Suppression of Microphthalmia Transcriptional Activity by Its Association with Protein Kinase C-interacting Protein 1 in Mast Cells*

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Ehud Razin‡§, Zhao Cheng Zhang‡, Hovav Nechushtan‡, Shahar Frenkel‡, Yu-Nee Lee‡, Ramachandran Arudchandran‡, and Juan Rivera‡

From the ‡Department of Biochemistry, Hebrew University-Hadassah Medical School, P. O. Box 12272, Jerusalem 91120, Israel and the §§Arthritis and Rheumatism Branch, NIAMS, National Institutes of Health, Bethesda, Maryland 20892-1820

Microphthalmia (mi) is a transcription factor that plays a major role in the regulation of growth and function in mast cells and melanocytes. Association of mi with other proteins is a critical step in the regulation of mi-mediated transcriptional activation. We found protein kinase C-interacting protein 1 (PKCI) specifically associated with mi in yeast two-hybrid screening. Immunoprecipitation of mi from quiescent rat basophilic leukemia cells or mouse melanocytes resulted in the specific co-immunoprecipitation of PKCI. This association was significantly reduced on engagement of the surface FceRI of mast cells or engagement of the Kit receptor on melanocytes. Hence, cell activation caused disengagement of mi from PKCI. Microphthalmia was previously shown to activate the mouse mast cell protease 6 (mMCP-6) promoter. Cotransfection of mi with PKCI in NIH 3T3 fibroblasts containing an mMCP-6 promoter-luciferase reporter demonstrated an up to 94% inhibition of mi-mediated transcriptional activation. PKCI by itself, although localized in the cytosol and nucleus of the cells, has no known physiological function and did not demonstrate transcriptional activity. Its ability to suppress mi transcriptional activity in the transient transfected fibroblast system suggests that it can function in vivo as a negative regulator of mi-induced transcriptional activation.

Microphthalmia (mi)† is a transcription factor of the basic helix-loop-helix leucine zipper (bHLH-Zip) type (1). Homozygous mutations at the mi locus were shown to cause the mouse microphthalmia phenotype (2). This phenotype is now known to contain more than 10 allelic variations (3) and is typified by a decrease in mast cell number, deafness, a defect in osteoclasts, small unpigmented eyes and skin, and a major depletion of inner ear melanocytes (3). In humans, mutation in this gene causes Wardenburg syndrome type II (4).

Northern blot of mRNA from different tissues and cell types showed that mi mRNA is present only in a limited number of tissues and cell types, those being heart, uterus, melanocytes, and mast cells (4). It was reported that mi regulates the expression of mouse mast cell protease 6 (mMCP-6) (5) and mouse c-Kit (6, 7) and that mi is involved in the expression of genes involved in pigmentation such as tyrosinase (albino locus), tyrosinase-related protein, and the pink-eyed Pmel 17 (silver) (8). Thus, down-regulation of mi expression plays a significant role in the decreased pigmentation observed in melanoma. We recently investigated the expression of mi in mast cells (9) and found that mi plays a crucial role in cell proliferation.

mi binds E-box-type enhancer elements (5), and, like many other transcription factors, mi’s activity is probably determined through its interactions with other proteins. It was observed that mi may heterodimerize with the related family members TFEB, TFE3 (10–12), and USF2 (9).

In the present work, we have identified other mi-interacting proteins, using a construct containing the mi bHLH-Zip domains as bait in yeast two-hybrid library screening. We identified one high affinity interacting protein as protein kinase C-interacting protein 1 (PKCI). This protein is a ubiquitous member of the histidine triad (HIT) protein family that is denoted by a conserved HIT sequence motif (13). This HIT family of proteins is highly conserved in nature, but the physiologic biological activity has not been identified for any of its members, although an in vitro study revealed that PKCI enzymatically hydrolyzes adenosine polyphosphates (13).

In the present study, we present clear evidence of a function for PKCI as a repressor of mi-induced transcriptional activity. Furthermore, we demonstrate that mi and PKCI interact in cell lysates and that this interaction is negatively regulated by cell surface receptor engagement. Thus, this study supports the idea that mi transcriptional activity is up-regulated in response to cell surface receptor engagement by dissociation from PKCI.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The bait was constructed in pAS2–1 by ligation of polymerase chain reaction-amplified cDNA covering the mi cDNA fragment coding the sequence from 608 to 1113 (bHLH-Zip) into the BamHI and PstI sites for use in the yeast two-hybrid screening. The cDNA library was prepared from resting rat basophilic leukemia (RBL) cells mRNA by reverse transcription and cloned in the EcoRI site of the pGAD10 vector. The insert size was on average 1.7 kilobase pairs and ranged from 0.3 to 3.5 kilobase pairs. The cDNA encoding the open reading frame of mi, PKCI, Rac (N17), and Drosophila melanogaster M7 were subcloned into the XbaI and HindIII sites of the pcDNA3.1 vector.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, Hebrew University-Hadassah Medical School, P. O. Box 12272, Jerusalem 91120, Israel. Tel.: 972-2-6758288; Fax: 972-2-6411663; E-mail: ehudr@cc.huji.ac.il.

‡ The abbreviations used are: mi, microphthalmia; bHLH-Zip, basic helix-loop-helix leucine zipper; mMCP-6, mouse mast cell protease 6; Ig, immunoglobulin; Ag, antigen; PKCI, protein kinase C-interacting protein 1; HIT, histidine triad protein; RBL, rat basophilic leukemia; GST, glutathione S-transferase; DNP, dinitrophenol; FITC, fluorocescin isothiocyanate; PI, propidium iodide; NRS, normal rabbit serum; G-CSF, granulocyte-colony-stimulating factor.
To generate the glutathione S-transferase (GST)-mi fusion protein, the cDNA of mi was subcloned into the SmaI site of the pGEX3x vector. Fidelity of all constructs was verified by direct sequencing. The luciferase reporter plasmid, pSP72, containing the mi binding region of the promoter and the first exon of the mouse mMCP-6 gene (−191 to +26), as well as a construct with a deleted mi binding site (−151 to +26) (5) were generously provided by Prof. Kitamura, Osaka, Japan.

**Yeast Two-hybrid Experiments**—Yeast transformation and two-hybrid screening and assays were performed according to the instructions for the MATCHMAKER two-hybrid system (CLONTECH). Yeast CG1945 was transformed with pAS2–1 mi (bHLH-Zip) in order to screen for proteins that were capable of interacting with mi. We screened the pGAD10 RBL cDNA library expressed in yeast and selected the transformed yeast by growth on synthetic medium lacking Trp, Leu, and His, and containing 0.25 mM 3-aminotriazole. Five days later one of the selected colonies was regrown on the same medium. The doubly selected colonies were then tested for galactosidase activity. The cDNA of positive clones was rescued in *Escherichia coli* DH5α and subsequently sequenced.

**Cells and Activation**—Melanocytes, B16S10.9 (9), were maintained at 37 °C in RPMI 1640 medium supplemented with 2 mM L-glutamine, 2 mM nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.), 50 μM β-mercaptoethanol (Fisher Scientific, Medford, MA), and 10% fetal calf serum (growth medium).

**Indirect Fluorescent Immunocytochemistry**—Treated cells were cytose- centrifuged and fixed with methanol (−20 °C) for 3 min. After 45 min blocking with normal goat serum, indirect fluorescent immunocytochemistry was carried out using normal rabbit serum (NRS), rabbit anti-myoosin II (kindly provided by S. Ravid, Hebrew University, Jerusalem), rabbit anti-mi (9), or rabbit anti-PKCI (13) antibodies and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). The cells were then counterstained with propidium iodide (PI) and mounted. Specimens were examined, analyzed, and photographed using laser scanning confocal microscopy.

**Suppression of mi Transcription by PKCI**

**Fig. 1.** Diagram of mi protein that was used as a bait in this study. Mouse cDNA encoding mi 160–328 was constructed into bait vector pAS2–1. This part of the mi protein contains a basic domain (b), a helix (H), a loop (L), and a leucine zipper (Zip).

**Fig. 2.** Association of mi with PKCI in RBL cells. A, Western blot of GST-mi-interacting PKCI isolated from RBL lysate. Cell lysates from non-activated RBL cells were incubated alone, with GST, or with GST-mi. Blots of the isolated proteins were probed with anti-PKCI antibody. B, Western blot of immunoprecipitation of PKCI with mi from RBL cells. Immunoprecipitation from lysate of non-activated RBL cells with either anti-mi- or NRS protein A. Blots were probed with anti-PKCI antibody. C, Western blot of immunoprecipitation of mi with PKCI from RBL cells. Immunoprecipitation from lysate of non-activated RBL cells with either anti-PKCI or NRS protein A. Blots were probed with anti-mi antibody. One representative of three experiments is shown.
three times with lysis buffer and then washed once with Tris-EDTA washing buffer. All buffers contained protease inhibitors. Proteins were solubilized in Laemmli sample buffer containing 0.5% SDS.

**Gel Electrophoresis and Western Blots**—Proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to 0.45-μm nitrocellulose membranes. After incubation with blocking buffer (5% fat-free milk, 0.1% Tween 20 in phosphate-buffered saline, pH 7.4), polyclonal antibodies were used at a dilution of 1:500 to detect PKCI or mi proteins. Visualization of reactive proteins was by enhanced chemiluminescence.

**RESULTS**

**mi Interaction with PKCI**—As described previously, the mi protein contains two putative transactivation domains in the amino terminus of the protein (1). Thus, to avoid background that might result from nonspecific interactions in the yeast, we used mi from which the two putative transactivating domains had been deleted (Fig. 1) to screen for mi-interacting proteins. As shown in Fig. 1, the expressed mi cDNA contained the bHLH-Zip motif. This mi cDNA fragment, which was subcloned in pAS2–1, did not activate transcription in yeast. From 1 × 10⁶ yeast transformants, 12 plasmids were isolated that encoded proteins that interact with mi. Most of these proteins will be described elsewhere. One of the isolated cDNA clones encoded the TFE3 transcription factor, which was previously shown by biochemical means to associate with mi (15). Another isolated cDNA encoded the PKCI protein. To ascertain the specificity of this interaction, the PKCI in the pGAD10 vector was transfected in yeast with an unrelated bHLH-Zip protein, the D. melanogaster M7 (14), cloned in the bait vector pAS2–1. No interaction was found between these two proteins.

To determine if mi could bind to PKCI derived from cell lysates, mi protein was fused to GST, immobilized on glutathione-Sepharose beads, and used to isolate PKCI from RBL cell lysates. Endogenously expressed PKCI was specifically retained on the beads coupled to GST as it was not bound to beads linked to GST alone (Fig. 2A). Thus, the interaction of mi with PKCI in cell lysates corroborated the results of the yeast two-hybrid interaction assay. We then investigated whether immunoprecipitation of mi from quiescent RBL cells would result in specific co-immunoprecipitation of PKCI. This was carried out by incubating the RBL lysate with either anti-mi or anti-PKCI prebound protein A. The recovered and resolved immune-complexes showed the co-immunoprecipitation of...
PKCI with mi using either immunoprecipitation approach (Fig. 2, B and C). We also analyzed if PKCI would co-immunoprecipitate with mi from mouse melanocytes. Immunoprecipitation of mi from quiescent melanocytes resulted in co-immunoprecipitation of PKCI. This was carried out by incubating the melanocyte lysate with anti-mi prebound protein A (Fig. 3). Thus, PKCI forms a complex with mi in yeast, mast cells, and melanocytes.

We assessed whether the interaction of these two proteins could be modulated by engagement of the IgE receptor by IgE-Ag on RBL mast cells. In three separate experiments, this stimulus dramatically reduced the amount of PKCI co-immunoprecipitated with mi by 80%, as determined by densitometric analysis (Fig. 4). We investigated if this was a more general mechanism regulating mi transcriptional activity by immunoprecipitating mi from melanocytes stimulated by Kit receptor engagement. Stimulation of melanocytes with Kit ligand also dramatically reduced the amount of PKCI that co-immunoprecipitated with mi (Fig. 3). Therefore, these results suggested that the dissociation of mi from PKCI could be a prerequisite for increased mi transcriptional activity in both mast cells and melanocytes.

**Regulation of mi Transcriptional Activity by PKCI**—As mentioned above, PKCI is well conserved throughout nature and expressed in almost all nucleated eukaryotic cells including NIH 3T3 fibroblasts (16). However, mi expression is tissue-specific (2, 3). To test the effect on PKCI interaction on mi transcriptional activity, we chose to work with cells that did not abundantly express endogenous mi. The expression of mi protein in NIH 3T3 fibroblasts was determined and, as shown in Fig. 5, mi protein could not be detected in these cells. NIH 3T3 fibroblasts were co-transfected with a luciferase reporter plasmid containing the mouse mMCP-6 promoter and pcDNA constructs of either mi or pkci and PKCI. The relative luciferase activity in NIH 3T3 cells cotransfected with 1 \( \mu \)g of mi in pcDNA together with 1 \( \mu \)g of the pcDNA vector alone was 3.1 \( \pm \) 0.9 (mean \( \pm \) S.E., \( n = 5 \)). However, cotransfection of 1 \( \mu \)g of mi in pcDNA together with 1 \( \mu \)g of PKCI in pcDNA showed a relative luciferase activity of only 1.5 \( \pm \) 0.2 (mean \( \pm \) S.E., \( n = 5 \)). Up to 94% inhibition of the luciferase activity was observed when the NIH 3T3 fibroblasts were cotransfected with 5 \( \mu \)g of mi and 5–20 \( \mu \)g of PKCI (Fig. 5). The extent of inhibition was dose-dependent. Cotransfection of mi with the pcDNA vector alone or with pcDNA constructs containing either the transcription factor Ptf1 from D. melanogaster or a dominant negative Rac1 (N17) showed no inhibitory effect on the mi-induced luciferase activity.

**Intracellular Localization of PKCI and mi**—The intracellular localization of mi and PKCI was determined by indirect fluorescent immunocytochemistry. The cells were labeled with FITC-conjugated secondary antibody (green) that recognized the highly specific anti-mi or anti-PKCI antibodies and counterstained with PI (red) to label their nuclei. All cells had mi and PKCI in their nuclei (Fig. 6). Cells stained with anti-myosin II showed only cytoplasmic staining, and normal rabbit serum staining showed practically no background staining. The presence of mi and PKCI in the nucleus was determined by optically scanning the samples at 0.6 mm increases through the z axis and counterstaining of the nucleus with PI. That ensured that the cells were viewed in a mid-section plane, thus guaranteeing that the nucleus was not overshadowed by the cytoplasm. The presence of both PKCI and mi in the nucleus corroborates the results from the cotransfection experiments that suggested that PKCI serves as a repressor of mi transcriptional activity.

**DISCUSSION**

We demonstrated here that the interaction of PKCI with mi was found to inhibit the transcriptional activity of mi. Thus, PKCI may serve as a negative regulator of cell proliferation and differentiation.

Sequence alignment of PKCI family members ranging from mycoplasma to plants to humans reveal that this family of proteins is extremely well conserved throughout nature (17). Although PKCI was originally identified as an in vitro inhibitor of the conventional protein kinase C isoforms (18), subsequent studies revealed that it is unlikely that this protein is a potent in vivo inhibitor of protein kinase C (16). Studies using x-ray crystallography and in vitro enzyme assays have elucidated the structural and functional aspects of PKCI, suggesting a possible nucleotidyl hydrolase or transferase activity (15). However, the in vivo function of PKCI is not known with certainty. Our above mentioned findings of this study along with the nuclear localization of PKCI and mi serve to promote the possibility that the inhibitory effects of PKCI might be mediated through sequestration of mi or through PKCI-mediated changes in the mi-binding target DNA.

Indirect evidence in support of the idea of mi as a key player in c-Kit-mediated signaling has been reported by Dubreil et al. (19). In this study it was shown that the G-CSF receptor could overcome a deficiency of Kit receptor in c-Kit-deficient mast cells, thus allowing these cells to proliferate in response to G-CSF. However, mast cells developed in culture from mi/mi mice did not proliferate when G-CSF receptor was engaged. Therefore, mi probably acts downstream of c-Kit in growth
factor-mediated signaling leading to mast cell proliferation. Thus, it would seem that mi plays a pluripotent role in mast cell activation both as a regulator of c-Kit gene expression and as an essential component in the Kit receptor-mediated signaling.

Our present findings suggest a possible mechanism for regulation of cellular proliferation and development by sequestration of mi from complexing with other transcription elements required for gene activation. This may be a more general mechanism, which may regulate mi association with other proteins such as TFE3, a bHLH-Zip transcription factor related to mi (11), which was previously shown to interact with mi and whose association with mi confirmed in our yeast two hybrid screening. The complexing of these two transcription factors has also been shown to play an important role in osteoclast function and possibly in age-related osteoclast homeostasis (15).

The expression of mMCP-6 is highly restricted to mast cells (20), and even fibroblasts in normal tissues do not express this protease (21, 22). However, not all mast cells express this tryptase. For example, during a helminth infection, mast cells that reside in the jejunal epithelium fail to express mMCP-6, whereas mast cells in the peritoneal cavity, skin, and heart express this tryptase (23). The mucosal mast cell analog, RBL (24), does not express substantial amounts of rat homolog of mMCP-6 (rMCP-6, 25). Therefore, it is possible that the expression of substantial amounts of PKCI in these cells may account for their failure to express significant levels of rMCP-6. mMCP-6 induces the selective extravasation of neutrophils into tissues (26) by a mechanism as of yet undefined. Because mMCP-6 induces the selective extravasation of neutrophils into tissues (26) by a mechanism as of yet undefined. Because mMCP-6 is potent, selective, and long-lived, multiple control mechanisms must exist to ensure that its expression is tightly regulated. This tight regulation is essential because mi is also expressed in cells other mast cells (1). Based on our results, it now appears that one such mechanism is PKCI to inhibit mi activity.

PKCI, however, represents a novel type of molecule that associates with mi, as it does not have transcriptional activity. Its nuclear localization and role in suppression of mi transcriptional activity indicate its physiological role as a co-repressor of mi. As it is a ubiquitously expressed protein, it may serve a similar role in regulating cell proliferation by interacting with cell-specific transcription factors. The observation that activation of mast cells and melanocytes causes a marked decrease in the association of PKCI with mi suggests that PKCI functions as a transcriptional repressor that associates with mi and prevents mi recruitment and/or activation of target genes until the appropriate stimuli is encountered. Hence, it is likely that mi participates in the coordination of expression of multiple genes by formation of specific complexes. These complexes are likely formed when mi dissociates from PKCI and is thus available to bind other transcription factors like TFE3 and USF2 that may provide both cellular and gene specificity.

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