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Identification and Categorization of Inducible Mast Cell Genes in a Subtraction Library

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Mast cells play an important role in allergic inflammation by releasing inducible proinflammatory cytokines. While many inducible genes have been identified, we hypothesized that a significant number remain to be identified. We thus constructed an activation-specific mast cell subtraction library to establish a profile of induced genes in mast cells following allergic stimulation. To date, we have sequenced 150 cDNA clones. Among them, we have isolated 22 known genes whose expression has not been reported in mast cells, and an additional 26 cDNA clones which do not have significant homology to known genes in the Genbank database. We next selected 10 cDNA clones with strong signals by differential plaque hybridization. Of these cDNA clones, five genes were induced in mast cells upon FcεRI-mediated stimulation. They are cofilin, annexinVI, interferon (IFN)-β, serglycin, and a novel inducible mast cell (IMC) gene, IMC-415. Characterization and relevant studies of this novel gene and other inducible known genes in mast cells will provide insight into the functions of mast cells in mammalian biology.

The mast cell is an essential effector cell in allergic inflammation through its 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).

In spite of potent biological activities, there appears much to be learned 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 new or novel genes in macrophages (8), lymphocytes (9), and nerve cells (10).

This is the first report of a profile of genes expressed in mast cells and 5 inducible genes including IMC-415 upon stimulation.

MATERIALS AND METHODS

Cells. The interleukin-3 (IL-3)-independent cloned mast cell line Cl.MC/C57.1 (kind gift of S. Galli, Harvard Medical School, Boston, MA) and IL-3-dependent primary bone marrow cultured mast cells (BMCMC) were maintained as described (11).

Stimulation conditions. For FcεRI-dependent activation, mast cells were adjusted to a density of 1×10^6 cells/ml in complete Dulbecco's Modified Eagle Medium (DMEM), 0.5 mg/ml of IgE anti-DNP was added, and the cells were incubated at 37°C for 60 min.

Cells were then centrifuged at 400 × g at 4°C for 10 min and the cell pellets resuspended in fresh media. Antigen-mediated degranulation was performed by addition of $DNP_{30\text{-}40}$- human serum albumin (HSA) (Sigma Chemical Co., St. Louis, MO) to a final concentration at 40 ng/ml. Where indicated, PMA (Sigma) was added at 50 ng/ml, A23187 (Sigma) at 0.5 mM, ionomycin (Sigma) at 1 mM, and recombinant murine stem cell factor (rmSCF) at 5 or 50 ng/ml. The reaction was terminated at a specific time point.

cDNA probes for Northern Blot analysis. cDNA fragments were labeled by random hexamer priming using primers and protocols suggested by the manufacturers (Stratagene) (12).

Northern Blot analysis. Twenty μg of whole cell RNA was isolated and then subjected to electrophoresis in 1.5% agarose-formaldehyde gels and transferred to nylon-reinforced nitrocellulose membranes (MSI, Westboro, MA) (13). Prehybridizations were performed at 37°C for 30 min. Hybridizations were performed at 37-42°C for 18-24 h in 50% formamide, 5 x Denhardt's solution, 0.55 M NaCl, 0.09 M $NaPO_4$ (pH 7.0), 0.4 mM Na_3EDTA, 0.09 M sodium pyrophosphate, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA.

Construction of the Subtractive cDNA Library. The subtraction library was constructed as described with minor modifications (14).

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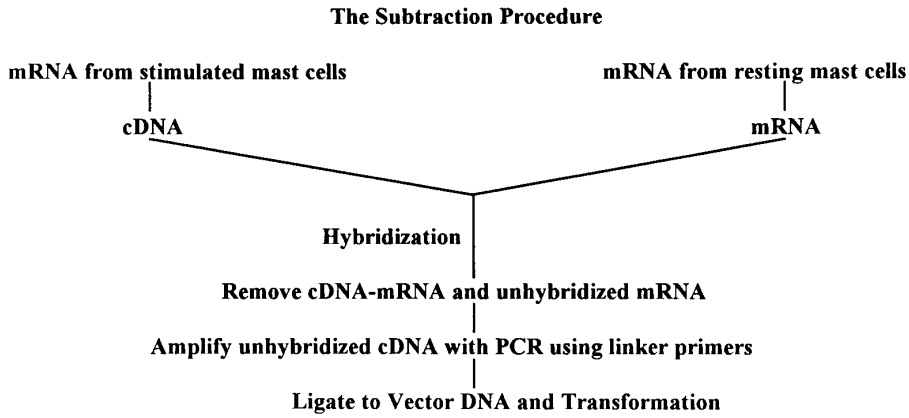

FIG. 1. Schematic illustration of the construction of a subtractive cDNA library. The subtractive hybridization employed oligo (dT)30-Latex.

a. Subtractive hybridization. Twenty μl of sense-strand DNA prepared from activated cells was hybridized with cDNA-Oligotex particles from resting cells in the hybridization buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 500 mM NaCl, 0.5% SDS) at 55°C for 50 min. This subtractive hybridization step was repeated four times in total.

b. cDNA Cloning in λPhage Vectors - Amplified cDNA was digested with *Eco*RI and *Xho*I, ligated with Uni Zap XR Vector, and in vitro packaging performed using packaging extract (Epicenter Technologies, Madison, WI).

c. cDNA library screening. Five hundred thousand clones were screened with ^{32}P-labeled cDNA probe from subtracted cDNA. Positive plaques were picked and rescreened as described above except that the number of plaques per plate were decreased to 500-1000. After tertiary screening, positive plaques were picked and resuspended in 1 ml of SM medium (15). Final screening was performed by dot blot analysis of the positive clones using probes from activated and resting cells. The plaques were spotted in triplicate and hybridized with these probes separately.

RESULTS

Construction and Screening of the cDNA Library

To identify genes induced during mast cell activation, a subtractive cDNA library was constructed with mRNA of a murine mast cell line, Cl.MC/C57.1. The mRNA was enriched by removing mRNA common to resting cells and cells stimulated with PMA and

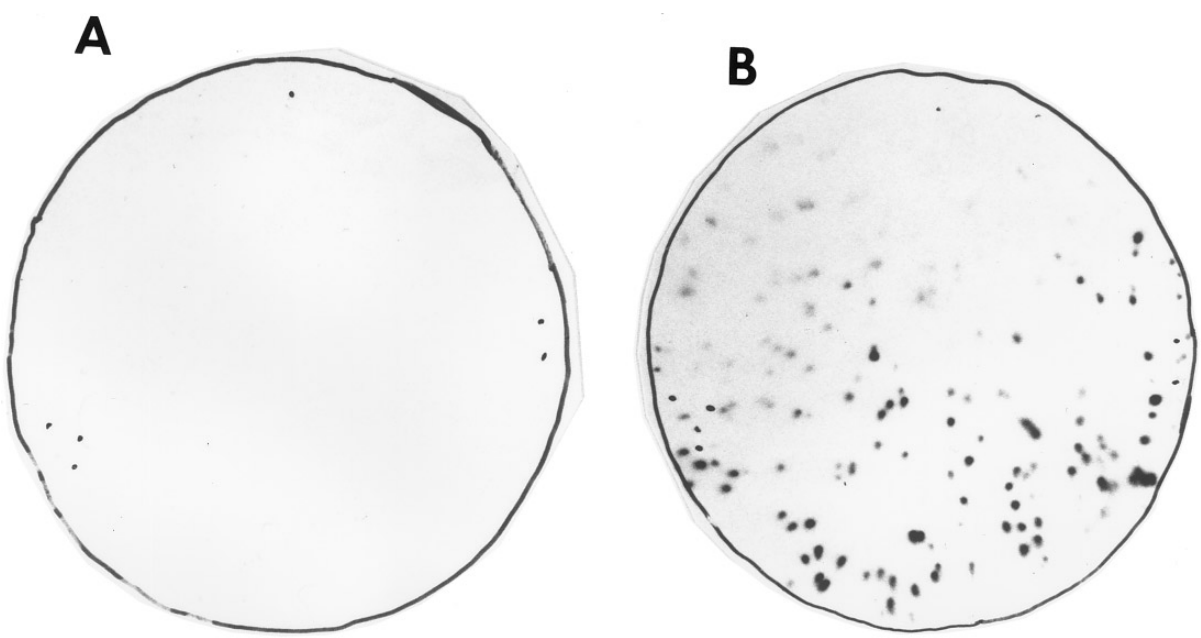

FIG. 2. Differential plaque hybridization. The subtractive cDNA library was screened for clones preferentially expressed in stimulated mast cells by successive differential plaque hybridizations. The positive clones at the second hybridization were spotted onto the bacterial lawn in duplicate. The plaque hybridization was then repeated with ^{32}P-labeled cDNA probes prepared from either resting (A) or stimulated (B) mast cells.

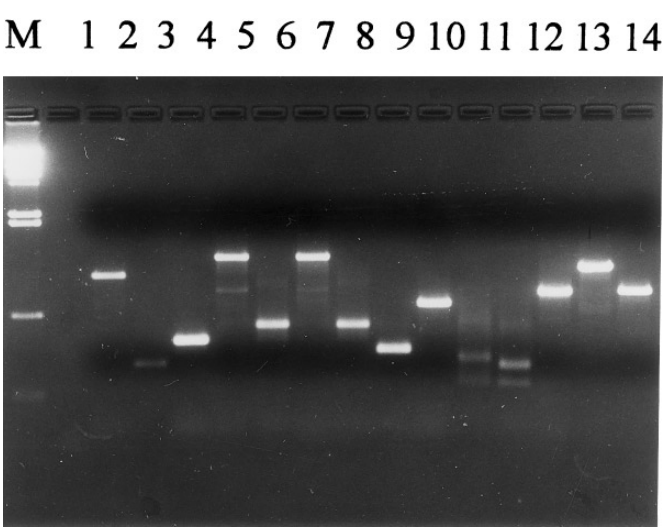

FIG. 3. Sizes of cDNA inserts. The plasmid, pBluescript carrying the cDNA insert, was generated from λZapII recombinants by co-transfection of *E. coli* XL1-blue with the f1 helper phage. The cell lysates and the plasmid DNA were prepared by heating the infected cells at 70°C for 15 min. *E. coli* XL1-blue was transfected with the lysates and the plasmid DNA were prepared from ampicillin resistant transformants. The DNA were PCR-amplified with T3 and T7 primers and electrophoresed in 1% agarose gels. The DNA fragments were stained with ethidium bromide. λ *Hin*dIII was used as a molecular marker.

A23187 (Fig. 1) (14). The mast cell line was used for its homogeneity and to avoid contamination with other cells. A combination of PMA and A23187 was used in order to achieve maximum stimulation of mast cells. The library was screened for phage colonies containing activation specific sequences by differential plaque hybridization with cDNAs probe prepared from resting and activated mast cells. Greater than 90% of the colonies showed preferential hybridization to the cDNA probe from activated mast cells (Fig. 2). To estimate average lengths of cDNA inserts, plasmid DNAs were excised from 14 randomly selected recombinant phages as described in Materials and Methods. The DNAs were PCR-amplified with T3 and T7 primers and analyzed on 1% agarose gels. The lengths of cDNA inserts ranged from 0.2 to 2 kb (Fig. 3).

DNA Sequence Analysis

One hundred and fifty colonies with insert sizes 200 bp or larger were selected and sequenced on both strands. Nucleic acid sequences obtained from these clones were compared to known sequences using the BLAST network of the NCBI (Table I). Cytokine genes have been isolated from mast cells (16). However, there is no profile of genes expressed in mast cells. In other

TABLE I

cDNA Clones Isolated from the Mast Cell Subtraction Library[a]

Known genes		Unknown genes	
Previously identified cDNAs in mast cells	cDNAs not previously identified in mast cells		
2 Lymphotactin	12 IFN-beta	22	671
3 MIP-1 alpha	21 G-protein-beta subunit	96	713
13 PCNA	23 Connexin 43	105	721
16 Ubiquitin	27 Mbh (myc basic motif homolog)-1	112	
20 MIP-1 beta	30 Sec 61	127	
54 TCA3	104 RNA helicase	143	
95 MMCP 5 (chymase 1)	107 Gamma-actin	154	
115 MMCP 1	142 Retinoic acid inducible E3	160	
116 Cathepsin	163 A-X actin	201	
123 Glyceraldehyde-3-phosphate	221 Endogenous murine leukemia proviral	230	
134 Aldolase A	flanking region & insertion DNA	241	
663 Hexokinase	226 TRAP (tranclocon associated protein)	253	
666 Tryptophan hydroxylase	248 Beta- galactoside binding protein	259	
686 c-raf	254 p24.6 protein	267	
699 Mast cell CPA	303 Alpha- tubulin isotype	346	
714 Phosphoglycerate kinase	311 Rab 7	415	
717 Serglycin	541 Teg 23	418	
	607 Cofilin	432	
	633 Annexin VI	562	
	650 PR264 (SC35)	591	
	672 TCP-1 beta subunit	596	
	679 MPR 46 mRNA	597	
	680 Thymosin beta-4	667	

[a] Numbers represent the specific cDNA clones isolated from subtraction library. Genes encoding mitochondrial DNA, ribosomal protein, and clones with unclear sequences are not included. When multiple clones represent the same gene, only one clone is shown.

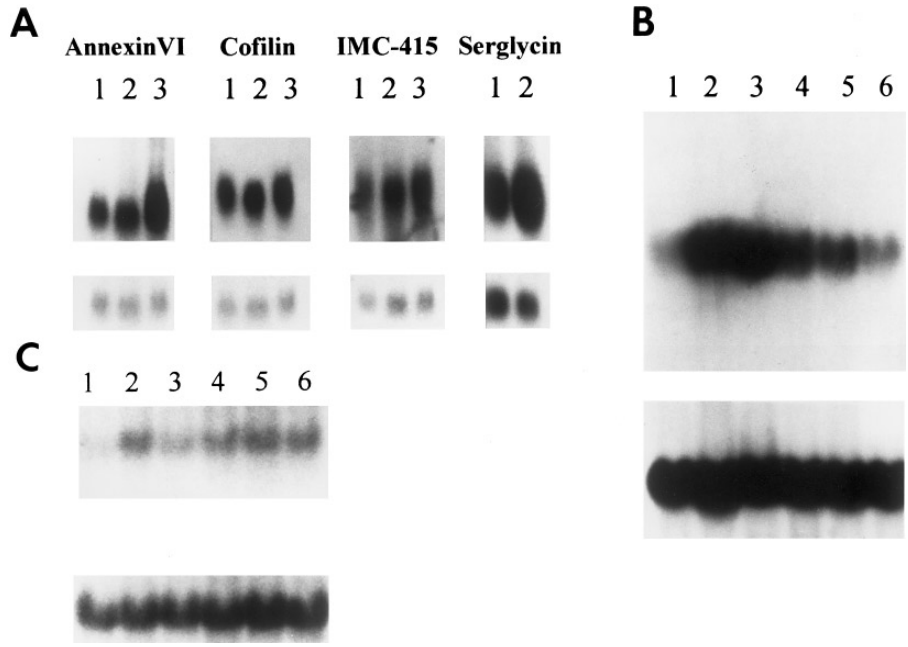

FIG. 4. (A). Expression levels of subtractive cDNA clones in resting and stimulated cells. Twenty μg of total RNA was subjected to Northern blot analysis. **Upper panel:** ^{32}P-labeled probes were prepared from cDNA encoding annexinVI, cofilin, IMC-415 and mRNA. RNA was loaded from resting mast cells (lane 1) or mast cells treated with PMA and A23187 (lane 2), or following FcεRI crosslinking (lane 3). **Lower panel:** Corresponding β-actin. (B). Kinetics of the IFN-β mRNA induction in mast cells. **Upper panel:** ^{32}P-labeled probes were prepared from cDNA encoding IFN-β mRNA. RNA was loaded from resting mast cells (lane 1) or mast cells treated with PMA and A23187 for 1 h (lane 2), 3 h (lane 3), 5 h (lane 4), or following FcεRI crosslinking for 3 h (lane 5), 5 h (lane 6). **Lower panel:** Corresponding β-actin. (C). Effect of SCF on the expression of serglycin mRNA. **Upper panel:** BMCMC were either left untreated (lane 1); or treated with PMA (lane 2), A23187 (lane 3), 5 ng/ml (lane 4) or 50 ng/ml (lane 5) of rmSCF for 4 h, or FcεRI crosslinking for 3 h (lane 6) before preparation of total RNA and analysis of serglycin mRNA levels by Northern hybridization. **Lower panel:** Corresponding β-actin.

cell types, subtracted libraries helped identify new genes and give indications about the function of the cell type (8). To date, we have thus isolated 48 expressed genes. Twenty two genes have not been reported previously as being expressed in mast cells. The other 26 cDNA clones do not have significant homology to known genes in the Genbank database (Table I). Chemokines were the major cytokines induced in activated mast cells, as has been reported in an activated macrophage cDNA library (17). Other mast cell cytokines such as TNF-α, interleukin-4 (IL-4) and IL-6 were not found in our activation-specific library and a mast cell library constructed without activation (18). These results suggest that mast cells may be an important source of chemokines during inflammatory reactions.

Isolation of Inducible cDNA Clones from Stimulated Mast Cells

From the genes in Table I, we selected 10 cDNA clones with strong signals by differential plaque hybridization. Of these cDNA clones, five genes were induced in mast cells upon FcεRI-mediated stimulation. They are cofilin, annexinVI, interferon (IFN)-β, serglycin, and IMC-415 (Fig. 4). Messages were upregulated following stimulation of mast cells. Rapid induction of cofilin, annexinVI, serglycin, and IMC-415 mRNA expression upon stimulation by FcεRI aggregation was similar to the induction pattern of most proinflammatory cytokines, suggesting that these genes may be important in mast cell function.

Cofilin (Table I, Fig. 4A) is a mediator of costimulatory signals via the accessory receptors CD2, CD4, and CD8 (19), suggesting that cofilin may have a role in mediating FcεRI-mediated stimulatory signals in mast cells. Similarly, it has been shown that annexinVI is phosphorylated in vitro by p56lck in a human T cell line (Jurkat) (20). T cell activation causes association of p56lck phosphoprotein with annexinVI, suggesting that annexinVI may also participate in signal transduction in mast cells upon stimulation.

IFN-β is a known proinflammatory cytokine in other cell types and the kinetics have been well described. In order to examine the kinetics of IFN-β message induction in mast cells in comparison to other cell types, a more detailed analysis was performed. IFN-β message was induced by 1 h in mast cells after treatment with PMA and A23187, maintained a high level at 3 h, and reduced significantly by 5 h (Fig. 4B). A pattern seen in other cell types where IFN-β is believed to play a

role in inflammation, IFN-β message was induced similarly in mast cells stimulated by FcϵRI aggregation.

Serglycin mRNA was induced by multiple signals, including rmSCF, in a dose responsive manner (Fig. 4C). Participation of proteoglycan (PG) has been implicated in the growth and differentiation of hematopoietic cells. Several reports have shown that PGs are extruded from mast cells after activation (21). rmSCF acts as a competence factor for mast cell progenitor growth and profoundly influences certain stages of mast cell differentiation (22). Here, we report that the levels of serglycin mRNA were increased following treatment with rmSCF as well as IgE receptor cross-linking (Fig. 4C). This observation is consistent with the need for activated mast cells to re-synthesize extruded components to restore (23).

In summary, we have sequenced 150 cDNA clones. Among them, twenty two genes which have not been reported in mast cells and the other 26 cDNA clones which do not have significant homology to known genes in the Genbank database have been isolated. This is the first reported profile of genes expressed and induced in activated mast cells. In the profile of induced genes in these cells, chemokines are especially prominent. We subsequently identified and partially characterized 5 inducible mast cell genes including a novel gene, IMC-415. Protein expression of the IMC-415 cDNA and antibody production may provide information about the biologic function. Results from this study provide insight into the biologic functions of mast cells in inflammation.

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