A Novel Macrophage Actin-associated Protein (MAYP) Is Tyrosine-phosphorylated following Colony Stimulating Factor-1 Stimulation*

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An ~37-kDa cytoplasmic protein is rapidly tyrosine-phosphorylated in the response of mouse BAC1.2F5 macrophages to colony stimulating factor-1 (CSF-1). pp37 was purified from the cytosolic fraction by anti-Tyr(P) affinity chromatography, size exclusion chromatography, and C4 reverse phase high pressure liquid chromatography. The sequences of four peptides derived from the purified protein matched portions of an expressed sequence tag (EST) sequence, and the EST clone was used to obtain cDNA clones encoding the pp37 protein, which shares sequence similarity with the PST PIP (proline, serine, threonine phosphatase interacting protein)/CDC15 family of protein-tyrosine phosphatase substrates. pp37 is predicted to contain a Fes/CIP4 homology (FCH) domain and an actin-binding domain-like sequence. It is expressed selectively in macrophages, macrophage cell lines, and at low levels in macrophage-containing tissues. pp37 is predominantly found in the cytosol, where it is associated with actin. However, ~4% of the pp37 resides in the membrane fraction, and the trace amount in the cytoskeletal fraction is increased by CSF-1 stimulation. Termed macrophage actin-associated tyrosine-phosphorylated protein (MAYP), p37 is the major F-actin-associated protein that is tyrosine-phosphorylated in macrophages and is likely to play a role in regulating the CSF-1-induced reorganization of the actin cytoskeleton.

The survival, proliferation, and differentiation of mononuclear phagocytic cells is regulated by the growth factor, colony stimulating factor-1 (CSF-1), via its specific interaction with the CSF-1 receptor (CSF-1R), a protein-tyrosine kinase encoded by the c-fms proto-oncogene product (reviewed in Refs. 1–3). Incubation of macrophages with CSF-1 causes CSF-1R dimerization, activation, and tyrosine phosphorylation, followed at 1 min after CSF-1 addition by the tyrosine phosphorylation of several, primarily cytoplasmic, proteins, usually associated in complexes with cytokinetic and/or signaling proteins (4–9). The tyrosine phosphorylation of the non-CSF-1R proteins may be directly or indirectly mediated by the CSF-1R kinase or may increase due to growth factor-induced inhibition of a protein-tyrosine phosphatase (PTP).

We have used direct purification and sequencing approaches, to identify several tyrosine-phosphorylated proteins, including the PTP, SHP-1, and Shc, as well as several cytokinetic and/or signaling molecules associated with them (9, 10). In this paper, we describe the characterization of the major macrophage F-actin-associated tyrosine-phosphorylated protein (MAYP). This 37-kDa protein shares sequence similarity with a family of PTP substrates, is selectively expressed in macrophages and following CSF-1 stimulation, exhibits increased tyrosine phosphorylation, and is increased in the cytoskeletal fraction.

EXPERIMENTAL PROCEDURES

Cell Culture, Protein Purification, and Sequencing—BAC1.2F5 macrophages (11) were cultured in 100-mm tissue culture dishes and stimulated with 13.2 nm CSF-1 (human recombinant macrophage colony stimulating factor, a gift from Chiron Corp.) at 4 or 37 °C, in the presence or in the absence of 8 μM iodoacetic acid (IAA, Fluka), as described previously (9). For purification of pp37, the CSF-1-stimulated cells for 2 h at 4 °C in the presence of IAA to increase the yield of Tyr(P) proteins, their subcellular fractionation, the isolation of the anti-Tyr(P) reactive fraction from the cytosol, its further fractionation by C4 reverse phase high performance liquid chromatography (RP-HPLC), the endoproteinase-Arg-C (endo-R-C) digestion of selected fractions, and the separation of resulting peptides for microsequencing were performed exactly as described previously (9, 10). Cell lines were obtained and bone marrow-derived macrophages (BMM) prepared as described previously (12, 13).

cDNA Cloning—p37 cDNAs were cloned by screening a previously described azaphil BAC1.2F5 cDNA library (Stratagene) (12) using a mixture of two 32P-labeled oligonucleotides 5′-cTTgAgACAgTgTCTgAATCCTCTACTgC-3′ and 5′-TggAAgAGACTCCCAgCACggCCTggCTgCg-3′ that were based on the expressed sequence tag (EST) vJ01 b01.r1 from the Washington University-Howard Medical Institute mouse EST project. Filters were hybridized in 6 × SSC, 20 mM NaH2PO4, 0.4% SDS, 500 μg/ml salmon sperm DNA at 55 °C for 18 h and washed stepwise to a final stringency of 0.5% SCC, 0.1% SDS at 55 °C. From the 1.3 × 106 phages screened, 36 positive clones were obtained and further analyzed by PCR using a combination of an antisense internal primer (5′-TTTgTCTgAATCCTgTACTgC-3′) with T3 or T7 primers. The six clones with the longest 5′ end sequences were recovered in pBluescript II SK− by phagemid excision. cDNA inserts were characterized by restriction analysis and by sequencing on both strands by the dyeoxy nucleotide chain termination method using an automatic sequencer (Applied Biosystems, model 373A). One clone (2.1 kb long) was shown to contain the full-length open reading frame. Transient Expression of MAYP—Semi confluent 293T cells were transfected by calcium-phosphate precipitation with a cytomegalovirus-based expression vector (pCMV5) containing the 2.1-kb clone. Eighteen

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1 The abbreviations used are: CSF-1, colony stimulating factor-1; CSF-1R, CSF-1 receptor; endo-R-C, endoproteinase-Arg-C; EST, expressed sequence tag; FCH, Fes/CIP4 homology; IAA, iodoacetic acid; MAYP, macrophage actin-associated tyrosine-phosphorylated protein; PST PIP, proline, serine, threonine phosphatase interacting protein; Tyr(P), phosphotyrosine; PTP, protein-tyrosine phosphatase; PTP-HSCF, PTP-hematopoietic stem cell factor; RP-HPLC, reverse phase high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; WASP, Wiskott-Aldrich syndrome protein; BMM, bone marrow-derived macrophages; PCR, polymerase chain reaction; kb, kilobase pair(s); HRP, horseradish peroxidase; ORF, open reading frame; aa, amino acids.
RESULTS

Identification of pp37 as a CSF-1-regulated Phosphotyrosyl Protein—When the anti-Tyr(P)-reactive cytosolic fraction of CSF-1-stimulated BAC1.2F5 macrophages is subjected to SDS-PAGE, pp37 is the major CSF-1-stimulated tyrosine-phosphorylated band of molecular mass < 50 kDa. Its tyrosine phosphorylation is increased 4–6-fold by CSF-1 stimulation. If the cells are preincubated with the PTP inhibitor, IAA, pp37 is constitutively tyrosine-phosphorylated to ~27 times the levels seen in unstimulated cells in the absence of IAA, suggesting that its CSF-1-induced tyrosine phosphorylation could be regulated by inhibition of its dephosphorylation by a PTP (Fig. 3 of Ref. 9). Two-dimensional gel electrophoretic analysis of the anti-Tyr(P)-reactive cytosolic fraction of cells incubated with IAA (Fig. 1) demonstrates that the pI of tyrosine-phosphorylated pp37 ranges from 6 to 9.5, consistent with its possible phosphorylation on multiple residues (upper and middle panels). This behavior of pp37 was subsequently confirmed by reprobing the immunoblot with anti-MAYP (lower panel).

Purification and Sequence Analysis of pp37—pp37 was purified from cytosol of IAA-treated and CSF-1-stimulated BAC1.2F5 cells by anti-Tyr(P) affinity chromatography, denaturing size exclusion chromatography (9, 10), and C8 RP-HPLC (Fig. 2). Ten percent of the purified pp37 from the C4 fraction was used for NH2-terminal sequence determination and 90% digested by endo-R-C. The resulting peptides were resolved by C8 RP-HPLC and sequenced.

Cloning and the Properties of the Cloned pp37 (MAYP)—A search of the Genbank database using the amino acid sequences of nine tryptic peptides derived from purified pp37 and the tblast.n algorithm yielded a single match with an EST clone. A single ORF in this clone matched four of the nine peptides. Two 50-mer oligonucleotides based on the sequence of that clone were used to screen a λZapiI BAC1.2F5 cDNA library. Thirty-six independent hybridizing clones were obtained. The corresponding primary phages were further analyzed by PCR using an EST internal antisense primer and T3 or T7 primers. The six clones containing the longest 5’ end sequences were rescued and sequenced. The sequence of the larger cDNA insert (2.1 kb) contained a full ORF of 1005 base
Fig. 3. The predicted amino acid sequence of p37 (MAYP) and its schematic comparison with the sequences of PST PIP and CDC15. A, the predicted sequence of the mature MAYP is aligned with the sequences of the endo-R-C peptides (lowercase) and shows the EST sequence (underlined), potential Ser, Thr, Tyr phosphorylation sites (asterisks, including those for cAMP- and cGMP-dependent protein kinases, protein kinase C, and casein kinase II) and SH3 domain binding sites (double underlined). Tyr residues conserved with PST PIP (C), and the peptide sequence used to prepare the anti-MAYP antiserum (overlined). This sequence data is available from the EMBL nucleotide sequence database under accession number Swiss-Prt Y18101. B, schematic relationship of the primary structures of MAYP, PST PIP, and CDC15, showing the region of sequence similarity (shaded, aa 5–295 of MAYP), including the shared FCH domain (filled, aa 10–98 of MAYP), the coiled-coil domains (heavy hatching, aa 95–121 of MAYP), and the shared basic and acidic residue-rich region (light hatching, aa 99–160 of MAYP). Also shown are the additional regions of high homology between PST PIP and CDC15 (cross-hatching and interrupted in CDC15 by a Pro-, Glu-, and Thr-rich (PEST) domain) and their SH3 domains (unfilled).
pairs. Consistent with the results of the two-dimensional gel electrophoresis (Fig. 1), the cDNA sequence predicts a 334-amino acid protein with a molecular mass of 38500.7 Da and a pI of 8.37 (Fig. 3A). The predicted protein sequence also contains the sequences of all other sequenced endo-R-C peptides (Fig. 3A). The sequence of MAYP shares high homology (87% identity and 95% similarity) to the EST sequence (underlined), potential Ser, Thr, Tyr phosphorylation sites (asterisks, including those for cAMP- and cGMP-dependent protein kinases, protein kinase C, and casein kinase II) and SH3 domain binding sites (double underlined). Tyr residues conserved with PST PIP (C), and the peptide sequence used to prepare the anti-MAYP antiserum (overlined). This sequence data is available from the EMBL nucleotide sequence database under accession number Swiss-Prt Y18101. B, schematic relationship of the primary structures of MAYP, PST PIP, and CDC15, showing the region of sequence similarity (shaded, aa 5–295 of MAYP), including the shared FCH domain (filled, aa 10–98 of MAYP), the coiled-coil domains (heavy hatching, aa 95–121 of MAYP), and the shared basic and acidic residue-rich region (light hatching, aa 99–160 of MAYP). Also shown are the additional regions of high homology between PST PIP and CDC15 (cross-hatching and interrupted in CDC15 by a Pro-, Glu-, and Thr-rich (PEST) domain) and their SH3 domains (unfilled).

Fig. 4. Cell, tissue, and subcellular distribution of MAYP. A, cells; B, tissues. The long exposure of the Western blot (WB) reveals the presence of small amounts of p37 MAYP and additional bands of unknown significance in several tissues. Cells or tissues were solubilized in SDS sample buffer and equal amounts of protein used. C, subcellular distribution. BAC1.2F5 macrophages were incubated with or without CSF-1 for 2 h at 4˚C in the presence and absence of IAA prior to separation into cytosolic, membrane, cytoskeletal, and nuclear fractions as described under “Experimental Procedures.” All samples were subjected to gradient SDS-PAGE and Western blotting with either anti-Tyr(P) or anti-MAYP antibodies.
MAYP was detected in the nuclear fraction. The amounts of MAYP in the cytosol and membrane fractions are apparently unchanged following CSF-1 stimulation, and despite its presence in the membrane fraction, no association of MAYP with the CSF-1R could be detected (data not shown). However, when cells are incubated with IAA in the absence or presence of CSF-1, the increased tyrosine phosphorylation of total MAYP (Fig. 1 of Ref. 9; data not shown) is associated with a 5-fold increase in the proportion of MAYP in the membrane fraction (Fig. 4C).

**CSF-1-stimulated MAYP Tyrosine Phosphorylation and Association with F-actin—Anti-Tyr(P) immunoblotting of MAYP immunoprecipitates of the cytosolic fraction of unstimulated macrophages and macrophages stimulated with CSF-1 at 37 °C for 1 min revealed that MAYP is only very slightly tyrosine-phosphorylated in unstimulated cells. However, in response to CSF-1 stimulation, the tyrosine phosphorylation of MAYP was increased by 6-fold (Fig. 5, upper panel). As expected from the results of the immunoblots in Fig. 4C, the amount of immunoprecipitable cytosolic MAYP was unchanged by CSF-1 stimulation (Fig. 5, middle panel). Previous studies have shown that phalloidin precipitation of F-actin from the cytosolic anti-Tyr(P)-reactive fraction co-precipitated a 37-kDa tyrosine-phosphorylated protein (9), that we have subsequently identified to be MAYP by immunoblotting with the antiserum used for the experiments shown in Fig. 5 (data not shown). Immunoprecipitation of cytosolic MAYP also co-precipitated actin (Fig. 5, lower panel). These results indicate that MAYP associates, directly or indirectly, with F-actin.

**DISCUSSION**

Previous phalloidin co-precipitation experiments indicated that pp37 was the most prominent F-actin-associated tyrosine-phosphorylated protein in the cytosolic fraction of CSF-1-stimulated macrophages (9). The present study establishes the identity of pp37 as MAYP and indicates that it is related to a group of PTP substrates, one of which has been shown to play an important role in regulation of the actin cytoskeleton. From its pattern of expression in cell lines, MAYP appears to be selectively expressed in macrophages (i.e. BAC1.2F5, BMM, and J774.2 cells), rather than less mature mononuclear phagocyte progenitor cells (i.e. M1 and WEHI-3 cells) and not at all in some other cell types, including fibroblasts, erythroid progenitors, and mast cells. MAYP exhibits a low level of tyrosine phosphorylation in unstimulated macrophages that is increased 4–6-fold by stimulation with CSF-1. Although the vast majority of MAYP resides in the cytosolic fraction, ~4% resides in the membrane fraction and a trace amount in the cytoskeletal fraction. Upon stimulation with CSF-1, the proportion of MAYP in the cytoskeletal fraction is rapidly increased. Treatment with IAA alone increases the proportion of tyrosine-phosphorylated MAYP by 27-fold and increases the proportion of membrane associated MAYP by 6-fold. It is therefore possible that stimulation with CSF-1 alone, which increases tyrosine phosphorylation by only ~5-fold, is associated with a movement of MAYP to the membrane that is below the level of detection and that membrane association may require tyrosine phosphorylation of MAYP. Interestingly, cytosolic MAYP is constitutively associated with actin (Fig. 5; Ref. 9). These data suggest that MAYP may be involved in regulating some of the fast, CSF-1-induced cytoskeletal changes that take place within minutes of macrophage stimulation with CSF-1 (11, 18, 19).

MAYP, PST PIP, and CDC15 share FCH domains, coiled-coil domains, and basic and acidic amino acid-rich regions. PST PIP was identified by a yeast two-hybrid screen with the PEST-type PTP, PTP hematopoietic stem cell fraction (PTP-HSCF) (20), which is expressed in hematopoietic stem/progenitor cells and fetal thymus, but not in more differentiated cells, including macrophages (21, 22). PST PIP appears to be the ortholog of the actin-associated Schizosaccharomyces pombe protein, CDC15p, a phosphorylated protein implicated in the assembly of the actin ring in the cytokinetic furrow (20, 23). Association of PST PIP with PTP-HSCF involves the proline-rich region of the phosphatase and the coiled-coil domains of PST PIP. In co-transfection experiments in COS cells, PST PIP was shown to be tyrosine-phosphorylated by v-Src and dephosphorylated by its associated PTP-HSCF. PST PIP is co-localized with F-actin-rich regions (cortical actin cytoskeleton, actin stress fibers, lamellipodia) in interphase cells and with cortical actin and the cytokinetic furrow in cells undergoing cytokinesis (20). More recently, its role in the regulation of the actin cytoskeleton was further emphasized by the demonstration that the interaction between the PIP SH3 domain and proline-rich regions of Wiskott-Aldrich syndrome protein (WASP) results in a loss of actin bundling activity by the COOH terminus of WASP and that tyrosine phosphorylation in the polyproline binding pocket of the SH3 domain of PST PIP inhibits binding of PST PIP and WASP, releasing WASP and PST PIP for their independent functioning elsewhere in the cell (24).

Three features of MAYP suggest that its function and regulation in cells differ from the function and regulation of PST PIP. First, MAYP appears to be selectively expressed in macrophages and within the mononuclear phagocytic lineage has the inverse expression pattern of PTP-HSCF, which is expressed in more primitive cells and not in macrophages (21, 22). For this reason, MAYP is unlikely to be regulated by PTP-HSCF. Second, MAYP differs from PST PIP/CDC15 in that it lacks the SH3 domain of PST PIP that appears to be critical for its modulatory effect on WASP. Third, the existence of a human EST sharing 95% sequence similarity with MAYP indicates that MAYP is not the mouse homolog of human PTP PIP.

Despite these differences, MAYP shares several features in common with PST PIP that suggest that they could function in a similar manner. First, they share a region of sequence similarity that includes an FCH domain, a coiled-coil domain, and a region rich in basic and acidic amino acids. Second, the increased tyrosine phosphorylation of MAYP in the presence of IAA could be due to inhibition of a closely associated PTP, akin to the regulation of PST PIP by PTP-HSCF. Third, MAYP binds F-actin, and PST PIP is clearly intimately involved in regulating the actin cytoskeleton.
The possibility that MAYP is involved in regulation of the actin cytoskeleton is appealing because of the presence of both the FCH domain and regions sharing sequence similarity with known actin-binding domains and because of the rapid reorganization of actin observed in response to CSF-1 (18, 19). The FCH domain has been found to occur at the extreme NH2 terminus of the nonreceptor tyrosine kinase FER, the Fujinami Sarcoma virus Fes/Fps family of proto-oncogene products, the RhoGAP protein p115, mouse proteins h74, and the growth arrest-specific gene product as well as two gene products from Caenorhabditis elegans (FO9E10.9 and F45E1.7) and one from S. cerevisiae. Several of these proteins have potential roles in organizing Rho proteins and the actin cytoskeleton. It has been suggested that the FCH domains may bind functionally related target molecules (25). The coiled coil domain of MAYP is highly conserved with the central coiled coil domain of PST PIP (67% identity). These domains are also rich in lysine and arginine residues and resemble sequences present in several actin-binding proteins (16, 17), including paramyosin, myosin heavy chain, and troponin. While PST PIP has not been reported to bind actin, its function is intimately involved in the regulation of F-actin. Thus the association of MAYP with F-actin and its close relationship to PST PIP are intriguing, particularly in view of the rapid reorganization of the cytoskeleton observed in response to CSF-1, that follows MAYP tyrosine phosphorylation. Obviously, it will be important to identify the PTP(s) and kinase(s) that regulate MAYP tyrosine phosphorylation, the mechanism by which MAYP associates with actin and its functional role in the CSF-1 response of macrophages.

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