Expression of Mona (monocytic adapter) in myeloid progenitor cells results in increased and prolonged MAP kinase activation upon macrophage colony-stimulating factor stimulation

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Abstract Mona is an SH3 and SH2 domain-containing adapter molecule that is induced during monocytic differentiation. Here we have first shown that M-CSFR is the major Mona partner in M-CSF signaling, the interaction being mediated through tyrosine 697 of the receptor. Next we asked whether Mona expression would alter the Ras/MAP kinase pathway since Mona is a likely competitor of Grb2 for binding to M-CSFR. We found that M-CSF induced late and massive phosphorylation of ERK molecules in Mona-expressing myeloid cells compared to non-expressing cells. These results suggest that Mona expression might modify M-CSF signaling during monocytic differentiation.

Key words: Macrophage colony-stimulating factor receptor; Mona; Grb2; Shc; MAPK kinase

1. Introduction

Macrophage colony-stimulating factor (M-CSF) is the principal regulator of monocyte/macrophage development [1]. Biological effects of M-CSF are mediated by a specific receptor (M-CSFR), encoded by the c-*fms* proto-oncogene [2], the expression of which is restricted to committed monocytic cells in the bone marrow [3]. Ectopically expressed M-CSFR can induce M-CSF-dependent proliferation of fibroblasts [4] or of hematopoietic cells [5–7], and differentiation towards the monocyte/macrophage lineage of myeloid progenitor cell lines such as FDC-P1 [8] or NFS-60 [9].

Ligand binding to the extracellular domain of M-CSFR induces receptor dimerization and autophosphorylation on several cytoplasmic tyrosine residues, of which three can recruit Src homology 2 (SH2) domain-containing signaling molecules [10]. Tyrosine 559, located in the juxtamembrane region, interacts with Src family members [11]. In the kinase insert (KI), tyrosine 697 interacts with two related adapters, Grb2 [12] and Mona [13], and tyrosine 721 binds either to the p85 adapter subunit of phosphatidylinositol 3′ kinase (PI3K) [14] or to phospholipase C-γ2 (PLCγ2) [15]. Thus it appears that two M-CSFR autophosphorylation sites, tyrosines 697 and 721, are bivalent. Following binding on tyrosine 721, PI3K transduces an anti-apoptotic signal through PKB/Akt activation [16], whereas PLCγ2 controls commitment of myeloid cells to terminal monocytic differentiation [15]. Binding of Grb2 to tyrosine 697 allows translocation of the nucleotide exchange factor Sos to the plasma membrane then activation of the Ras-MAP kinase (MAPK) pathway in fibroblasts and hematopoietic cells [12,17]. However, the role of Mona in myeloid cells is still unclear.

We originally identified Mona as a molecule that interacts with activated M-CSFR, using a yeast two-hybrid screen [13]. Mona contains one SH2 domain and two SH3 domains closely related to the corresponding domains of Grb2, and a unique proline-rich region. It is expressed only in hematopoietic cells, including monocytes and T lymphocytes [13]. In lymphoid cells, Mona (also called Gads, Grf-40 or GrpL) interacts with SLP-76 to activate the nuclear factor of activated T cells (NF-AT) and is believed to play an important role in TCR-mediated signaling [18–20]. In the monocytic lineage, Mona expression is restricted to maturing cells and circulating monocytes [13]. Moreover, Mona overexpression strongly inhibits M-CSF-dependent monocyte/macrophage production in vitro [13]. Collectively, these data strongly suggested the involvement of Mona in M-CSF signaling and/or monocytic differentiation.

Since Mona could compete with Grb2 for binding onto Tyr-697 [13], the present studies were undertaken to clarify the role of Mona in M-CSF signaling. The Mona SH2 domain appears to have a specificity similar to that of Grb2, thereby binding to M-CSFR [13], LAT [18,19], Shc and the Bcr-Abl fusion protein [21]. Since Grb2 associates with both M-CSFR and Shc [17], we compared the interactions of Mona and Grb2 with M-CSFR or Shc in M-CSF-stimulated myeloid cells. Here we show that Mona binds essentially to M-CSFR and very weakly to Shc and that Mona overexpression results in sustained and elevated MAPK activation in response to M-CSF.

2. Materials and methods

2.1. Cells

The M-CSFR-expressing derivatives of FDC-P1 cells (FD/Fms) [22] used in these studies were maintained in Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Roche Diagnostics, Mannheim, Germany) and 1% X63-IL-3 cell conditioned medium [23] as a source of interleukin-3 (IL-3). To obtain FD/Y697F/Mona cells, FD/Y697F cells were cultivated on a layer of ψ-2/L(Mona)SN producer cells [13] for 24 h, then selected for 1 week by 1 mg/ml of G418 (Life Technologies).

Supernatants from Sf9 insect cells expressing murine recombinant

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M-CSF from a baculovirus vector [24] were used as a source of M-CSF. Original stock contained 150 000 U/ml M-CSF, one unit being able to stimulate formation of one colony from 50 000 bone marrow cells plated in standard agar cultures.

For immunoprecipitation studies, cells were washed in IMDM and cultivated for 15 h in IMDM supplemented with 1% FBS and 1% X63-IL-3 cell conditioned medium. After two washes in IMDM, cells were resuspended in IMDM-10% FBS then stimulated or not with M-CSF (15 000 U/ml) for 90 s at 37°C or 1 h at 4°C, as specified in the figure legends. For MAPK activation studies, cells were washed in IMDM, incubated for 3 h in IMDM supplemented with 1% FBS, then stimulated or not with M-CSF (15 000 U/ml) for various durations as indicated in the figure legends.

2.2. Immunoprecipitation and immunoblotting

Polyclonal rabbit antisera to Mona (#L1) and the murine Fms cytoplasmic domain (#4599B) have been previously described [13,25]. Polyclonal rabbit antiserum to Shc (#1403H) and monoclonal antibody to phosphotyrosine (clone 4G10) were kindly provided Drs. L.R. Rohrschneider (Fred Hutchinson Cancer Research Center, Seattle, WA, USA) and P. Dubreuil (INSERM U119, Marseille, France), respectively. Monoclonal antibody to murine Grb2 was from Transduction Laboratories. Anti-pERK and anti-pan-ERK were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

Cells were lysed as previously described [13]. For immunoprecipitation, equalized cell lysates were incubated with antibodies, as specified below, for 1 h at 4°C. Subsequently, protein G-Sepharose beads (Sigma, Saint-Louis, MO, USA) were added to samples for 1 h incubation at 4°C with gentle rotation. Immune complexes were washed five times in lysis buffer, and bound proteins eluted by boiling for 5 min in Laemmli sample buffer. Immunoblotting procedures have been described previously [13].

2.3. GST fusion proteins and in vitro binding assays

Glutathione-*S*-transferase (GST)–Mona SH2 fusion protein has been previously described [13]. To obtain GST-Mona fusion protein, the Mona cDNA insert obtained by PCR from L(Mona)SN retroviral vector [13] was fused to GST cDNA in the pGEX3 vector (Amersham Pharmacia Biotech). Pull-down experiments were conducted as described in [13].

3. Results

3.1. Mona associates with M-CSFR in vivo

Using pull-down experiments, we previously showed that Mona expressed as a GST fusion protein binds to phosphorylated tyrosine 697 of M-CSFR [13]. To examine this interaction in a physiological context, co-immunoprecipitation experiments were performed. FDC-P1 cells expressing both M-CSFR and Mona (FD/wtFms/Mona cells) were stimulated or not with M-CSF. Anti-Mona immunoprecipitates from cell lysates were probed with either anti-Fms or anti-phosphotyrosine antibodies. Fig. 1A shows that M-CSFR was detected in immunoprecipitates only when cells were stimulated with M-CSF (left panel) and also suggests that M-CSFR is the major tyrosine-phosphorylated molecule associating with Mona in M-CSF stimulated cells (right panel). Furthermore, substituting tyrosine 697 for phenylalanine was sufficient to almost completely abrogate Mona–M-CSFR interaction (Fig. 1B).

3.2. Association of Mona and Shc is marginal in FDC-P1 cells

M-CSF stimulation of FD/wtFms cells results in rapid Shc phosphorylation and association with Grb2 through its SH2 domain [17]. As shown in Fig. 2A, full-length Mona, as well as its SH2 domain, expressed as GST fusion proteins associated with both phosphorylated isoforms of Shc, $p52^{Shc}$ and $p46^{Shc}$ in a M-CSF-dependent manner. Unexpectedly, anti-Mona immunoprecipitates from FD/wtFms/Mona cells contained only small amounts of Shc (Fig. 2B). Non-precipitated material was then used for immunoprecipitation with anti-Shc antibodies, revealing the presence of abundant Shc-Grb2 complexes in material not retained with anti-Mona antibodies (Fig. 2B). To confirm these data, Shc was immunoprecipitated by anti-Shc antibody from lysates from M-CSF-stimulated FD/wtFms/Mona cells and immunoprecipitates were probed using anti-Mona or anti-Grb2 antibodies. Grb2 was readily detected in these immunoprecipitates whereas Mona was not detectable (Fig. 2C). These results show that, in vivo, Shc associates with Grb2 rather than with Mona following M-CSF stimulation.

3.3. The kinetics of MAPK activation is altered in FD/Fms/Mona cells

M-CSF triggers rapid activation of the Ras/Raf-1/MEK/MAPK pathway in myeloid 32D cells [26]. These effects may be mediated through association of Grb2/Sos complexes to activated M-CSFR and Shc [17]. Since Mona associates preferentially with M-CSFR, we asked whether this would impinge on MAPK activation in response to M-CSF. FD/

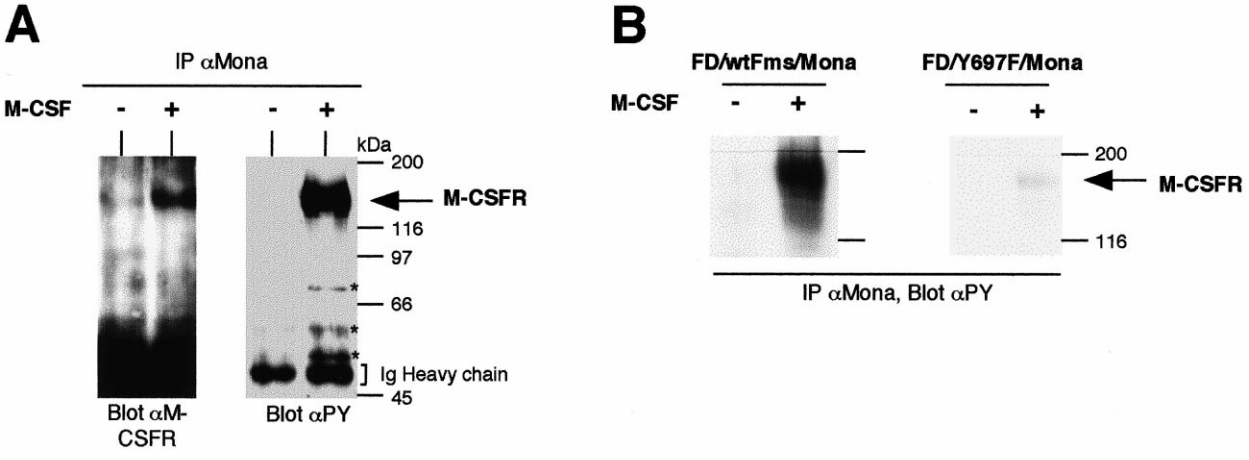

Fig. 1. Mona associates with activated M-CSFR. A: Starved FD/wtFms/Mona cells were stimulated (+) or not (−) for 1 h at 4°C with M-CSF (15 000 U/ml). Cell lysates were incubated with anti-Mona antibody, then immunoprecipitates were run on a 7.5% polyacrylamide gel, blotted and probed with anti-phosphotyrosine antibody or anti-Fms antibody as indicated. B: FD/wtFms/Mona cells and FD/Y697F/Mona cells were treated as above, except that anti-Mona immunoprecipitates were probed with anti-phosphotyrosine antibody.

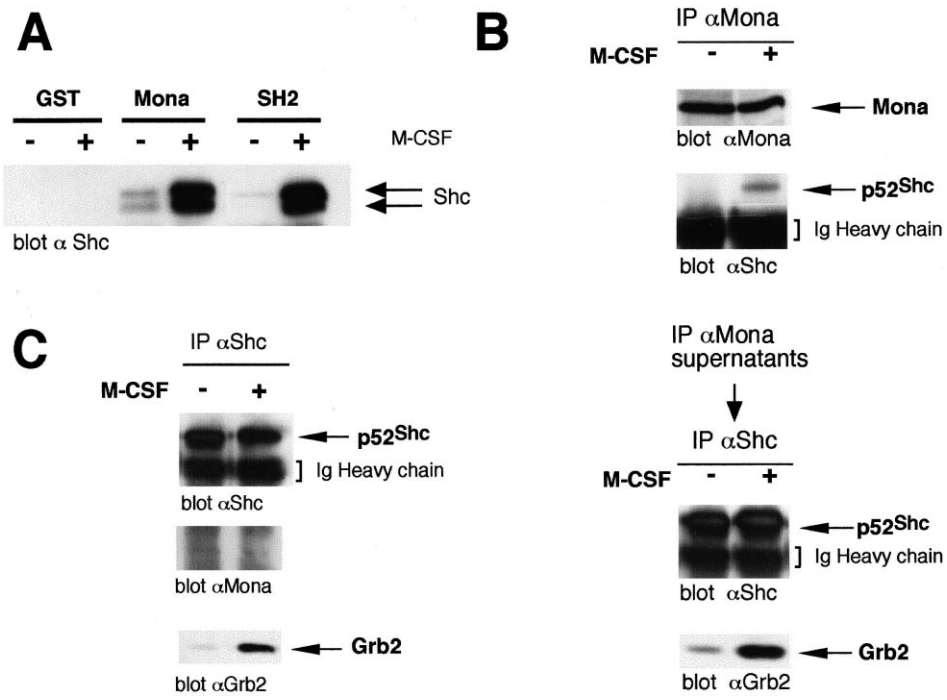

Fig. 2. Analysis of the interaction between Mona and Shc in FD/wtFms/Mona cells. A: Lysates of FD/wtFms/Mona cells, stimulated (+) or not (−) for 1 h at 4°C with M-CSF (15 000 U/ml), were mixed with GST-Mona full-length (Mona) or GST–Mona SH2 (SH2) fusion proteins immobilized on glutathione–Sepharose. Bound proteins were run on a 12% polyacrylamide gel, blotted and probed with anti-Shc antibody. B: Starved FD/wtFms/Mona cells were stimulated (+) or not (−) for 90 s at 37°C with M-CSF (15 000 U/ml). Lysates were used for immunoprecipitation with anti-Mona antibody. Immunoprecipitates were run on a 7.5% polyacrylamide gel, blotted and probed with anti-Mona antibody or anti-Shc antibody. Supernatants were used for a second immunoprecipitation with anti-Shc antibody and precipitated proteins were then immunoblotted with anti-Shc antibody or anti-Grb2 antibody. C: As for B, but lysates were used for a single immunoprecipitation with anti-Shc antibody. Immunoprecipitates were immunoblotted with anti-Shc antibody, anti-Mona antibody or anti-Grb2 antibody.

wtFms and FD/wtFms/Mona cells were therefore stimulated with M-CSF for different durations, and activation of the endogenous MAPK p42 (ERK2) and p44 (ERK1) was determined using a specific antibody detecting the phosphorylated form of ERK1 and ERK2 (pERK). Total ERK expression being monitored using a pan-ERK antibody, pERK bands were quantitated and normalized to signals obtained with anti-pan ERK antibody (Fig. 3).

In FD/wtFms cells, M-CSF clearly induced biphasic activation of MAPK (Fig. 3A, left panel). The early phase occurred between 1 min 30 s and 10 min whereas the late phase started after 1 h, MAPK activation returning to basal levels between 30 min. and 1 h after the addition of M-CSF (Fig. 3B). In sharp contrast, M-CSF-stimulated FD/wtFms/Mona cells exhibited profound changes in the kinetics and magnitude of MAPK activation especially during the second wave (Fig. 3A, right panel; Fig. 3B). Firstly, FD/Fms/Mona cells maintained a high level of MAPK activation between 30 min and 4 h of M-CSF stimulation. Secondly, pERK levels at between 1 and 15 h of stimulation were much higher in FD/wtFms/Mona cells than in FD/wtFms cells (Fig. 3B). These results were confirmed in three independent experiments (Fig. 3C). Interestingly, FD/wtFms and FD/wtFms/Mona cells showed identical kinetics of MAPK phosphorylation in response to IL-3 (data not shown), suggesting that Mona alters MAPK phosphorylation through recruitment to activated M-CSFR.

4. Discussion

This study investigated the role of Mona in M-CSFR signaling, using myeloid FDC-P1 cells ectopically expressing M-CSFR (FD/wtFms cells) or M-CSFR and Mona (FD/wtFms/Mona cells). Expression of M-CSFR confers upon FDC-P1 cells the property of differentiating to macrophages in response to M-CSF, thus providing a valuable model for studying the M-CSF signal in a nearly physiological context [8,15,17].

Mona and Grb2 SH2 domains exhibit identical specificity in vitro for binding to M-CSFR [13] or Shc [21]. Indeed, we have shown here that Mona interacts in vivo with M-CSFR via phosphorylated tyrosine 697, like Grb2, as previously suggested [13]. However, we have also demonstrated that Shc preferentially associates with Grb2 in vivo, its association with Mona being marginal (Fig. 2). Thus, in myeloid progenitor cells, Mona associates with M-CSFR upon M-CSF stimulation whereas Grb2 binds to both M-CSFR and Shc. Grb2 exists in a constitutive complex with Sos in FD/wtFms cells [17]. Upon M-CSF stimulation, preformed Grb2-Sos complexes bind to phosphorylated M-CSFR and Shc and both types of complex may contribute to activation of the Ras-MAPK pathway [17]. Previous investigations and data reported here combine to suggest that Mona and Grb2 might have different roles in M-CSFR signaling. Firstly, Mona selectively binds to M-CSFR via its SH2 domain whereas Grb2 binds to both M-CSFR and Shc. Secondly, Mona binds to SLP-76 but not to Sos in vivo whereas Grb2 binds to Sos, not to SLP-76 [18,20]. Finally, time-course studies have revealed that FD/wtFms/Mona cells exhibit sustained and increased MAPK activation in response to M-CSF compared to control FD/wtFms cells (Fig. 3).

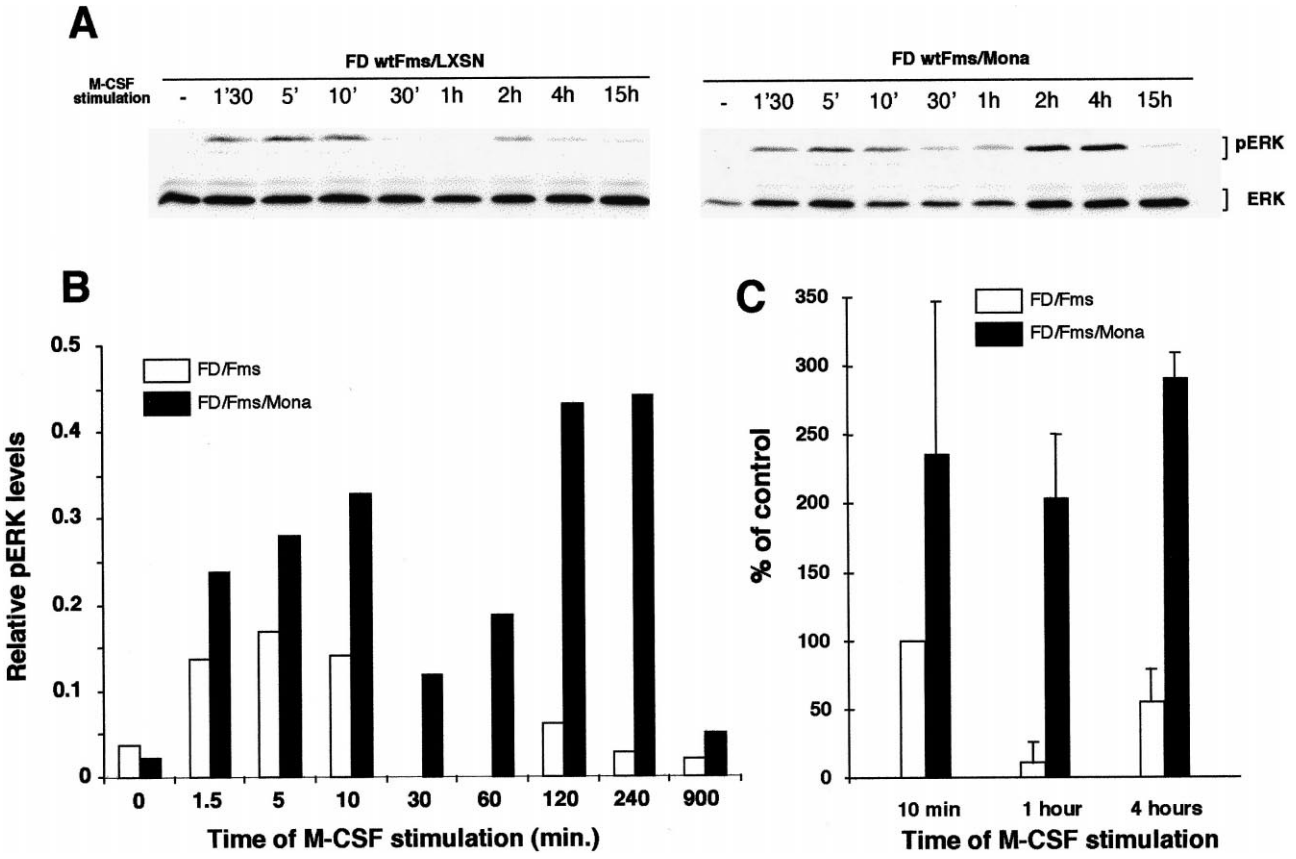

Fig. 3. Time-course studies of MAPK activation. A: FD/wtFms cells and FD/wtFms/Mona cells were stimulated for various times at 37°C with M-CSF (15 000 U/ml). Cell lysates were run on a 12% polyacrylamide gel and immunoblotted with anti-phosphorylated ERK or anti-pan-ERK antibody. B: As in A, pERK and ERK bands were quantitated and data expressed as the ratio of ERK signal to pERK signal. C: Results from four independent experiments were quantitated as in B then normalized to relative pERK levels by 10 min of M-CSF stimulation. Results are shown as mean ± S.E.M.

Persistent MAPK activation has been associated with the differentiation process in neuronal [27] or megakaryocytic [28] differentiation. Similarly, expression of the activated Ha-*ras* oncogene leads to macrophage differentiation in FDC-P1 cells [29]. However, we here observed that macrophage differentiation proceeded identically in FD/wtFms and FD/wtFms/Mona cells (data not shown), although they differed markedly in their levels of activated MAPK (Fig. 3). It is noteworthy that FD/wtFms cells show a rapid, uniform and dramatic differentiation response to M-CSF [22] which could preclude observation of any positive effects on the differentiation of FD/wtFms/Mona cells.

M-CSF clearly stimulated two waves of MAPK activation in FD/wtFms and FD/wtFms/Mona cells (Fig. 3). Temporal regulation of MAPK activation notably involves dual specificity phosphatases [30] which are then potential targets of signaling pathways downstream of Mona. Alternatively, Mona may operate on signaling pathways that positively regulate MAPK activation. It has been shown recently that PI3K activity is essential to IL-3-triggered MAPK activation in FDC-P1 cells [31]. Tyrosine phosphatases SHP-1 and SHP-2 may also be involved in the Ras-mediated activation of MAPK through as yet undetermined mechanisms [32,33]. Thus, our current studies, which aim at identifying Mona partners, should help to elucidate the role of the protein in M-CSFR signaling and also shed further light on the regulation of MAPK activation during monocytic differentiation.

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