Mona, a novel hematopoietic-specific adaptor interacting with the macrophage colony-stimulating factor receptor, is implicated in monocyte/macrophage development

Roland P.Bourette, Sylvie Arnaud, Gary M.Myles¹, Jean-Paul Blanchet, Larry R.Rohrschneider¹ and Guy Mouchiroud²

Centre de Génétique Moléculaire et Cellulaire, UMR CNRS 5534, 43 Boulevard du 11 novembre 1918, 69622 Villeurbanne Cedex, France and ¹Division of Basic Science, Fred Hutchinson Cancer Research Center, B2-152, 1100 Fairview Avenue North, Seattle, WA 98109-1024, USA

²Corresponding author
e-mail: gmouchir@biomserv.univ-lyon1.fr

The production, survival and function of monocytes and macrophages are regulated by the macrophage colony-stimulating factor (M-CSF or CSF-1) through its tyrosine kinase receptor Fms. Binding of M-CSF results in Fms autophosphorylation on specific tyrosines that act as docking sites for intracellular signaling molecules containing SH2 domains. Using a yeast two-hybrid screen, we cloned a novel adaptor protein which we called 'Mona' for monocytic adaptor. Mona contains one SH2 domain and two SH3 domains related to the Grb2 adaptor. Accordingly, Mona interacts with activated Fms on phosphorylated Tyr697, which is also the Grb2-binding site. Furthermore, Mona contains a unique proline-rich region located between the SH2 domain and the C-terminal SH3 domain, and is apparently devoid of any catalytic domain. Mona expression is restricted to two hematopoietic tissues: the spleen and the peripheral blood mononuclear cells, and is induced rapidly during monocytic differentiation of the myeloid NFS-60 cell line in response to M-CSF. Strikingly, overexpression of Mona in bone marrow cells results in strong reduction of M-CSF-dependent macrophage production *in vitro*. Taken together, our results suggest an important role for Mona in the regulation of monocyte/macrophage development as controlled by M-CSF.

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Introduction

Signaling by receptor tyrosine kinases plays a major role in regulation of developmental processes. Once activated by their ligands, these transmembrane receptors are the starting point of a cascade of events initiated by recruitment and/or activation of intracellular molecules into multi-protein signaling complexes (Ullrich and Schlessinger, 1990; Fantl *et al.*, 1993). In such a context, the specificity of receptor expression and the innate responsiveness of the cell, including the nature and concentration of the signaling molecules, will govern cell fate (Pawson and Scott, 1997). Defining all components of the initial sig-

naling complexes is thus necessary in order to understand how a receptor controls specific biological responses at different stages of development.

Activation of macrophage colony-stimulating factor (M-CSF, also called CSF-1) receptor offers an interesting example of the inherent complexity of regulating opposing processes such as cell proliferation and terminal differentiation. During hematopoiesis, M-CSF is the principal regulator of the production of monocytes and macrophages both *in vivo* and *in vitro* (Metcalf, 1989). Osteopetrotic mutant mice lack functional M-CSF and are deficient in osteoclasts and macrophages, but can be cured by injection of M-CSF (Wiktor-Jedrzejczak *et al.*, 1991). *In vitro*, M-CSF enables proliferation and differentiation of committed bone marrow progenitors, the colony-forming unit macrophage (CFU-M), into macroscopic colonies of macrophages (Stanley *et al.*, 1978). M-CSF also stimulates survival of monocytes and macrophages, and enhances their specialized functions (Stanley, 1981; Tushinsky *et al.*, 1982).

M-CSF receptor, encoded by the *c-fms* proto-oncogene (Sherr *et al.*, 1985; Woolford *et al.*, 1985), is a member of the type III receptor tyrosine kinase family, which includes α and β platelet-derived growth factor (PDGF), stem cell factor (SCF) and Flt3/FLK2 receptors. M-CSF binding to its receptor (Fms) induces dimerization of the tyrosine kinase domain of the receptor, resulting in autophosphorylation of the cytoplasmic domain on specific tyrosine residues used as binding sites for Src homology 2 (SH2) domain-containing molecules that will initiate various signaling cascades (Rohrschneider, 1995). Four such proteins interacting directly with Fms have been described. Src family members can associate with phosphotyrosine 559 in the juxtamembrane Fms region (Alonso *et al.*, 1995). Three molecules interact with the Fms kinase insert (KI): Grb2, the p85 subunit of phosphatidylinositol 3' kinase (p85-PI3K) and phospholipase C- γ 2 (PLC γ 2). Grb2 binds to phosphotyrosine 697 (van der Geer and Hunter, 1993; Lioubin *et al.*, 1994) and translocates the nucleotide exchange factor mSOS to the plasma membrane (Egan *et al.*, 1993; Li *et al.*, 1993). Both p85-PI3K and PLC γ 2 bind to phosphotyrosine 721 (Reedijk *et al.*, 1992; Bourette *et al.*, 1997). In addition, Tyr706, another autophosphorylation site located in the KI, is necessary for STAT1 activation, although its direct interaction with STAT1 remains to be demonstrated (Novak *et al.*, 1996). Other molecules not binding directly to Fms participate in early signaling complexes whose interactions are not well understood. Such molecules include Shc and Crk-II adaptors, SH2-containing inositol phosphatase SHIP and c-Cbl (Lioubin *et al.*, 1994, 1996; Husson *et al.*, 1997).

Based on the numerous biological effects of M-CSF in monocytic development, we speculated that additional

signal transduction proteins interact with activated Fms. We previously performed a yeast two-hybrid screen using the phosphorylated cytoplasmic domain of murine Fms as a bait, resulting in the demonstration of PLC γ 2 interaction with phosphotyrosine 721 and its role in the differentiation signal (Bourette *et al.*, 1997). In the same two-hybrid screen, we also identified a new SH2 domain related to Grb2. We have now cloned the full-length cDNA and characterized a novel signaling molecule of 38 kDa, whose expression is restricted to hematopoietic tissues in adult mouse (spleen and peripheral blood mononuclear cells). Furthermore, its expression was induced during monocytic differentiation of the bipotential hematopoietic cell line NFS-60. Due to the presence of an SH2, two SH3 domains and the absence of any catalytic domain, we postulate that this protein is an adaptor molecule involved in protein-protein interaction during M-CSF-dependent monocytic development. We have termed this protein 'Mona' for monocytic adaptor. Our results indicate that Mona interacts with Fms and participates in monocytic differentiation.

Results

Isolation of Mona, a novel adaptor interacting with the M-CSF receptor

The yeast two-hybrid screen (Bourette *et al.*, 1997) used a LexA-Fms cytoplasmic domain fusion protein as a bait and two different VP16 target libraries containing cDNAs derived from mouse embryos (Hollenberg *et al.*, 1995) or from the murine hematopoietic cell line EML (Tsai *et al.*, 1994). From this screen, four independent clones encoding a new SH2 domain with significant sequence similarity to the Grb2 SH2 domain were isolated from both libraries. Whereas three of these cDNAs encoded only the SH2 domain and short flanking sequences, a larger clone contained an extended open reading frame (ORF) 3' to the SH2 domain followed by a large 3'-untranslated region. Since all four clones lacked the starting methionine, 5' RACE-PCR was carried out on total RNA isolated from the NFS-60/MAC monocyte/macrophage cell line (Bourette *et al.*, 1993) and mouse peripheral blood mononuclear cells (PBMCs), two cell types expressing Fms, using a specific oligonucleotide primer corresponding to the N-terminal end of the SH2 domain. A 400 bp cDNA was then recovered with an ATG codon in an accurate context for translation initiation.

Combining all sequence information led to a cDNA of 2929 nucleotides (accession No. AF055465) with a single ORF encoding a 322 amino acid protein (Figure 1A) with a calculated mol. wt of 36 787 Da. This protein contains an N-terminal SH3 domain (amino acids 1–56), an SH2 domain (amino acids 59–150), a proline-rich region (amino acids 167–216) with two P-XX-P putative SH3-binding sites, and a C-terminal SH3 domain (amino acids 264–322). No known catalytic domain was identified. This protein is most closely related to the human Grb2-related adaptor Grap (Feng *et al.*, 1996) and the murine Grb2 adaptor (Suen *et al.*, 1993), with 47 and 49% amino acid identities, respectively (Figure 1B). Interestingly, homologies concerned only the SH2 and both SH3 domains, whereas the proline-rich region is specific to our new protein. Based on structural features characteristic of adaptor proteins, its expression in a monocytic cell line

Fig. 1. Amino acid sequence analysis of Mona. (A) Translation of the Mona-coding sequence with a schematic representation of the protein. Underlined bold characters represent the two putative SH3 domain-binding motifs (PxxP). (B) Alignment of the Mona sequence with murine Grb2 and human Grap proteins was performed using the ClustalX program. Regions of identity are outlined by gray boxes. Note that alignment is interrupted between the SH2 and C-terminal SH3 domains due to the proline-rich domain that is unique to Mona.

and its functional properties as described below, this new protein was named Mona. During review of the manuscript, a DDBJ/EMBL/GenBank database homology search revealed a new cDNA sequence (accession No. AF053405) encoding a protein named Gads. Reported Mona and Gads cDNAs have different lengths (2929 and 1362 bp, respectively) but contain highly homologous (>99.5%) 1.3 kb sequences encoding identical proteins. Thus, Mona and Gads are probably products of the same gene.

In order to characterize Mona further, an antiserum was generated after injecting rabbits with a bacterial glutathione S-transferase (GST)-Mona SH2 fusion protein, while the entire Mona-coding sequence was inserted into the LXS retroviral vector (Miller and Rosman, 1989) and transfected into ψ -2 packaging cells. By Western blotting with the antiserum, a protein of the expected size (38 kDa) was detected in ψ -2 cells transfected with L(Mona)SN vector but not in cells transfected with the empty vector (Figure 2). Furthermore, supernatants of ψ -2 cells were used to infect various hematopoietic cell lines, including the SCF-dependent pluripotent cell line EML (Tsai *et al.*, 1994), interleukin-3 (IL-3)-dependent FDC-P1 cells overexpressing wild-type Fms (FD/wtFms cells; Bourette *et al.*, 1995) and the M-CSF-dependent BAC1.2F5 cell line (Morgan *et al.*, 1987). Western blotting analysis indicated

Fig. 2. Immunoblot identification of Mona cDNA product in fibroblasts and various hematopoietic cell lines. Lysates of cells transfected with LXSN or L(Mona)SN vectors were run on a 12% polyacrylamide gel, blotted and probed with anti-Mona antiserum.

that Mona could be expressed efficiently as a 38 kDa protein in hematopoietic cells using retroviral gene transfer (Figure 2).

Mona SH2 domain interacts *in vitro* with activated Fms via phosphorylated Tyr697

Using the GST–Mona SH2 domain fusion protein, we investigated the interaction between Mona and Fms. FD/wtFms cells were starved of growth factors and then either stimulated with M-CSF or left unstimulated. Cell lysates were mixed with the GST–Mona SH2 domain fusion protein coupled to glutathione–Sepharose beads, and interacting proteins were precipitated and probed with an anti-Fms antibody (Figure 3A). Under these conditions, the mature form of Fms was detected only when the GST fusion protein was mixed with lysate of M-CSF-stimulated FD/wtFms cells, showing that the Mona SH2 domain interacts *in vitro* with activated Fms. Accordingly, activated Fms was detected in Mona immunoprecipitates obtained from lysates of FD/wtFms cells overexpressing Mona (not shown).

To determine which Fms autophosphorylation site is involved in this interaction, we performed similar experiments using lysates from M-CSF-stimulated FDC-P1 cells expressing various Fms autophosphorylation site mutants (Bourette *et al.*, 1995). Cell lysates containing equal amounts of Fms protein were used (Figure 3B, upper panel). Mutations of tyrosines 706, 721 or 807 had no effect on Fms interaction with the Mona SH2 domain, but mutation of Tyr697 totally abolished the interaction (Figure 3B, lower panel), suggesting that Tyr697 is the Mona-binding site.

In order to confirm this result, we performed the mirror experiment using GST fusion proteins containing either the wild-type Fms KI or KI mutated at different autophosphorylation sites (Y697, Y706 and Y721). Fusion proteins were expressed in the Epicurian TKX1 bacterial strain that enables tyrosine phosphorylation. Then, phosphorylated fusion proteins immobilized on glutathione–Sepharose were mixed with cell lysates of FDCP-1 cells or Mona-overexpressing ψ-2 cells. The presence of Mona and known Fms KI-binding molecules (PLCγ2, p85-PI3K and Grb2) among precipitated proteins was then examined by Western blotting using the relevant antibodies (Figure 3C). As expected, mutation of Tyr697 abolished Fms interaction with Grb2 adaptor (van der Geer and Hunter, 1993), and mutation of Tyr721 abolished Fms interaction with both p85 (Reedijk *et al.*, 1992) and PLCγ2 (Bourette *et al.*, 1997). In addition, consistent with the results shown in Figure 3B, mutation of Tyr697 abolished the

Fig. 3. The Mona SH2 domain interacts with phosphorylated Tyr697 of the M-CSF receptor. (A) Lysates of FD/wtFms cells, stimulated (+) or not (–) for 1 h at 4°C with M-CSF, were mixed with GST–Mona SH2 fusion proteins immobilized on glutathione–Sepharose. Bound proteins were run on a 7.5% polyacrylamide gel, blotted and probed with anti-Fms antibody. (B) As in (A), except that FDC-P1 cells expressing different M-CSF receptors mutated on autophosphorylation sites were also used. For each cell lysate, immunodetection of Fms was performed on whole-cell lysate (upper panel) and on GST–Mona SH2-bound proteins (lower panel). (C) GST–KI Fms fusion proteins were produced as phosphoproteins in Epicurian cells, immobilized on glutathione–Sepharose and incubated with various cell lysates: FDC-P1 cells for PLCγ2 and p85 analysis, and Mona-overexpressing ψ-2 cells for Grb2 and Mona analysis. Bound proteins were then subjected to Western blot analysis as specified in the figure.

interaction between Fms and Mona, demonstrating that Tyr697 is necessary and sufficient for binding of Mona to activated Fms.

Mona expression is restricted to some hematopoietic cells

Various tissues and cell lines were probed for Mona expression by Western blotting using anti-Mona SH2 antibody. Among all samples tested, only spleen and PBMCs expressed the 38 kDa protein, whereas testis and liver unexpectedly expressed a 44 kDa protein (Figure 4A). In order to establish the relationship between this 44 kDa protein and Mona, RT–PCR analyses were performed on a selected set of tissues, using the primers specific to the Mona SH2 domain since our antiserum recognizes the same Mona protein region. Only those tissues expressing the 38 kDa protein (PBMCs and spleen) gave a positive signal, whereas 44 kDa protein-expressing tissues (testis and liver) or non-expressing

Fig. 4. Western blotting analysis of Mona expression in various murine tissues and cell lines. (A) Expression in selected mouse tissues (PBMCs, peripheral blood mononuclear cells). Other tissues tested that were negative for Mona expression included bone, heart, intestine, muscles, ovary, placenta, skin, stomach and uterus. (B) Expression in various cell lines, including multipotential cell lines (EML, FDCP-Mix and NFS-60), committed myeloid (FDCP-1, 32D), erythroid (E31, MEL), lymphoid (FL5.12, BaF/3 and CTLL-2) and monocyte/macrophage (NFS-60/MAC and BAC1.2F5) cell lines, fibroblasts (Rat-2 and NIH 3T3), pheochromocytoma cells (PC12), stroma cells (MS-5) and hepatocytoma cells (HepG2). (C) Expression in highly enriched T lymphocytes (CD4⁺ or CD8⁺ cells) and monocytes. All analyses were performed with 20 µg of protein loaded per lane, except for CD4⁺ cells (14 µg) and CD8⁺ cells (8 µg).

tissues (brain and kidney) were found to be negative (Figure 5A). We conclude that Mona is expressed *in vivo* as a single 38 kDa protein, and that the 44 kDa protein found in testis and liver is different from Mona, although both proteins are immunologically related.

Since it is apparently restricted to some hematopoietic tissues, Mona expression was examined using a large panel of hematopoietic cell lines, including multipotential cells and committed erythroid, myeloid or lymphoid cells. Western blot (Figure 4B) and RT-PCR (Figure 5A) analyses showed that Mona is expressed in the T-lymphoid cell line, CTLL-2, and in the monocyte/macrophage cell

Fig. 5. Analysis of Mona mRNA expression in tissues and cell lines. (A) RT-PCR detection of transcripts was performed using primers specific to the Mona SH2 domain. HPRT was used as a control. (–) indicates analysis performed in the absence of RNA. (B) Northern blot analysis performed in 20 µg of total RNA loaded per lane. Membrane was hybridized with a 1.1 kb ³²P-labeled probe corresponding to the entire Mona-coding sequence. The estimated size of the various transcripts is indicated on the right (in kb). The lower panel shows ethidium bromide staining of the gel before transfer onto nylon membrane and indicates that the PBMC sample was underloaded.

line NFS-60/MAC, an M-CSF-dependent derivative of bipotential NFS-60 cells (Bourette *et al.*, 1993). Accordingly, PBMC sorting enabled us to confirm Mona expression in T cells (CD4⁺ cells and CD8⁺ cells) and monocytes (Figure 4C).

Finally, Mona expression was assessed by Northern blotting using full-length Mona cDNA as a probe. Four mRNA species (5.0, 3.9, 3.3 and 1.5 kb) hybridized with this probe but exhibited a specific expression pattern (Figure 5B). The 3.9 and 1.5 kb species were found in all samples, whereas the 5.0 and 3.3 kb species were detected in Mona-expressing cells (PBMCs, NFS-60/MAC and CTLL-2 cells), but not in Mona-negative cells (brain, testis, fibroblasts and NFS-60 cells). This clearly indicates that 5.0 and 3.3 kb mRNAs are products of Mona gene transcription. Moreover, since Mona and Grb2 cDNAs are strongly homologous (69% overall) and Grb2 is ubiquitously expressed as two mRNA species of 3.9 and 1.5 kb (Suen *et al.*, 1993), these mRNAs may represent Grb2 mRNAs.

Mona expression is induced during monocytic differentiation of NFS-60 cells

Mona is expressed in the NFS-60/MAC cell line, an M-CSF-dependent variant of the NFS-60 line, which otherwise does not express Mona (Figures 4B and 5). Interestingly, whereas NFS-60 cells are IL-3-dependent bipotential progenitor cells and do not express Fms, NFS-60/MAC cells exhibit a monocyte/macrophage phenotype and strongly express Fms (Bourette *et al.*, 1993), suggesting that Mona expression is induced during monocyte/macrophage differentiation. To test this hypothesis, we used NFS-60 cells transduced with murine Fms

Fig. 6. Mona expression is induced during monocytic differentiation of N-Fms cells. (A) Cells were starved of IL-3, cultivated in the presence of M-CSF (2500 U/ml) for different times as indicated, then lysed and analyzed for Mona or Grb2 expression by Western blotting (Grb2 was used as a control). (B) Cells were starved of IL-3, stimulated for different times at 37°C with M-CSF (2500 U/ml), and then total RNA was extracted. Mona transcripts were detected by RT-PCR performed using primers specific to the Mona SH2 domain. PBMNCs were used as a positive control for Mona expression. HPRT was used as a standard.

(N-Fms cells), which can differentiate to monocytes within 5 days of culture in the presence of M-CSF (Bourette *et al.*, 1993; G.Pawlak, C.Valadoux-Delplanque, V.Revol, R.P.Bourette, J.P.Blanchet and G.Mouchiroud, in preparation). When maintained in the presence of IL-3, N-Fms cells, like parental NFS-60 cells, do not express Mona protein (Figure 6A, t_0). However, Mona protein expression was induced in N-Fms cells after 1 day of culture in the presence of M-CSF, and then increased until day 5 (Figure 6A). RT-PCR showed that Mona expression was indeed induced very rapidly during the first day of culture, with a significant and reproducible expression signal after 8 h in the presence of M-CSF (Figure 6B). Induction of Mona in N-Fms cells was not a consequence of IL-3 withdrawal prior to M-CSF stimulation since it was also obtained in the response to both IL-3 and M-CSF (data not shown). Taken together, these observations suggest a role for Mona during development of the monocytic lineage as controlled by M-CSF.

Overexpression of Mona in mouse bone marrow cells strongly decreases macrophage production *in vitro*

In order to look for its possible role during monocytic differentiation, Mona protein was first overexpressed in M-CSF-responsive cell lines that do not normally express the protein, such as BAC1.2F5 and FD/wtFms. Although high expression levels were obtained (Figure 2), Mona overexpression had no effect on cell response to M-CSF, whether it was proliferation or differentiation which was assessed (not shown). We then used the physiologically more relevant experimental system provided by bone marrow progenitor cells differentiating

Fig. 7. Overexpression of Mona in mouse bone marrow cells (BMCs) strongly decreased macrophage production *in vitro*. BMCs were infected with viral supernatant of Ψ -2/LXSN or Ψ -2/L(Mona)SN cells. Infected BMCs were washed and plated in agar ($1-4 \times 10^5$ cells/ml) or liquid (1.5×10^6 cells/flask) cultures containing 10% FBS, G418 (1 mg/ml) and M-CSF (1000 U/ml). Macrophage colonies (A) and bone marrow-derived macrophages (B) were counted after 10 days. Colony or macrophage numbers were normalized based on the ratio between the viral titers of LXSN and L(Mona)SN. Data represent mean values $\pm$ standard error of the mean of three independent experiments.

in vitro to macrophages in response to M-CSF. Differentiation of monocyte/macrophage progenitors (CFU-M) can then be assessed by macrophage colony formation in agar culture (Stanley *et al.*, 1978) or by the production of macrophages in liquid cultures (Tushinsky *et al.*, 1982). For these experiments, murine bone marrow cells (BMCs) infected by Mona-expressing vector [L(Mona)SN], or by empty LXSN vector as a control, were plated in agar cultures. Viral titers determined on NIH 3T3 cells were 5×10^5 and 2×10^5 viral particles/ml for Ψ -2/LXSN or Ψ -2/L(Mona)SN cells, respectively. This 2.5:1 ratio of LXSN and L(Mona)SN titers was also obtained using myeloid FDC-P1 cells as a target. Infected BMCs were plated in soft agar cultures containing G418 and M-CSF (1000 U/ml), and macrophage colonies were counted after 10 days. Colony numbers were significantly lower in L(Mona)SN cultures than in LXSN cultures. Taking into account the difference in virus titers between LXSN and L(Mona)SN Ψ -2 cell supernatants, we calculated an average 6.6-fold decrease in CFU-M development when Mona was overexpressed (Figure 7A). In liquid cultures containing G418 and M-CSF (1000 U/ml), infected BMCs proliferated and differentiated into adherent macrophages which were enumerated after 10 days of culture. Similarly to the agar assay results, we found an 8.5-fold reduction in macrophage number in L(Mona)SN cultures as compared with LXSN control cultures (Figure 7B). Examination of May-Grünwald-Giemsa-stained preparations revealed no significant morphological difference between macrophages obtained from these cultures (data not shown). These results demonstrate that overexpression of Mona in mouse bone marrow cells strongly decreases macrophage production *in vitro*.

Discussion

We have isolated a novel M-CSF receptor-binding molecule in a yeast two-hybrid screen. This 38 kDa protein was named Mona for monocytic adaptor because: (i) its SH3- and SH2-containing structure, closely related to that of Grb2, is typical of a molecular adaptor; (ii) its SH2 domain interacts with the M-CSF receptor which controls monocytic development; and (iii) its expression is induced during monocytic differentiation.

Mona contains one SH2 domain and two SH3 domains closely related to the corresponding domains of Grb2 and Grap adaptors (Suen *et al.*, 1993; Feng *et al.*, 1996). No homology to known catalytic domains was found. However, an additional proline-rich region located between the SH2 and the C-terminal SH3 domain is unique to Mona. This region contains two PxxP motifs which represent the minimum binding site for an SH3 domain (Alexandropoulos *et al.*, 1995). This would raise the interesting possibility that Mona may be able to establish more complex molecular interactions with signaling molecules than can Grb2 or Grap. Alternatively, recognition of these polyproline motifs by either Mona SH3 domain would provide a mechanism for regulating Mona activity by intramolecular interactions, as recently shown for the Hck kinase (Moarefi *et al.*, 1997).

Using GST fusion proteins, we demonstrated that Mona associates with Fms Tyr697, which is also the Grb2-binding site, suggesting that Mona may compete with Grb2 for binding to activated Fms. Due to their structural similarity, Mona and Grb2 might also compete for downstream signaling molecules, as previously demonstrated for the Grb2 isoform Grb3-3 that acts as an inhibitor of Grb2 and has a dominant-negative effect on the Ras pathway by forming inactive heterodimeric complexes with Sos (Fath *et al.*, 1994). Since Grb2 binding to Fms results in translocation of Sos to the membrane and subsequent Ras activation (van der Geer and Hunter, 1993; Lioubin *et al.*, 1994), our biochemical data raise the possibility that Mona functions as a Grb2 competitor thereby modulating the Ras pathway. It will now be important to determine the respective affinities of Grb2 and Mona for Fms and to analyze the interactions of Mona with known Grb2-binding partners to validate this competition model.

Several hematopoietic-specific adaptor proteins have been reported recently, such as Lnk (Huang *et al.*, 1995), LAT (Zhang *et al.*, 1998) and TSad (Spurkland *et al.*, 1998). Similarly, Mona is expressed only in spleen and PBMCs of adult mice. Furthermore, using a representative selection of hematopoietic cell lines, we detected Mona in the monocyte/macrophage NFS-60-MAC cell line, but also in the T-lymphoid CTLL-2 cell line. Consistent with this observation, we found Mona in peripheral blood T cells and monocytes. Since it is expressed in circulating T cells, but not in thymocytes, Mona could be involved in T-lymphocyte development. This suggests an interaction with receptors other than Fms since Fms is not expressed in normal T cells (Dubreuil *et al.*, 1988). In contrast, Mona is physiologically expressed together with Fms in monocytes. However, Mona was not detected in BMCs by Western blotting (see Figure 4A), which may be accounted for by the low proportion (<5%) of Fms-

expressing cells, all belonging to the monocytic lineage (Guilbert and Stanley, 1980; Byrne *et al.*, 1981). On the other hand, we also failed to detect Mona transcripts by RT-PCR (data not shown). Thus, Mona may be induced during the latest steps of monocytic differentiation *in vivo*, consistent with our observation on monocytic differentiation of NFS-60 cells. Furthermore, Mona was not detected in peritoneal macrophages arising from blood monocytes and harvested a few hours after peritoneal agar injections (data not shown), nor in bone marrow-derived macrophages (data not shown) or in BAC1.2F5 cells (see Figure 4). Thus, the physiological expression of Mona seems to be restricted to a specific window of the monocyte/macrophage lineage: precisely, in circulating mature monocytes.

Since our results strongly suggest that Mona is involved in monocyte/macrophage differentiation, we tested whether its overexpression would alter proliferation and/or differentiation of myeloid progenitors in response to M-CSF. We found that Mona overexpression strongly reduced the *in vitro* development of macrophages from bone marrow progenitors stimulated by M-CSF. Such a negative effect appeared to be specific for native monocytic cells, and thus dependent on the cellular background, since Mona overexpression did not impede the growth of bone marrow-derived mastocytes cultivated for 2 weeks in IL-3, SCF-dependent EML cells, IL3-dependent FD/wtFms cells or M-CSF-dependent BAC1.2F5 cells (data not shown). These observations also argue against a non-specific toxic effect of Mona.

This effect of Mona overexpression on bone marrow-derived macrophage production may be explained along several lines. (i) Mona overexpression could result in more rapid proliferation and differentiation of monocyte progenitors. This seems very unlikely since daily examination of M-CSF-stimulated cultures did not reveal any premature production of macrophages in liquid cultures or early appearance of macrophage colonies in agar cultures. Furthermore, no accumulation of immature monocytic cells was observed in these cultures (not shown), ruling out the possibility that Mona overexpression blocks monocyte/macrophage differentiation. (ii) A proapoptotic effect of Mona is also unlikely since Mona can be overexpressed in various hematopoietic cell lines, including M-CSF-dependent cells. In addition, Mona is naturally co-expressed with M-CSF receptor in NFS-60/MAC cells and, interestingly, in monocytes, i.e. in a cell lineage and at a differentiation stage that is normally not affected by apoptosis. (iii) Premature overexpression of Mona may have an adverse effect on proliferation of bone marrow monocytic progenitors, resulting in decreased macrophage production and inhibition of macrophage colony formation. Interestingly, physiological expression of Mona occurs at a stage of the monocytic lineage where >90% of the cells have lost the capacity for cell division (van Furth, 1992). It is thus tempting to postulate that Mona is involved in cell cycle regulation when cells differentiate to monocytes. SHIP inositol phosphatase (Lioubin *et al.*, 1994, 1996), tyrosine phosphatase SHPTP1 (Chen *et al.*, 1996) and Dab2 (Xu *et al.*, 1998) represent good candidates for Mona partners in the relevant signaling pathways since these molecules that negatively regulate cell growth are expressed in monocytic cells and bind to

Grb2. More detailed studies on Mona partners are thus required to elucidate the physiological role of the protein in monocytic development and in Fms signaling.

The central position of adaptor proteins in the formation of multiprotein complexes has become more and more apparent over recent years (Pawson and Scott, 1997). In particular, an interesting aspect of these molecules lies in their regulatory role in cell signaling. Mona might form part of the signaling pathways that appear during cell differentiation to decrease the M-CSF growth signal. Identifying the molecules downstream of Mona should help to elucidate molecular mechanisms used by the M-CSF receptor to control the monocyte/macrophage development and thus shed further light on processes altering the balance between proliferation and differentiation during formation of specialized cells.

Materials and methods

Yeast two-hybrid screen

The yeast two-hybrid screen in which the Mona SH2 domain was isolated has been described previously (Bourette *et al.*, 1997). Briefly, a LexA–cytoplasmic domain of murine Fms receptor fusion protein was used as a bait for screening two VP16 target libraries containing cDNA fragments from 9.5- and 10.5-day-old mouse embryos (Hollenberg *et al.*, 1995) or from the hematopoietic EML cell line (Tsai *et al.*, 1994). DNAs from positive colonies for transcriptional activation of *his3* and *lacZ* reporter genes were isolated, sequenced by PCR using VP16-specific sense and antisense oligonucleotide primers (Vojtek and Hollenberg, 1995), and compared with existing DNA sequence databases.

Isolation and cloning of Mona

Based on the ORF of the VP16 fusion protein, four independent VP16 cDNA clones containing sequences coding for a new SH2 domain, with significant homology with the SH2 domain of Grb2, were isolated from the murine embryonic library (MEL) (clone 10) and the EML library (clones 71, 96 and 152). MEL10, EML71 and EML96 clones contain only sequences encoding the SH2 domain and short flanking sequences, whereas the EML152 clone (2500 bp) contains 3' to the SH2 domain a sequence encoding a proline-rich region and an SH3 domain, followed by a stop codon and ~1700 bp of 3'-untranslated region. The sequence missing 5' to the SH2 domain was isolated by 5' RACE using the 5'/3' RACE kit (Boehringer Mannheim). Templates were total RNA from mouse PBMCs and from the NFS-60/MAC cell line (Bourette *et al.*, 1993). For the RACE-PCR, the manufacturer's instructions were followed using standard PCR conditions and an antisense oligonucleotide primer specific to the SH2 domain sequence (5'-ATTCTTAGG-CACATATCCTTCTTGGC-3'). The final product was cloned into pCR2.1-TOPO vector (Invitrogen) and sequenced. All the various sequences were combined using the SIM program (www.expasy.hcuge) resulting in a cDNA of 2929 bp.

RNA preparation and Northern blot analysis

Total RNA was extracted from frozen tissues of adult OF1 mice (IFFA-CREDO) or frozen pellets (5×10^6 cells) of different cell lines using the RNeasy kit (Qiagen), according to the manufacturer's instructions. Samples (20 µg) of total RNA were size-fractionated on a 1% agarose gel/formaldehyde denaturing gel, transferred to nylon membrane (Hybond-N+ Extra nitrocellulose, 0.45 µm; Amersham) and heated for 2 h at 80°C. The membrane was pre-hybridized overnight at 42°C (50% formamide, 5% Denhardt's solution, $5 \times$ SSPE, SDS 0.1%, 0.1 mg/ml sheared salmon DNA) and hybridized in the same solution for 48 h with a PCR-amplified cDNA probe corresponding to the full-length coding sequence of Mona (1.1 kb), labeled by [α - 32 P]dCTP using a random priming kit (Amersham). The membrane was washed extensively with $2 \times$ SSC, SDS 0.1% at room temperature and exposed to film at -70°C with an intensifying screen.

Cells and culture conditions

PBMCs were isolated by centrifugation of blood cells on Lympholyte-M reagent (Cedarlane) according to the manufacturer's instructions. For T-cell purification, PBMCs were incubated for 45 min on ice with

phycoerythrin-conjugated monoclonal antibody (MoAb) to mouse CD4 or with fluorescein-conjugated MoAb to CD8 (Caltag). CD4⁺ cells and CD8⁺ cells were then sorted using a FACScalibur system (Becton-Dickinson). For monocyte purification, PBMCs were incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC)-labeled bacteria (Molecular Probes), then brightly labeled cells were sorted using FACScalibur. The purity of CD4⁺ and CD8⁺ cells was >93% as assessed by FACS analysis of sorted cells, and that of the monocyte fraction was >85% as assessed by morphological examination.

NFS-60 cells expressing wild-type murine Fms (N-Fms) (Bourette *et al.*, 1993; G.Pawlak, C.Valadoux-Delplanque, V.Revol, R.P.Bourette, J.P.Blanchet and G.Mouchiroud, in preparation) were maintained in Iscove's modified Dulbecco's medium (IMDM; Gibco-BRL) supplemented with 10% fetal bovine serum (FBS, Boehringer Mannheim) and 1 ng/ml of recombinant murine IL-3 (R&D Systems). The fibroblast ψ -2 producer and NIH 3T3 cells were maintained in IMDM containing 5% FBS. FDC-P1 cells and their Fms-expressing derivatives (Bourette *et al.*, 1995) were maintained in IMDM supplemented with 10% FBS and 1% conditioned X63-IL-3 cell medium (Karasuyama and Melchers, 1988) as a source of growth factor. EML cells were maintained in IMDM supplemented with 20% horse serum and 10% MKL cell supernatant as a source of SCF (Tsai *et al.*, 1994). BAC1.2F5 cells were maintained in IMDM supplemented with 10% FBS and M-CSF (1000 U/ml). M-CSF was partially purified from supernatants from Sf9 insect cells expressing M-CSF from a baculovirus vector (Wang *et al.*, 1993). Original stock contained 140 000 U/ml M-CSF, one unit being able to stimulate formation of one colony from 50 000 BMCs plated in standard agar cultures.

Retroviral vector and infections

An *EcoRI*–*Bam*HI PCR-amplified full-length Mona cDNA was inserted into the corresponding restriction sites of LXSN (Miller and Rosman, 1989) retroviral vector. Empty vector or L(Mona)SN was transfected in the ψ -2 producer cells using the calcium phosphate method. Cells were then selected using 1 mg/ml G418. Viral supernatants were titrated on NIH 3T3 cells. Cells were infected by different dilutions of supernatant of ψ -2/LXSN or ψ -2/L(Mona)SN in the presence of 4 µg/ml of polybrene, selected by 1 mg/ml of G418 (Gibco), and clones were counted after 2 weeks.

For infection of BMCs, 2×10^7 cells were resuspended in 10 ml of ψ -2/LXSN or ψ -2/L(Mona)SN supernatant medium supplemented with 10% FBS, 10% spleen conditioned medium (SCM) and 8 µg/ml of polybrene. Four rounds of infection were performed with change of medium every 12 h. Different concentrations of infected BMCs were washed and then plated in 0.3% agar or liquid cultures containing 10% FBS, G418 (1 mg/ml) and M-CSF (1000 U/ml).

GST fusion proteins

GST–Mona SH2 fusion protein was obtained as follows. A yeast DNA miniprep of pVP16-MEL10 plasmid was prepared and amplified in *Escherichia coli* XL-1 Blue (Stratagene). The Mona SH2 cDNA insert was recovered after digestion with *Bam*HI and *Eco*RI, purified from an agarose gel, and ligated in the same sites of pGEX3 vector (Pharmacia). GST–Mona SH2 fusion protein was affinity purified from cell lysates of *E.coli* BL21 (Pharmacia) by adsorption to glutathione–Sepharose beads (Pharmacia) as previously described (Lowenstein *et al.*, 1992).

The entire KI region of the murine Fms receptor (amino acids 668–746), mutated or not on various tyrosines, was cloned in pGEX1 (Pharmacia). GST–KI fusion proteins were tyrosine phosphorylated by an inducible tyrosine kinase expressed in the *Epicurian* TKX1 bacteria strain (Stratagene) according to the manufacturer's instructions, and then purified as described above. Tyrosine phosphorylation of the GST–KI fusion proteins was verified by Western blotting using anti-phosphotyrosine antibody (Transduction Laboratories).

Antibodies and immunoblotting

Rabbit polyclonal antiserum to Mona (CovalAb, France) was produced by injecting purified GST–Mona SH2 fusion protein which had been eluted from glutathione–Sepharose beads with 5 mM glutathione (Sigma). Monoclonal antibodies to murine Grb2, and the p85 subunit of PI3K were from Transduction Laboratories. Rabbit polyclonal anti-PLC γ 2 antibody was from Santa-Cruz Biotechnologies (Santa Cruz, CA). Polyclonal rabbit antiserum 4599B to the murine Fms cytoplasmic domain has been described previously (Carlberg and Rohrschneider, 1994). For Western blot analysis, tissues or cells were lysed in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM KCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100), containing protease inhibitor cocktail

(Boehringer Mannheim). After 10 min on ice, lysates were spun at 25 000 g for 20 min, and pellets containing nuclei and other insoluble material were discarded. Samples were run on SDS–7.5% or –12% polyacrylamide gels and transferred to nitrocellulose membranes (Protran 85, Schleicher & Schull) for 1 h at 1.5 mA/cm² with a semi-dry blotting apparatus. After transfer, membranes were blocked overnight at 4°C with block buffer (10 mM Tris–HCl pH 9.5, 150 mM NaCl, 0.25% Tween-20, 3% non-fat dry milk). Nitrocellulose membranes were incubated for 1 h at room temperature with primary antibody diluted in block buffer. Antibody binding was visualized using horseradish peroxidase-conjugated secondary antibodies (Sigma) and enhanced chemoluminescence reagent (ECL+, Amersham).

Accession number

The Mona cDNA sequence has been deposited in the DDBJ/EMBL/GenBank nucleotide database under the accession No. AF055465.

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