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Identification and Characterization of the Inducible Murine Mast Cell Gene, *imc*-415

Seong H. Cho,* Jeong-Je Cho,* Il S. Kim,* Harissios Vliagoftis,†
Dean D. Metcalfe,† and Chad K. Oh*,[1]

**Division of Allergy and Immunology, Department of Pediatrics, Harbor–UCLA Medical Center, Torrance, California 90509; and †Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-1881*

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Activation of mast cells results in the generation and release of bioactive mediators which in turn initiate allergic inflammation. Mast cell function is enhanced following stimulation in part because of the induction of specific genes and their products. To identify additional genes induced in mast cells that support this process, we thus constructed an activation-specific mast cell subtraction library. To date, we have isolated 26 novel inducible murine mast cell (*imc*) cDNA clones. Among them, a full-coding region of the murine gene *imc*-415 was found to have a greater than 90% nucleotide sequence homology and a 97.5% amino acid sequence homology to both a human β_4 integrin-binding protein ($p27^{BBP}$) and a human translation initiation factor 6 (eIF6), which in turn are identical. *In vitro* translation of the *imc*-415 gene yielded a band of an approximately 26 kDa. This is the same as the calculated molecular weight of murine IMC-415 protein based on the predicted amino acid sequence and is the molecular weight of $p27^{BBP}$/eIF6. Murine *imc*-415 message was also induced in inflamed lung tissues in a mouse model of asthma. These results suggest a role for murine *imc*-415 in allergic inflammation where it may enhance protein synthesis. Human eIF6/$p27^{BBP}$ may also play a role in allergic diseases based on the similarities in sequence and in gene expression patterns.

Mast cells are essential effector cells in allergic inflammation through their capacity to respond to IgE-dependent activation with release of both preformed and newly generated mediators (1). Mast cells also participate in the modulation of physiologic processes, including delayed type hypersensitivity reactions (2), fibrosis (3), autoimmune processes (4), neoplasia (5), inflammation in the rheumatoid synovium (6), and in inflammatory bowel diseases (7).

While a number of the features attendant to the activation of mast cells in disease are well described (1-7), more remained to be understood about the expression and function of inducible mast cell genes and proteins in response to allergic stimulation. We thus constructed a cDNA subtraction library which was enriched by removing mRNA common to resting cells and cells stimulated by PMA and the ionophore A23187. Subsequently, isolated genes were tested for inducibility upon FcεRI-mediated stimulation. Similar approaches have been used to identify novel genes in macrophages (8), lymphocytes (9), and nerve cells (10). We have reported a group of novel genes induced in mast cells (11).

Among the genes we have isolated, *imc*-415 is of particular interest for its significant homology to the human genes, $p27^{BBP}$ (12) and eIF6 (13). In the present study, we report the full-coding sequence of the *imc*-415 gene and the translation of the *imc*-415 gene *in vitro*. We further characterize both murine *imc*-415 and human eIF6/$p27^{BBP}$ gene expression.

MATERIALS AND METHODS

Cells. The interleukin-3 (IL-3)-independent cloned murine mast cell line Cl.MC/C57.1 (a kind gift of S. Galli, Harvard Medical School, Boston, MA) (14, 15). Briefly, Cl.MC/C57.1 cells were cultured in DMEM with 10% heat inactivated fetal calf serum (FCS), 50 mM β-mercaptoethanol, 4 mM L-glutamine, and 100 μg/ml penicillin/streptomycin (complete DMEM) in a humidified 5% CO_2 incubator. The cell number was adjusted to 5 x 10^5 cells/ml twice weekly by adding fresh media.

Stimulation conditions. For FcεRI-dependent activation, Cl.MC/C57.1 murine mast cells were adjusted to a density of 1 x 10^6 cells/ml in complete DMEM, 0.5 μg/ml of IgE anti-DNP was added, and the cells were incubated at 37ºC for 60 min in humidified 5% CO_2/95% air with gentle rocking. Cells were then centrifuged at 400 x g at 4°C for 10 min and the cell pellets resuspended in fresh media. EL-4 (murine T cell line) and Raw 264.7 (murine macrophage cell line) cells were cultured in RPMI 1640 media with 10% FCS, 50 mM

[1] To whom correspondence should be addressed. Fax: (310) 212-7440. E-mail: chad_oh@humc.edu.

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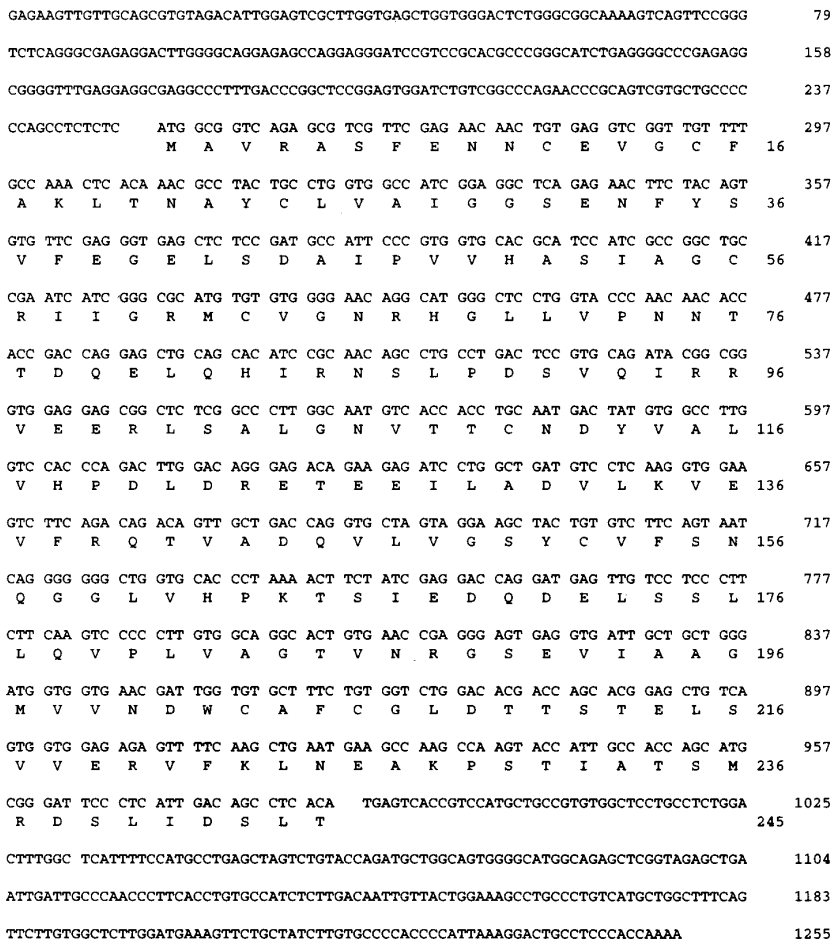

FIG. 1. Nucleotide sequence of murine *imc*-415 mRNA with the amino acid sequence of the open reading frame. Detection and translation of the open reading frame were completed using the MacVector program.

β-mercaptoethanol, 4 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 100 μg/ml penicillin/streptomycin (complete RPMI) in a humidified 5% CO_2 incubator. Cell numbers were adjusted to 5 x 10^5 cells/ml twice weekly with fresh media. When indicated, PMA (Sigma) was added at 50 ng/ml, A23187 (Sigma) at 0.5 mM, calcium ionomycin (Sigma) at 1 mM, CHX (Sigma) at 10 μg/ml, CsA (Sigma) at 2 μg/ml, Dex (Sigma) at 0.1 or 1 μM, and recombinant murine stem cell factor (rmSCF) at 5 or 50 ng/ml.

cDNA probes for Northern blot analysis. A plasmid construct containing cDNA was isolated by coinfection of an *E. coli* strain with the f1 helper phage from the subtractive cDNA library according to the manufacturer's protocol (Uni-Zap XR kit, Stratagene, La Jolla, CA). Following digestion of the plasmid with *Eco*RI and *Xho*I restriction enzymes, whose sites were used for construction of the library, the isolated cDNA fragment was labeled by random hexamer priming using primers and protocols suggested by the manufacturers (Stratagene) (16).

Northern blot analysis. Northern blot analysis was performed with total cellular RNA isolated from Cl.MC/C57.1 mast cells by a guanidine thiocyanate-cesium chloride gradient centrifugation as described (11, 17).

Mice. Female Balb/c mice, age 6-10 weeks, were obtained from in-house sources. All experiments involving mice were under a protocol approved by the NIH Animal care and Use Committee.

Sensitization and challenge. These procedures were performed as described (18). Briefly, mice were first sensitized by intraperitoneal injection of 1 mg chicken egg albumin (OVA, Sigma) and 5 mg alum in 0.1 ml phosphate buffered saline (PBS). Mice were lightly anaesthetized by inhalation of methoxyflurane (Metofane) and subjected to a single intranasal challenge with 100 μl of 1% OVA on the tenth day. Mice were killed by CO_2 asphyxiation at time 0 (immediately prior to antigen challenge); and 100 min, 6 h, and 24 h after antigen challenge for analysis of lung tissue.

Reverse transcription (RT) PCR. Total RNA was isolated as described (17). One μg of RNA was reverse transcribed with avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI) at 37°C for 1 h using random hexamers in a total volume of 50 μl. Two μl of the cDNA was amplified with 0.2 mM dNTPs, and 2.5 units of *Taq* DNA polymerase in the buffer recommended by the supplier (Promega). Amplification consisted of denaturation at 94°C for 5 min followed by 30 cycles at 94°C for 1 min, at 55°C for 2 min, and at 72°C for 3 min; and was ended by a 10 min extension at 72°C. The PCR reaction consisted of the amplification of a murine *imc*-415 cDNA fragment of 242 bp corresponding to exons 2 to 3 of the *imc*-415 gene with the following primers: sense (forward, 5′ AACTTCTACAGTGTGTTCGAGGG 3′) and antisense (reverse, 5′ TAGTCATTGCAGGTGGTGACATT 3′). Amplification of a fragment of the cDNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (239 bp) was performed in the same PCR reaction as the internal control,

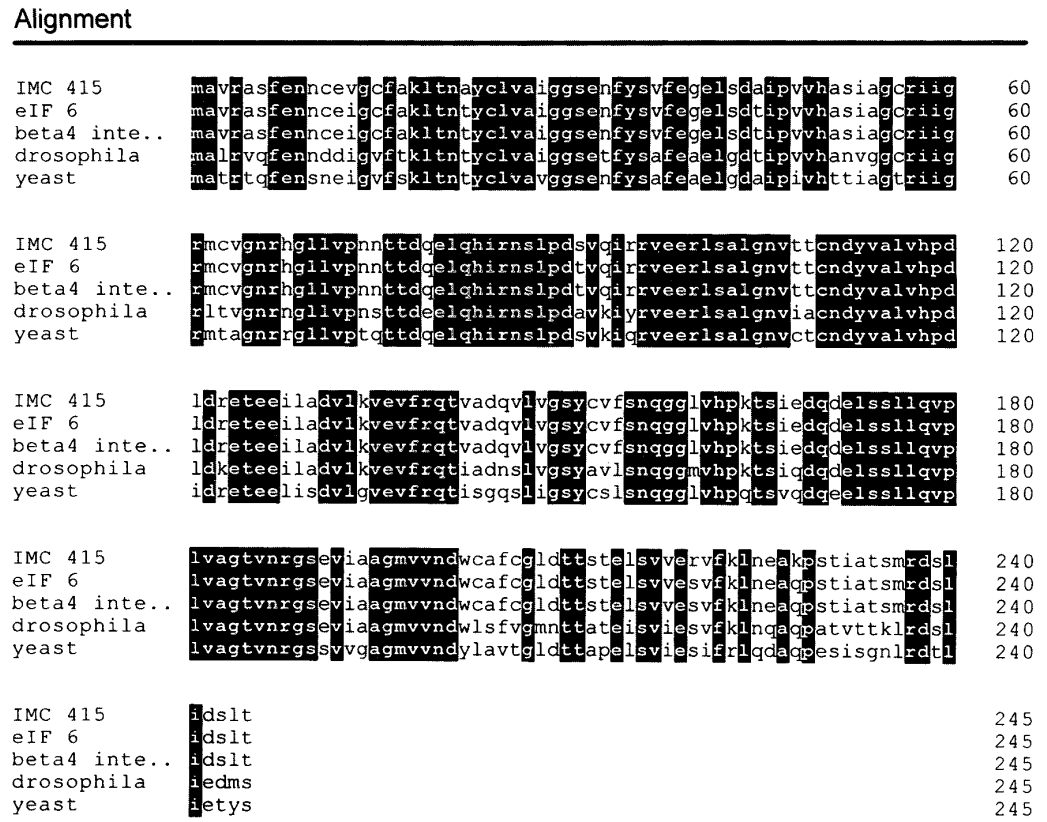

FIG. 2. Homology analysis of amino acid sequence of murine *imc*-415 with human $p27^{BBP}$, human eIF-6, *Drosophila b(2)gcn*, and a hypothetical protein YPRO16c (*Saccharomyces cerevisiae*) yeast gene. Analysis was performed with the Entrez system through Internet access.

with the following primers: sense (forward, 5′ GAGTCTACTGGT-GTCTTCACC 3′) and antisense (reverse, 5′ GTCATGAGCCCTTC-CACA ATGC 3′). Half of each reaction mixture was analyzed by agarose gel electrophoresis. Similar results were obtained with two other experiments.

Construction of the subtractive cDNA library. The subtraction library was constructed as described (11, 19).

In vitro translation. *In vitro* translation reactions were performed according to the manufacturer's instructions (Promega). Briefly, 1 μg of the *imc*-415 DNA template was mixed with rabbit reticulocyte lysate, 1 mM amino acid mixture minus methionine, 10 μCi ^{35}S-methionine, and 40 units of ribonuclease inhibitor with and without canine pancreatic microsomal membranes. Following incubation at 30°C for 2 h, translated products were separated by SDS-PAGE gel and exposed to Kodak X-OMAT-AR film for 16 h to obtain autoradiographs.

RESULTS

Sequence analysis of imc-415. Murine clone *imc*-415 cDNA consists of 1252 nucleotides with a poly A+ tail (approximately 30 As). An open reading frame of this gene codes for 245 amino acids (Fig. 1). Nucleotide and amino acid sequence analyses of *imc*-415 revealed 89% nucleotide sequence homology and 97.5% amino acid sequence homology to $p27^{BBP}$ and eIF6, which themselves are identical. *imc*-415 also shows greater than 70% amino acid sequence homology to the benign (2) gonial cell neoplasm [*b(2)gcn*] gene in *Drosophila* and a yeast gene, respectively (Fig. 2). The identification of the murine *imc*-415 sequence may be of particular interest in determining the *in vitro* and *in vivo* function of this gene, allowing knockout or transgenic animal models to be developed.

Expression of imc-415 in different cell types. To determine whether *imc*-415 is induced in murine mast cell-, T cell- and macrophage cell types, Northern blot analysis was performed before and following activation by PMA and A23187. As can be seen in Fig. 3, murine *imc*-415 message was present in all cells and increased in mast cells following activation. Murine *imc*-415 message did not change significantly in T cells or macrophages upon activation. Until now, inducibility of eIF6 has not been demonstrated, although other eIFs, such as eIF4, are known to be induced in cells such as T cells and fibroblasts (20). These data suggest that expression of *imc*-415 gene is differentially regulated among immune cells.

In vitro translation of imc-415. To express the murine *imc*-415 gene product, a construct which contains a coding sequence of murine *imc*-415 gene was transcribed and translated in a reticulocyte extract with or

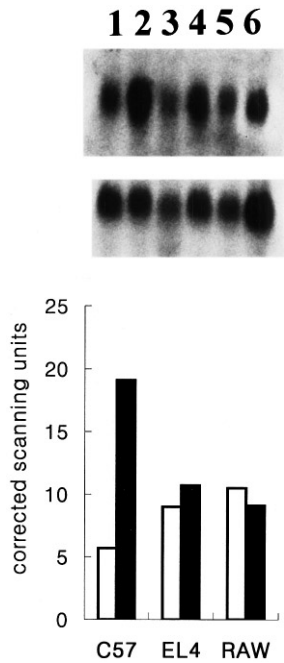

FIG. 3. Expression of murine *imc*-415 mRNA in different cell types by Northern blotting. Upper panel: twenty μg of whole cell RNA from unstimulated mast cells (lane 1), T cells (lane 3), macrophages (lane 5); or mast cells (lane 2), T cells (lane 4), macrophages (lane 6) stimulated with PMA and A23187 for 3 h. ^{32}P-labeled probe was prepared from cDNA encoding murine *imc*-415. Corresponding β-actin is shown. Lower panel: Bars indicate the relative gene expression as determined by densitometric analysis and normalization to the β-actin control gene. Blank bars represent resting cells and solid bars represent cells activated by exposure to PMA and A23187. C57 = Cl.MC/C57.1 murine mast cell line; EL-4 = EL-4 murine T cell line; Raw = Raw 264.7 murine macrophage cell line. Three repeat experiments showed similar results.

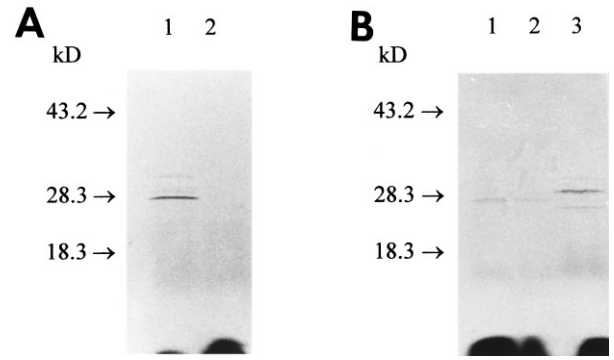

FIG. 4. SDS-PAGE of the *in vitro* translation product of murine *imc*-415. *imc*-415 mRNA was transcribed and translated *in vitro* from cDNA in the pBluescript phagemid using reagents and protocols supplied by Promega. A. Sense (lane 1) and antisense (lane 2) mRNAs were transcribed. B. Sense RNA was transcribed without (lane 1) and with (lane 2) canine microsomal membranes. β-lactamase was used as a positive control (lane 3). Two repeat experiments showed similar results.

without canine microsomal membranes. Fig. 4A demonstrates the synthesis of a protein with a MW of approximately 26 kDa, which is the same as the calculated molecular weight of murine IMC-415 based on the predicted amino acid sequence. Addition of the microsomal membranes did not increase the mobility of the translation product, suggesting that murine IMC-415 is not secreted (Fig. 4B). These results are in agreement with conclusions that the p27^{BBP} protein is highly insoluble and associated with the intermediate filament cytoskeleton both in the cytoplasm and in the nucleus (12).

Induction of imc-415 mRNA in lung tissues of mouse asthma. Asthma is characterized by airway inflammation. The airway inflammation involves activation of multiple cell types including mast cells. We thus examined murine *imc*-415 message levels during an antigen-specific allergic inflammatory response in a murine asthma model (18). Expression of mRNA for murine *imc*-415 in lung extracts was determined by semi-quantitative RT-PCR as a function of time (0 to 24 h) after initiation of the challenge. As can be seen in Fig. 5, 242 bp bands appeared 100 min after challenge and remained through 24 h. No band was observed from unchallenged lung RNA. Presence of similar amounts of RNA for each time point was confirmed by demonstration of GAPDH bands of similar intensity. These data support the hypothesis that induction of murine *imc*-415 gene occurs in asthmatic inflammation.

In summary we report a full coding sequence of *imc*-415 gene and that the *imc*-415 gene is a murine homolog of human eIF6/p27^{BBP}. *imc*-415 is induced in mast cells upon stimulation and may enhance protein synthesis. We also expressed a non-secreted IMC-415 protein *in vitro.* Furthermore, induction of *imc*-415 in lungs of murine asthma suggests a role of murine *imc*-415 in allergic inflammation. Human eIF6/p27^{BBP} may also enhance protein synthesis in human allergic

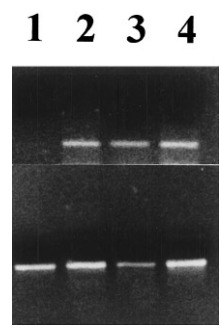

FIG. 5. RT-PCR analysis of *imc*-415 expression during allergic airway inflammation. Whole lung RNA was isolated from mice at 0 (lane 1), 100 min (lane 2), 6 h (lane 3), and 24 h (lane 4) after challenge. Five μg of RNA were reverse transcribed in each sample of the cDNA amplified with imc-415 and murine β-actin primers. The resultant products were examined on a 2% agarose gel containing ethidium bromide and visualized by UV transillumination. Two repeat experiments showed similar results.

diseases based on the similarities in sequences and gene expression patterns.

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