimulation. Because p56^{dok-2} suppresses cytokine-mediated cell proliferation, it becomes clear that p56^{dok-2} can act in a feedback loop that negatively regulates cytokine-mediated cell proliferation. Although we found that p62^{dok} was not induced in macrophages by M-CSF (see Figure 1A), the assessment of p62^{dok} or Dok-L/Dok-3 gene as a cytokine-inducible gene will be clarified further by analyses with other cell types and other cytokines.

Although the induction of p56^{dok-2} expression in M-NFS-60 cells by M-CSF was obvious (Figure 1A), that by either IL-3, G-CSF or SCF was detectable but slight (data not shown). Since M-NFS-60 cells proliferate well in response to M-CSF and weakly in response to IL-3, G-CSF or SCF (Nakoinz *et al.*, 1990), the expression of the p56^{dok-2} gene may be regulated by a signal transduction pathway that transmits proliferative signal.

Based on its structural features, p56^{dok-2} has been thought to act as a multiple docking protein downstream of receptor or non-receptor tyrosine kinases. However, the role of p56^{dok-2} in tyrosine kinase-mediated signal transduction is largely unknown. p56^{dok-2} and p62^{dok} were first noted for their association with rasGAP upon phosphorylation (Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997; Di Cristofano *et al.*, 1998; Jones and Dumont, 1998; Nelms *et al.*, 1998). Since rasGAP is

capable of negatively regulating ras by enhancing its intrinsic GTPase activity, one attractive model might be that p56^{dok-2} links rasGAP to the attenuation of ras signaling. However, in contrast to p56^{dok-2} and p62^{dok}, Dok-L/Dok-3, the third member of the *dok* family, does not bind rasGAP (Cong *et al.*, 1999; Lemay *et al.*, 2000). Furthermore, p56^{dok-2} was shown to attenuate epidermal growth factor-stimulated activation of MAP kinase independently of its association with rasGAP (Jones and Dumont, 1999). Thus, the inhibitory effect of p56^{dok-2} on cell proliferation may be independent of its association with rasGAP and ras signaling. In this study, we found that the induction of *c-jun* or *c-fos* in response to M-CSF was not inhibited by the constitutive overexpression of p56^{dok-2} (Figure 5). Since the induction of *c-jun* or *c-fos* has been shown to be dependent on ras function (Barone and Courtneidge, 1995), p56^{dok-2} is thought to be involved in a signaling pathway other than the ras pathway.

We found that the induction of *c-myc* in response to M-CSF was severely impaired in p56^{dok-2}-overexpressing cells (Figure 5). The induction of *c-myc* has been shown to be required for M-CSF-dependent cell proliferation based on the following observations. One of the major autophosphorylation sites within ligand-stimulated M-CSF receptor is tyrosine 809, which is located in the activation loop of the kinase domain (Roussel *et al.*, 1990). Substitution of this residue with phenylalanine (Y809F) leads to reduction but not abolition of receptor protein tyrosine kinase activity (Davis *et al.*, 1997). However, NIH 3T3 cells expressing the mutant M-CSF receptor (Y809F) fail to proliferate in response to M-CSF (Roussel *et al.*, 1990) and to induce *c-myc* (Roussel *et al.*, 1991). Finally, enforced expression of *c-myc* restores the ability of the cells expressing the mutant M-CSF receptor to proliferate in response to M-CSF (Roussel *et al.*, 1991). Therefore, the finding that the induction of *c-myc* is severely impaired in p56^{dok-2}-overexpressing cells, which show a reduced proliferation rate, is consistent with the notion that *c-myc* is a key regulator of M-CSF-dependent cell proliferation.

Src family kinases have been shown to activate a pathway that mediates the induction of *c-myc* and to be required for M-CSF-dependent cell proliferation based on the following observations: (i) the constitutive expression of *c-myc* rescues a block to DNA synthesis elicited by a dominant-negative form of Src (Barone and Courtneidge, 1995); (ii) Src kinases associate with and are activated by the M-CSF receptor (Courtneidge *et al.*, 1993); and (iii) the microinjection of antibodies to Src kinases inhibits DNA synthesis in response to M-CSF (Roche *et al.*, 1995). However, Src family kinase-mediated signaling pathways that regulate the expression of the *c-myc* gene have remained largely unresolved. Our data indicate that the overexpression of p56^{dok-2} affected the cascade downstream of Src family kinases rather than the activation of the kinases itself (Figure 6). Biochemical and genetic evidence has also indicated the importance of Shc in initiating the signaling cascade by Src kinases. For example, there is a loss of tyrosine phosphorylation of Shc in *lyn*-deficient cells (Nagai *et al.*, 1995; Ptasznik *et al.*, 1995) and overexpression of Fyn is associated with the formation of a complex between Fyn and Shc (Li *et al.*, 1996). Shc participates in early signaling complexes of the M-CSF receptor but does not bind directly to the receptor

(Lioubin *et al.*, 1994), suggesting that Shc is a substrate of Src kinases activated by the M-CSF receptor but not of the receptor itself. p56^{dok-2} is also a substrate of Src kinases (Lock *et al.*, 1999; Figure 6A). We found that tyrosine phosphorylation of Shc was severely reduced in p56^{dok-2}-overexpressing cells (Figure 7A). These findings raise the possibility that p56^{dok-2} competes with Shc for Src kinases and thereby inhibits the Src kinase-initiating pathway. However, more studies will be needed to clarify the inhibitory mechanism of p56^{dok-2} in the Src kinase-*c-myc* pathway.

The phosphorylation of Shc by Src family kinases leads to the association of Shc with Grb2-Sos guanine nucleotide exchange complex (Rozakis-Adcock *et al.*, 1992; Myers *et al.*, 1994; Salcini *et al.*, 1994). This molecular linkage results in the accumulation of activated ras-GTP, which activates raf kinase, which in turn initiates the MAP kinase cascade. In fact, although an earlier study suggested that Src kinases induced *c-myc* in a ras/MAP kinase pathway-independent manner (Barone and Courtneidge, 1995), a more recent study raises the possibility that the induction of *c-myc* by Src family kinases is in part dependent on the ras/MAP kinase pathway (Cheng *et al.*, 1999). Consistent with this notion, we observed a reduction in the association of Shc with Grb2 (Figure 7A) and in the activation of ERK2 in M-CSF-stimulated p56^{dok-2}-overexpressing cells (Figure 7B). However, the reduction in the activation of ERK2 was modest when compared with the reduction in *c-myc* induction (Figure 5). Therefore, it is obvious that unidentified Src kinase-mediated signaling pathways that regulate the induction of *c-myc* are also affected by p56^{dok-2}.

In this study, we identify p56^{dok-2} as a molecule that negatively regulates cell proliferation and signal transduction mediated by cytokines in a feedback loop. The CIS/JAB/SOCS/SSI family proteins are also known as inhibitors that act in a similar manner. The inhibitory effect of these proteins appeared to be specific for the JAK/STAT pathway (Yoshimura *et al.*, 1995; Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997). A recent study showed that the overexpression of CIS-1, SOCS-1, SOCS-2 or SOCS-3 in murine myeloid leukemia M1 cells had no effect on M-CSF-mediated cellular response such as tyrosine phosphorylation of STAT3, whereas the overexpression of SOCS-1 or SOCS-3 in the cells inhibited IL-6-mediated cellular response (Novak *et al.*, 1999). The result is in contrast to our finding that overexpression of p56^{dok-2} inhibited M-CSF-mediated cell proliferation and signal transduction. Therefore, the inhibitory effect of p56^{dok-2} may be different from that of CIS/JAB/SOCS/SSI family proteins. More studies will be needed to understand fully the inhibitory mechanism of p56^{dok-2}.

Materials and methods

Library subtraction

The murine bone marrow macrophages were prepared by culturing femoral bone marrow cells from C57Bl/6 mice (Charles River) with 100 ng/ml M-CSF for 7 days (Suzu *et al.*, 1997). Cells were factor depleted for 12 h in RPMI 1640 medium containing 10% fetal calf serum (FCS) and then treated with 100 ng/ml M-CSF for 3 h. Poly(A) RNA from untreated cells or from those treated with M-CSF was prepared using a mRNA separator kit (Clontech). cDNA library construction and library subtraction were performed according to the manufacturer's instructions

(PCR-Select cDNA subtraction kit, Clontech). The cDNA fragments of the subtracted cDNA library were cloned into pCR2.1 vector (Invitrogen). Randomly isolated clones were further analyzed by direct sequencing and by northern hybridization using total RNA from unstimulated and M-CSF-stimulated bone marrow macrophages.

Cell culture and transfection

The factor-dependent murine myeloid leukemia M-NFS-60 cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 100 ng/ml M-CSF (Nakoinz *et al.*, 1990; Suzu *et al.*, 1997). The immortalized macrophage-like tumor J774A.1 cells were maintained in RPMI 1640 medium supplemented with 10% FCS (Fan *et al.*, 1993). The murine p56^{dok-2} cDNA was obtained by RT-PCR using the primers 5'-AGGGCAGAGGCAATGGCAGTGGGGA and 5'-CCACCA TTTCCCTCCACAGCTTCC (DDBJ/EMBL/GenBank accession No. AF030627). The murine p56^{dok-2} cDNA containing the entire coding region was subcloned into an expression vector, pCEF, which was made by inserting the promoter region of polypeptide chain elongation factor-1 (Mizushima and Nagata, 1990; Kimura *et al.*, 1994; Suzu *et al.*, 1998) into pRc/CMV vector (Invitrogen). The resulting plasmid DNA or pCEF vector containing no insert was transfected into M-NFS-60 cells by using Lipofectamine reagent (Gibco-BRL). The transfected cells were selected in medium containing 200 µg/ml G418 (Geneticin; Gibco-BRL) and M-CSF, and were screened by immunoblotting using anti-p56^{dok-2} antibody (kindly provided by Dr W.E.Paul). Alternatively, the murine p56^{dok-2} cDNA containing the entire coding region was subcloned into pCEF vector in the antisense orientation. Then, the plasmid or pCEF vector containing no insert was transfected into J774A.1 cells as described above. The transfected cells were selected in medium containing 400 µg/ml G418. Expression levels of p56^{dok-2} were determined by immunoblot analysis.

Cell proliferation assay

M-NFS-60 cells were seeded into 6-well 35 mm plates at a density of 1×10^4 cells/ml and cultured for 2 or 3 days in RPMI 1640 medium containing 10% FCS and 100 ng/ml M-CSF. At the end of the cultures, cells were harvested and counted by the trypan blue dye exclusion method. Alternatively, the cells were seeded into 96-well plates (2×10^3 cells/well) and cultured for 72 h in the presence of SCF (100 ng/ml; Genzyme), G-CSF (10 ng/ml; R&D Systems) or IL-3 (10 ng/ml; Gibco-BRL), or in the absence of factor. Cells were then pulsed with [³H]thymidine for 4 h and the incorporated radioactivity was measured by liquid scintillation counting (Suzu *et al.*, 1998). J774A.1 cells were seeded into 6-well 35 mm plates at a density of 1×10^4 cells/ml and cultured for 3 or 4 days in RPMI 1640 medium containing 10% FCS. At the end of the cultures, cells were harvested and counted.

Northern blot analysis

The bone marrow macrophages were prepared as described above. Murine peritoneal macrophages were prepared by culturing peritoneal cells from C57Bl/6 mice with RPMI 1640 medium containing 10% FCS and 100 ng/ml M-CSF for 7 days. These cells were factor depleted for 12 h in RPMI medium containing 10% FCS. M-NFS-60 cells were factor depleted for 4 h in RPMI medium containing 1% bovine serum albumin (BSA). Cells were then treated with either M-CSF (100 ng/ml), GM-CSF (10 ng/ml) or IL-3 (10 ng/ml) for various periods. J774A.1 cells were serum depleted for 24 h in RPMI 1640 medium. Total RNAs from these cells were isolated using RNazol B reagent (Tel-Test), electrophoresed on agarose gels and transferred to a nylon membrane (Hybond N⁺, Amersham). The membrane was hybridized with radiolabeled cDNA probes (Suzu *et al.*, 1998). The murine p56^{dok-2} cDNA containing the entire coding region described above was used as a probe. A murine p62^{dok} cDNA was prepared by RT-PCR using the primers 5'-ATGGACGGGCTGTGATGGAGGGTC and 5'-CACCCCATTGG ACCTCCTATCAGCA (DDBJ/EMBL/GenBank accession No. U78818). cDNAs for murine *c-myc*, *c-jun*, *junB* and *c-fos* were also prepared by RT-PCR and used as probes. Primers for *c-myc* and *c-jun* were purchased from Clontech, and primers for *c-fos* were from Stratagene. Primers for *junB* were 5'-AACAGCCTTTCTATCAGCAGCTC and 5'-GGTTCA TCTGTGTCAGTCCGTCAG (DDBJ/EMBL/GenBank accession No. NM008416). A cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Clontech.

Immunoblot analysis

M-NFS-60 cells were factor depleted for 4 h in RPMI 1640 medium containing 1% BSA. In one experiment, cells were treated with the Src kinase inhibitor, PP2 (10 µM; Calbiochem), for the last 30 min. Cells

were then treated with 100 ng/ml M-CSF at 37°C for various periods or left untreated, and were solubilized with lysis buffer (1% Nonidet P-40, 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na₂VO₄, 1 mM NaF) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstatin). J774A.1 cells were also solubilized with the lysis buffer. The cell lysates were centrifuged, and the resulting supernatants were subjected to immunoblot analysis and immunoprecipitation. The protein concentrations of the cleared lysates were determined using the BCA protein assay reagent (Pierce). The cleared cell lysates containing equal amounts of protein were resolved by SDS-PAGE under reducing conditions and the proteins were transferred to a nylon membrane (Hybond-P, Amersham). The membrane was probed with antibodies to p56^{dok-2} (provided by Dr W.E.Paul), rasGAP (B4F8; Santa Cruz), total ERK (K-23; Santa Cruz) or activated ERK (E-4; Santa Cruz). The antibodies were visualized with horseradish peroxidase-coupled anti-immunoglobulin (Santa Cruz) using the Enhanced Chemiluminescence Western Blotting Detection System (Amersham) according to the manufacturer's instructions. For immunoprecipitation, the cleared cell lysates containing equal amounts of protein were precleared by treatment with excess protein A/G-agarose (Santa Cruz), then incubated with antibodies to p56^{dok-2} (M-20; Santa Cruz), Shc (H-108; Santa Cruz) or Grb2 (C-23; Santa Cruz). The immune complexes were precipitated with protein A/G-agarose, washed four times with the lysis buffer, and then subjected to immunoblot analysis with antibodies to phosphotyrosine (PY99; Santa Cruz), p56^{dok-2} (provided by Dr W.E.Paul), Shc (clone 30; Transduction Laboratories) or Grb2 (clone 24; Transduction Laboratories).

Peritoneal cell analysis of hr/hr mice

hr/hr and +/hr HRS/J mice were obtained from The Jackson Laboratory. Peritoneal cells were collected from mice by lavage with phosphate-buffered saline. Cells were stained for FACS by treatment with phycoerythrin-conjugated anti-Mac-1 antibody (clone M1/70.15; Caltag Laboratories) and analyzed using EPICS PROFILE II (Coulter). Alternatively, cytocentrifuge preparations of peritoneal cells were stained with Wright-Giemsa, and cells with macrophage morphology were enumerated. Total bone marrow cells and spleen cells were obtained from hr/hr and +/hr mice. Total RNAs were isolated from the cells using RNazol B reagent and analyzed for p56^{dok-2} gene expression by northern hybridization.

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