Regulation of interleukin 3 mRNA expression in mast cells occurs at the posttranscriptional level and is mediated by calcium ions

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ABSTRACT Interleukin 3 (IL-3) is transiently produced by murine bone marrow-derived mast cells in response to antigen stimulation of the high-affinity immunoglobulin E receptors. We have studied the postreceptor signaling pathways involved in regulating expression of the IL-3 gene in the murine mast cell line PB-3c. Large amounts of IL-3 mRNA accumulated after exposure of cells to calcium ionophore A23187, a reagent that increases intracellular Ca²⁺. Phorbol 12-myristate 13-acetate, which stimulates protein kinase C, did not induce IL-3 mRNA accumulation, although it did potentiate the effect of A23187. Nuclear run-on analysis showed that the IL-3 gene is constitutively transcribed in unstimulated cells and that treatment with A23187 and/or phorbol ester has no influence on its transcription rate. The effect of A23187 was found to be due to stabilization of the IL-3 mRNA. In cells maintained in the presence of A23187 the IL-3 mRNA was stable during 3 hr of incubation with actinomycin D, whereas removal of A23187 under the same conditions resulted in rapid degradation of the mRNA. These results indicate that control of expression of the IL-3 gene in mast cells is primarily at the posttranscriptional level and that the Ca²⁺-dependent signaltransduction pathway plays an important role in this process. Synthesis of granulocyte/macrophage colony-stimulating factor mRNA in response to A23187 and phorbol ester was found to be subject to both transcriptional and posttranscriptional regulation.

Interleukin 3 (IL-3) is important for the development and function of the hemopoietic system. This lymphokine promotes growth and differentiation of pluripotent stem cells and of progenitor cells committed to the myeloid lineages of granulocytes, macrophages, eosinophils, megakaryocytes, and erythrocytes; it also specifically supports proliferation of mature mast cells (1, 2). The biological properties of IL-3 observed in vitro have been confirmed in vivo by demonstrating its strong effect on the formation of blood cells in hemopoietic organs, following administration of recombinant IL-3 to mice (3, 4). However, the function of IL-3 in hemopoiesis is difficult to assess because this lymphokine has not been detected under physiological conditions in animal serum or tissue extracts (5). It is conceivable that to fulfill the requirements of the hemopoietic system, IL-3 is synthesized only transiently in response to specific stimuli and that tight control mechanisms exist which allow rapid modulation of its levels. Understanding of the regulatory mechanisms underlying this control would help to clarify the function of IL-3 in vivo.

For a long time T lymphocytes were considered the only normal, untransformed cells that produce IL-3. They secrete IL-3, together with several other lymphokines, in response to in vitro stimulation with mitogens or antigens (1, 6, 7). We and others have recently demonstrated that activated mast cells also serve as a source of IL-3 and other lymphokines (8, 9). With murine bone marrow-derived mast cells, antigen-induced crosslinking of the high-affinity receptors for IgE (Fc, R) resulted in the transient accumulation of IL-3 and granulocyte/macrophage colony-stimulating factor (GM-CSF) mRNAs and secretion of the two factors (8). As the Fc, R-mediated activation reflects the physiological response of mast cells to allergens (10), production of lymphokines accompanying cell activation may be of importance for immune reactions in allergic disease, in particular for recruitment of lymphoid and other hemopoietic cells (8, 9).

In this work we have analyzed the intracellular mechanisms regulating expression of IL-3 and GM-CSF genes in mast cells. There is evidence (10, 11) that Fc, R are coupled to the phospholipase C effector system that controls two distinct signal-transduction pathways, one regulated by Ca²⁺ ions and the other by protein kinase C (PKC) (12, 13). The intracellular concentration of Ca²⁺ is of particular importance for the function of mast cells in allergic response; aggregation of Fc, R, cell degranulation, and the release of allergic mediators are always associated with an increase in cytoplasmic Ca²⁺ (10, 11). We have compared the roles of Ca²⁺-dependent and PKC-dependent signaling pathways in expression of lymphokine genes in the murine mast cell line PB-3c. These signaling pathways were selectively stimulated with calcium ionophore A23187 or with phorbol 12-myristate 13-acetate (PMA), both of which act in a receptor-independent fashion. We present evidence that intracellular Ca²⁺ specifically controls IL-3 expression in mast cells by regulating stability of IL-3 mRNA. Expression of GM-CSF mRNA, unlike that of IL-3, is controlled by the two pathways at both the transcriptional and the posttranscriptional level.

MATERIALS AND METHODS

Cell Culture and Cell Activation. PB-3c cells, an IL-3-dependent murine mastocyte line (14), were cultured as described (15) in Iscove’s modified Dulbecco’s medium supplemented with a concentrated conditioned medium from WEHI-3B cells, which served as a source of IL-3 (16). Incubation of cells with 5 μM A23187 (Calbiochem) or 30 nM (20 ng/ml) PMA (Sigma) was for 3 hr, except when indicated otherwise. Actinomycin D (Calbiochem; 5 μg/ml) and cycloheximide (Calbiochem; 10 μg/ml) were used as indicated.

Isolation of Cytoplasmic RNA and Northern Blot Analysis. RNA was isolated according to Chomczynski and Sacchi (17). Poly(A)⁺ RNA was selected by one cycle of oligo(dT) -cellulose chromatography. For Northern analysis, RNA sam-

Abbreviations: IL-3, interleukin 3; GM-CSF, granulocyte/macrophage colony-stimulating factor; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; Fc, R, high-affinity IgE receptor(s).

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amples were fractionated in 1.1% agarose/1 M formaldehyde gels and transferred to nitrocellulose filters (Schleicher & Schuell). Complementary RNA probes were synthesized using SP6 polymerase and [α-32P]GTP (NEN; 800 Ci/mmole; 1 Ci = 37 GBq) (18) and, as templates, plasmids pSP65-multi CSF, containing a 369-base-pair (bp) HindIII-XbaI fragment of IL-3 (multi-CSF) cDNA (19), and pSP65-GM-CSF, containing a 398-bp BamHI-EcoRI fragment of GM-CSF cDNA (20). Hybridization was carried out at 55°C overnight in 50% (v/vol) formamide/5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0)/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/5 mM EDTA/10 mM Pipes/NaOH, pH 6.4, containing sonicated salmon sperm DNA (200 µg/ml), heparin (100 µg/ml), and one of the probes (≈2 × 106 cpm/ml; specific activity, ≈2 × 105 cpm/µg). Filters were washed with 0.1× SSC/0.1% SDS at 65°C. Kodak XAR-5 films and intensifying screens were used for autoradiography. Amounts of RNA loaded in each lane were verified either by rehybridization of filters with an actin probe or by acridine orange staining.

**RNase Protection Assay.** Cellular RNA was hybridized with 20,000 cpm of the 32P-labeled antisense RNA probes used for Northern analysis. Hybridization was set at 45°C for 16 hr and hybrids were digested with a RNase A/T1 mixture (Boehringer) (18). RNA was recovered by phenol extraction and ethanol precipitation and analyzed by electrophoresis in a 6% polyacrylamide/8 M urea gel.

**Transcription in Isolated Nuclei.** Nuclei were prepared (21), resuspended in aliquots (=2 × 106 nuclei per 90 µl), and stored in liquid N2. Transcription reactions were performed (21) and 32P-labeled RNA was isolated (17). The following plasmid probes were used: pBR322; pBR/actin4, containing a 567-bp PstI fragment of a β-actin cDNA (22); pMuS.1, containing a 0.6-kilobase (kb) EcoRI fragment of IL-3 cDNA (20); pGM3.2, containing a 0.75-kb BamHI-EcoRI fragment of GM-CSF cDNA (20); and pUC/c-myc, containing a 4.8-kb XbaI-BamHI fragment of c-myc (23). Linearized and denatured plasmids (3 µg) were spotted on 9-mm nitrocellulose filters and hybridized with labeled RNA (≃2 × 106 cpm/ml) at 45°C for 48–72 hr in the same solution as used for Northern analysis. Filters were washed with 2× SSC/0.1% SDS/20 mM Tris, pH 7.5, at 20°C and then with 0.1× SSC/0.1% SDS/20 mM Tris, pH 7.5, at 60°C and 65°C for 1 hr and 30 min, respectively.

**RESULTS**

**Induction of IL-3 and GM-CSF mRNAs.** We measured the effect of calcium ionophore (A23187) and phorbol ester (PMA) on the level of IL-3 mRNA in the PB-3c mast cell line. Northern blot analysis did not reveal IL-3 mRNA in untreated control cells (Fig. 1A, lanes 1 and 5), consistent with previous results (15). IL-3 mRNA was also not detected in the cells treated with PMA (lanes 2 and 6), irrespective of concentration (5–100 ng/ml) and time of treatment (1–16 hr) (data not shown). However, a strong specific signal corresponding to ≈1 kb IL-3 mRNA was observed following exposure to A23187 (lane 3). The strongest response was found after 3 hr of incubation of cells with 5 µM A23187. Although PMA had no effect when used alone, it potentiated by about 2-fold the effect of A23187 (lane 4). The level of IL-3 mRNA observed after treatment with A23187 was considerably higher than that found in the WEHI-3B leukemia cell line (lane W), which expresses IL-3 mRNA constitutively (16). Primer extension analysis (data not shown) mapped the 5‘ end of IL-3 mRNA induced by A23187 in mast cells to the proposed transcription start of IL-3 mRNA (24).

Calcium ionophore was also a very potent inducer of GM-CSF mRNA (Fig. 1B, lane 3), and PMA enhanced its effect by about 3.5-fold (lane 4). Induced levels of GM-CSF mRNA were always higher than those of IL-3 mRNA. Unlike IL-3 mRNA, the GM-CSF mRNA could be induced, though much less effectively, by PMA alone (lanes 2 and 6; note different amounts of RNA in these lanes). The results of Northern analysis were confirmed by RNase A/T1 mapping (Fig. 2). The 368-bp-long protected fragment specific for IL-3 mRNA (lane W) was observed using RNA isolated from PB-3c cells treated with A23187 (Fig. 2A, lane 3), but not with PMA (lane 2). A 398-bp-long fragment specific for GM-CSF mRNA was detectable in response to A23187 and to PMA (Fig. 2B, lanes 2 and 3).

To further analyze the contribution of Ca2+ and PKC-dependent pathways in regulating IL-3 and GM-CSF mRNA levels, the response to A23187 was measured in cells that were deprived of active PKC by prolonged exposure to PMA (25). Both IL-3 and GM-CSF mRNAs were strongly induced by A23187 in such cells (Fig. 3A and B, lanes 3), indicating that accumulation of these mRNAs can occur independently
of PKC activity; however, the levels of induction were slightly lower than in control cells (compare lanes 2 and 3), consistent with the weak synergism between PMA and A23187 seen in Fig. 1. That the prolonged exposure of PB-3c cells to PMA had indeed activated the PKC was confirmed by accumulation of GM-CSF mRNA following 3-hr (Fig. 3B, lane 4), but not 48-hr (lane 5), treatment with PMA.

**Control of IL-3 mRNA Expression.** Increased levels of IL-3 and GM-CSF mRNAs in cells exposed to calcium ionophore and/or to PMA could result either from an increased rate of transcription or from more efficient processing or enhanced stability of the RNA. To distinguish between transcriptional and posttranscriptional regulation, we measured transcription of the IL-3 and GM-CSF genes by in vitro run-on assays in nuclei isolated from control and stimulated PB-3c cells. In a control experiment, we verified that such run-on assays accurately reflect transcription activity in PB-3c nuclei (see Fig. 4B and its legend).

Although IL-3 mRNA was not detectable in unstimulated cells by Northern analysis, IL-3 gene transcription was reproducibly observed in nuclei from these cells (Fig. 4A). Interestingly, treatment with A23187 for 15 min, 1 hr, or 2 hr did not lead to an increase of IL-3 gene transcription. Transcription was also not enhanced by treatment of cells with PMA alone or with PMA and A23187.

Since calcium ionophore did not influence IL-3 gene expression at the transcriptional level, we investigated its effect on stability of IL-3 mRNA (Fig. 5a-d). Following treatment of cells with A23187 and/or PMA for 3 hr, actinomycin D, an inhibitor of transcription, was added and the levels of IL-3 mRNA were measured in cells harvested 0, 1, 2, or 3 hr later. The results indicate that IL-3 mRNA is stable in cells exposed to A23187 (Fig. 5a); even 3 hr after actinomycin D addition, there was no sign of mRNA decay. Similar high stability of IL-3 mRNA was observed in cells treated simultaneously with A23187 and PMA (Fig. 5b). The continuous presence of A23187 was required for stabilization of
treated with cycloheximide in the presence of actinomycin D addition, IL-3 mRNA decayed very rapidly and only traces of it were visible after 30 min of incubation (Fig. 5c).

Control of GM-CSF mRNA Expression. Run-on assays (Fig. 4A) revealed the presence of GM-CSF transcripts in nuclei from unstimulated cells; their level was comparable to that of IL-3 transcripts. Treatment of cells with A23187 had a weak stimulatory effect on GM-CSF gene transcription, while PMA had no effect when used alone. When A23187 and PMA were used together, they strongly increased the GM-CSF transcription rate, which reached the level of transcription of the actin gene. Stability of GM-CSF mRNA was also analyzed (Fig. 5 e–i). GM-CSF mRNA was stabilized most effectively in the presence of both A23187 and PMA (Fig. 5g) and less effectively by either of the reagents alone (Fig. 5 e and f). Similar to IL-3 mRNA, the level of GM-CSF mRNA declined sharply upon removal of A23187 (Fig. 5h). The results indicate that expression of GM-CSF mRNA in PB-3c cells is influenced by calcium ionophore and phorbol ester at both transcriptional and posttranscriptional levels.

Effect of Cycloheximide. To determine whether induction of IL-3 and GM-CSF mRNA by calcium ionophore requires ongoing protein synthesis, cycloheximide was used to inhibit translation (Fig. 6). Cells were preincubated for 1 hr with cycloheximide (10 μg/ml) and then incubated further in its presence with A23187. The levels of IL-3 mRNA were identical in cells treated with A23187 in the absence or presence of cycloheximide (Fig. 6A, lanes 2 and 3) and the level of GM-CSF mRNA was increased by cycloheximide (Fig. 6B, lanes 2 and 3). Treatment of cells with cycloheximide alone did not lead to accumulation of IL-3 mRNA (Fig. 6A, lane 5) but resulted in accumulation of low amounts of GM-CSF mRNA (Fig. 6B, lane 5), as previously seen in macrophages (27). Cycloheximide had no effect on transcription of IL-3 and GM-CSF genes, as analyzed by run-on assays (data not shown). However, cycloheximide did affect the stability of IL-3 and GM-CSF mRNAs; its presence partially prevented rapid decay of mRNAs taking place after removal of A23187 from the culture medium (Fig. 5 d and f).

**DISCUSSION**

We have demonstrated that expression of the IL-3 gene in the murine PB-3c mast cell line is regulated at the posttranscriptional level and that intracellular Ca²⁺ specifically modulates stability of IL-3 mRNA. The IL-3 gene is constitutively transcribed in PB-3c cells, but IL-3 transcripts must be rapidly degraded, as IL-3 mRNA is not detectable in unstimulated cells. The accumulation of IL-3 mRNA in cells treated with calcium ionophore A23187 is due to stabilization of RNA and not due to an increased rate of transcription of the IL-3 gene. To our knowledge, Ca²⁺-dependent regulation of RNA stability has not been reported before. It is not known whether expression of IL-3 mRNA in T lymphocytes is regulated in a similar fashion.

GM-CSF gene expression in mast cells is regulated differently from that of IL-3. Increases in both gene transcription and RNA stability are responsible for accumulation of GM-CSF mRNA in cells treated with A23187 and PMA. Unlike IL-3 gene expression, GM-CSF gene expression has been studied in a variety of cell types. Constitutive transcription of the gene, which we find in mast cells, has also been noted in macrophages (27), some myeloid cell lines (28), and fibroblasts (29). In human T cells, treatment with phorbol ester strongly stabilized GM-CSF mRNA (30), whereas in human fibroblasts its stabilizing effect was less pronounced. In fibroblasts, phorbol ester or tumor necrosis factor enhanced both RNA stability and transcription (29), as did A23187 and PMA in mast cells. On the other hand, induction of GM-CSF mRNA in macrophages by lipopolysaccharide occurred without enhancement of gene transcription (27).

No common regulatory pattern emerges from studies on the signaling pathways controlling expression of various lymphokine mRNAs. It appears that the same lymphokine may be regulated differently in various cell types; it also seems that selective mechanisms coordinate production of multiple lymphokines in one cell type. Studies on expression of IL-3 and GM-CSF provide evidence for existence of such selective regulatory mechanisms. The genes encoding these two factors in mouse and human (31, 32) are localized in close proximity on the same chromosome and are thought to be coordinately expressed (31, 33). Both GM-CSF and IL-3 are secreted by T lymphocytes activated via the T-cell antigen receptors and by mast cells following activation of FcR. The degree of involvement of the specific postreceptor signaling pathways in regulation of IL-3 and GM-CSF gene expression in these two cell types is, however, different. In T lymphocytes, both Ca²⁺-dependent and PKC-dependent pathways contribute to the induction of either lymphokine, as strong synergism between phorbol ester and calcium ionophore has been observed (19). In mast cells, the contribution of the PKC-dependent pathways is less pronounced; treatment with PMA did not induce expression of IL-3 mRNA at all, either in the PB-3c cell line or in bone marrow-derived mast cells (8), and induction of GM-CSF mRNA was rather inefficient.

The strong dependence of lymphokine expression on the Ca²⁺ pathway in mast cells is most likely related to the particular importance of these ions for the function of mast cells during allergic and immune responses. Allergen-induced activation of FcR requires a transient increase in intracellular Ca²⁺, which is maintained for as long as the receptors remain aggregated (10). Based on the results of this work we believe that the transient accumulation of IL-3 and GM-CSF mRNAs, observed by us previously in bone marrow-derived mast cells activated by crosslinking of the FcR (8), is associated with a transient increase of Ca²⁺ in these cells.

The regulation of mRNA turnover is recognized as an important step in the control of lymphokine levels. The clusters of A+U-rich motifs present in the 3' untranslated regions of transiently expressed mRNAs, including IL-3 and GM-CSF mRNAs, have been proposed as major determinants of mRNA instability (30, 34). The 3' terminal retention in GM-CSF mRNA has been identified as responsible for the short half-life of this RNA (30). Derepression of mRNA turnover resulting in constitutive expression of otherwise
transiently produced growth factors may contribute to the development of neoplastic transformation (35). The selective stabilization of GM-CSF mRNA has been described in monocytic tumors and involvement of the 3' untranslated region in GM-CSF mRNA in this process has been suggested (28).

Work in our laboratory has shown that PB-3c cells can progress to IL-3-producing autocrine mastocytomas after introduction of the v-Ha-ras oncogene (15). In addition, production of IL-3 and GM-CSF can be induced in PB-3c cells transfected with the vector expressing the EJ c-Ha-ras oncogene at high levels (36). Overexpression of a transforming ras p21 protein, which shares structural homology with guanine nucleotide-binding proteins (37), could influence transmembrane receptor signaling; indeed, introduction of v-Ha-ras into PB-3c cells enhanced expression of IL-3 mRNA in response to A23187 and PMA (A.W.-F., unpublished data). We have also observed that PB-3c cells infected with a viral vector that expresses high levels of IL-3 (obtained from C. Stocking and W. Ostertag) are highly tumorigenic. IL-3 transcripts expressed from this vector are devoid of 3'-terminal A+U-rich motifs and have a half-life of ≈2 hr (A.W.-F., unpublished data). Together, these results suggest that neoplastic transformation of PB-3c cells results from the increased stability of IL-3 mRNA and that the A+U-rich 3'-untranslated region of IL-3 mRNA plays a role in its turnover.

It is possible that structural motifs in IL-3 mRNA that are important for stabilization are recognized by protein factor(s) that are regulated by Ca2+. Because inhibition of protein synthesis in PB-3c cells by cycloheximide does not prevent induction of IL-3 mRNA by calcium ionophore, the putative regulatory protein(s) would have to be modified, rather than synthesized de novo, in response to increased intracellular Ca2+.

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