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Novel expression pattern of a new member of the MIP-1 family of cytokine-like genes

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Granulocyte/macrophage colony-stimulating factor (GM-CSF) specifically induces the growth of myeloid progenitors and their maturation into neutrophils and macrophages. We have identified a series of previously uncharacterized hematopoietic-specific mRNAs that are expressed in myelopoietic mouse bone marrow cultures stimulated by GM-CSF. One of these messages, C10, encodes a new member of the family of cytokine-like genes related to macrophage inflammatory protein-1 (MIP-1). Members of this family are all induced by one or more stimuli related to inflammation, wound repair, or immune response. In contrast, C10 mRNA showed little or no accumulation in response to such activating agents and was greatly reduced on activation of a T-cell line. On the other hand, C10 mRNA, unlike MIP-1, was acutely stimulated during the first day of bone marrow culture in GM-CSF, and it was also strongly elevated during the induction of neutrophilic differentiation of 32D cl3 cells by granulocyte colony-stimulating factor. The implications of this unusual expression pattern are discussed.

Introduction

Mammalian blood cells are continually turned over and are replenished by the proliferation and differentiation of precursor cells located in the bone marrow. A series of secreted proteins (cytokines) have been identified that can individually induce one or more of the developmental pathways that make up this process (Arai *et al.*, 1990). Many of these molecules can subserve multiple functions, including not only hemopoietic development but also regulation of the functional state of mature cells. Thus the challenge of host defense cells with inflammatory stimuli often induces the synthesis of cytokines, which may then initiate a network of interactions enhancing both the production and the functional activation of a variety of hemopoietic cell types (Arai *et al.*, 1990). This inducibility has often been a key feature of the strategies used in the isolation of novel cytokines. Recently, for example, a new family of cytokine-like molecules have been isolated as products induced on activation of macrophages or lymphocytes (Burd *et al.*, 1987; Schall *et al.*, 1988; Brown *et al.*, 1989; Kawahara and Deuel, 1989; Miller *et al.*, 1989; Wolpe and Cerami, 1989; Leonard and Yoshimura, 1990; Ohmori and Hamilton, 1990). The family is defined by sequence similarity highlighted by a set of conserved cysteine residues. The prototypical members of this family are the two gene products that comprise macrophage-inflammatory protein 1 (MIP-1),[1] MIP-1α, and MIP-1β (Wolpe and Cerami, 1989). The MIP-1 family, in turn, belongs to a superfamily that includes a second set of products related to MIP-2 (Wolpe and Cerami, 1989; Farber, 1990; Sporn *et al.*, 1990; Tekamp-Olson *et al.*, 1990; Vanguri and Farber, 1990). The functional significance of the MIP-1 family is not yet entirely clear. However, MIP-1 has been shown to function both as a hemopoietic regulator and an inflammatory mediator (Broxmeyer *et al.*, 1989; Davatelis *et al.*, 1989; Graham *et al.*, 1990), and chemotactic activity has been demonstrated for both MIP-1 and two other members (Wolpe *et al.*, 1988; Leonard and Yoshimura, 1990; Schall *et al.*, 1990).

Although the members of the MIP-1 family differ in their expression patterns, they are all characterized by acute stimulation on treatment of lymphocytes and/or macrophages with activating agents (Kaczmarek *et al.*, 1985; Introna *et al.*, 1987; Davatelis *et al.*, 1988; Brown *et al.*, 1989; Miller *et al.*, 1989; Zipfel *et al.*, 1989a). In contrast, we report here the identification of a

[1] Abbreviations: ConA, concanavalin A; FCS, fetal calf serum; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL2, interleukin-2; LPS, lipopolysaccharide; MIP-1 and MIP-2, macrophage-inflammatory proteins 1 and 2; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PBS, phosphate-buffered saline.

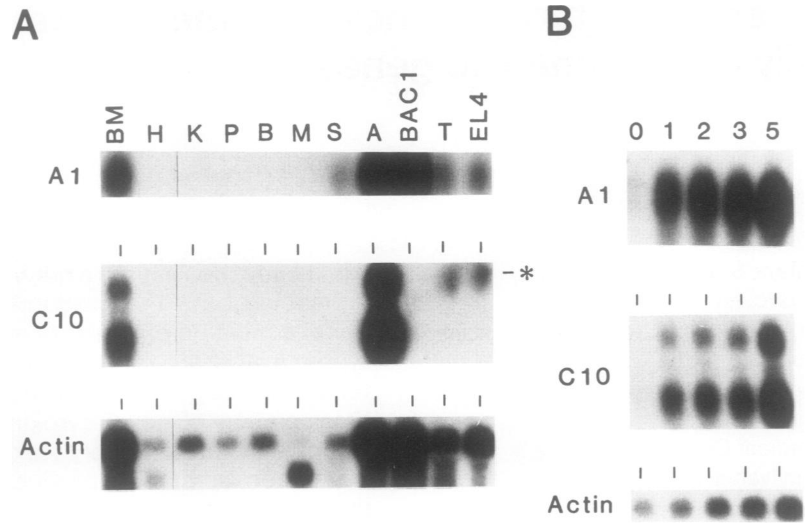

Figure 1. **Expression of inducible hematopoietic-specific mRNAs.** (A) Tissue-specific expression: replicate Northern blots of total RNA (10 μg/lane) from various mouse tissues and cell lines were hybridized with the indicated novel cDNA probes and then reprobed with β-actin (the β-actin probe cross-hybridizes to α-actin in skeletal muscle). Spleen RNA was loaded at ~2 μg as estimated by visualization of rRNA bands. The asterisk indicates an artifact. Exposures were for 4–7 (C10, actin) or 18 h (A1). BM, bone marrow cultured for 3 d in GM-CSF; H, heart; K, kidney; P, pancreas; B, brain; M, skeletal muscle; S, spleen; A, adherent fraction of day 9 bone marrow cultures; BAC1, BAC12F5 cells stimulated for 4 h with r-h-CSF-1; T, thymus. Estimated sizes of the novel mRNAs are A1, 0.8 kb; C10, 1.4 and 0.9 kb. (B) Induction by GM-CSF: replicate Northern blots of total RNA (10 μg/lane) from nonadherent bone marrow cells cultured for the indicated number of days in 10 ng/ml recombinant murine GM-CSF were hybridized as for A. Exposures were for 0.3 h (actin), 4 h (C10) or 18 h (A1). The gradual increase in actin mRNA has been observed previously (Jaffe *et al.*, 1988).

new member of this family that does not appear to obey this rule. Although strongly inducible in differentiating myeloid cultures, this candidate cytokine shows an extremely weak response in activated macrophages and is negatively regulated in a helper T-lymphocyte cell line.

Results

Isolation of the C10 cDNA clone

As part of an effort to identify novel genes associated with myeloid differentiation, we characterized a series of clones from a cDNA library, previously described (Moscinski and Prystowsky, 1990), prepared from mouse bone marrow cultured for 3 d in granulocyte/macrophage colony-stimulating factor (GM-CSF), a cytokine that specifically drives the proliferation and differentiation of macrophage and neutrophil precursors in these cultures. To isolate sequences specific to myeloid cells, the library had been differentially screened for clones expressed in day 3 bone marrow cultures but absent or weak in the T-lymphoid cell line, EL4. Of 72 clones that had survived two rounds of screening (Moscinski and Prystowsky, 1990), 45 were partially sequenced and compared with the GenBank and EMBL databases. Seven distinct cDNAs were identified that showed no similarity to any known sequence and were verified by Northern blot to be absent or weak in EL4 (not shown). Six of these seven were strongly specific for expression in hematopoietic tissues when examined on Northern blots containing a variety of mouse tissues (e.g., Figure 1A). Of these six novel, tissue-specific cDNAs, two clones, A1 and C10, had the intriguing property of dramatic induction during the first day of culture in GM-CSF (Figure 1B), suggesting a possible involvement in the myeloid differentiation induced by this cytokine. A1 has not been characterized further.

C10 is a member of the MIP-1 family of cytokine-like genes

The complete sequence of the C10 cDNA, combined with that of overlapping upstream clones subsequently isolated, revealed a single long open reading frame encoding a potential peptide of 116 amino acids (Figure 2). Although there are no in-frame stop codons upstream of the initiating ATG in this sequence, an overlapping genomic clone contains such a codon, as well as a possible TATA box, suggesting that the cDNA sequence is very nearly full length (data not shown). The potential peptide is likely

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                                                     Met Arg Asn Ser Lys Thr
GAGGAGTGAGCAAAAATTCTCAGACCAGCTGGGCCTGTCCTCCAGGAGG ATG AGA AAC TCC AAG ACT     67

 7  Ala Ile Ser Phe Phe Ile Leu Val Ala Val Leu Gly Ser Gln Ala|Gly Leu Ile
    GCC ATT TCA TTC TTT ATC CTT GTG GCT GTC CTT GGG TCC CAG GCT|GGC CTC ATA   121

25  Gln Glu MET Glu Lys Glu Asp Arg Arg Tyr Asn Pro Pro Ile Ile His Gln Gly
    CAA GAA ATG GAA AAA GAA GAT CGT CGC TAT AAC CCT CCA ATA ATT CAT CAA GGC   175

43  Phe Gln Asp Thr Ser Ser Asp Cys Cys Phe Ser Tyr Ala Thr Gln Ile Pro Cys
    TTT CAA GAC ACT TCT TCA GAC TGC TGC TTC TCT TAT GCC ACA CAG ATC CCA TGT   229

61  Lys Arg Phe Ile Tyr Tyr Phe Pro Thr Ser Gly Gly Cys Ile Lys Pro Gly Ile
    AAA AGA TTT ATA TAT TAT TTC CCC ACC AGT GGT GGG TGC ATC AAG CCG GGC ATC   283

79  Ile Phe Ile Ser Arg Arg Gly Thr Gln Val Cys Ala Asp Pro Ser Asp Arg Arg
    ATC TTT ATC AGC AGG AGG GGA ACC CAG GTC TGT GCC GAC CCC AGC GAT CGG AGA   337

97  Val Gln Arg Cys Leu Ser Thr Leu Lys Gln Gly Pro Arg Ser Gly Asn Lys Val
    GTT CAG AGG TGC CTA AGC ACC CTG AAG CAA GGC CCA AGA TCT GGG AAC AAG GTC   391

115 Ile Ala
    ATT GCT TGA GAAGGAGGGCAGGCATTGTCACCCACTTTCTTCTGTCTTCCCCAGTGACCGCCTGCCT    458

    AGGAGACCTTGTTTTTATAGATATTTAAAGCATTTATCCTTCTGTTCAGGTTTAGAGCAGTCAACAGTAT     528

    TCATGTGGACTCCGCCTGACACGGTTAGAGCCATCTGGAGTTGTAAACATCAAGATTGTCTTTGAGTAAT     598

    TGTTGGGTTTTTTTTCGGTTTCTCAGCAGATTATAAATGGATACATTATTAGGGTAGTCTTTGGGGCTTT     668

    GGAATGTGTCTGGTTCTGATACAAGCTTAAGCCGGGTAATATCTAGCTGAGATGAAATCAATTTTGCCCT     738

    AGGCCATACATATGTCCAGCTTTGTGGGTTCCCAGTTGTTCGCCCTGCCACAATAGAGCAATGAGTGCCC     808

    CCAATAAAGTCCACTCCATGTAGCCACAGGACTGTCTCTTTCTTCAGATTCATAGAACTACGGGGCCAGT     878

    CTGAAACTGGGCTCTTGGGGTGAAATTATCTCACTCACTCACCTTGAGTACAGGAGGGAGATGGGACCAT     948

    ATAACTTTGAATATTCTAAACCAAAAGACATCATGGTATAATTTTTAAAAATTAAGAACACGGTTTATTC    1018

    CTCTGAGCTTGGTGCAAAACAGAGGAATACCTTTTCCAGCAGGGCGTCTTCTTCTCTGCCTGACTTTTAT    1088

    CTGCGGAACCTGACCTTCACACCCTCTACCTGAGGAAGTTCAGGTAGTTGTTGGCAAATCTACAAGTAAG    1158

    AACCTGCACACCAAGTATCTGGGATTCCTGGAATGCCTCTCCATGCAAATGAGGCATTCCCAGTACTTTA    1228

    AACTTCATCTAGTAATTTACATTTACCCTGAAAACTCCTTCCTATACTCCAAGGTTCATGTATAGCCCTG    1298

    GTTTACCCCAATAAAGTGTATGCACACAAGCTGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA          1362
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***Figure 2.* Sequence and open reading frame of the C10 cDNA.** The C10 cDNA, which begins at nt 38, was completely sequenced on both strands. The first 37 nt were found in each of two overlapping cDNA clones as well as an overlapping genomic clone (not shown). The four potential destabilizing sequences (Shaw and Kamen, 1986) are underlined. The two polyadenylation signals are boxed. An arrow indicates the point at which a poly-A tail begins in a second, overlapping cDNA clone (not shown). A vertical bar indicates the predicted point of cleavage of a signal peptide (von Heijne, 1983). This sequence has been submitted to GenBank and has been assigned accession number M58004.

to be a secreted molecule, because it contains an apparent signal peptide, the cleavage of which would be expected to yield a mature protein of 95 amino acids (M_r = 10 749) (Figure 2). The expected pI of the mature product is 9.4. There are no *N*-glycosylation sites. The cDNA sequence contains four copies of the sequence ATTTA, which is found in many acutely regulated mRNAs and is thought to mediate rapid turnover of these messages (Shaw and Kamen, 1986). In addition, there are two consensus polyadenylation signals (boxed), and we have isolated additional cDNA clones that employ the upstream site, thus probably accounting for the two bands observed on Northern blots.

Although the C10 sequence showed no apparent similarity to any sequence when compared with DNA and protein databases, its membership in the MIP-1 family of inducible cytokine-like genes is readily apparent (Figures 3 and 4). In Figure 3, C10 is compared to the known mouse MIP-1 members. For comparison, three of the four known mouse members of the closely related MIP-2 family are shown as well. The MIP-2 family, members of which have been implicated in inflammation and repair processes, is characterized by an intervening amino acid that separates the first two conserved cysteines (Wolpe and Cerami, 1989). C10 is clearly more closely related to the MIP-1 family than to the MIP-2 family by the criterion of overall sequence similarity (Figure 4). C10 is distinguished from the other mouse family members by an amino-terminal extension containing a cluster of charged residues and by the presence of two unique cysteines (Figure 3). TCA3 also has a pair of nonconserved cysteines located similarly to those of C10.

Five human members of the MIP-1 family have been identified, of which four have been assigned as probable homologues of the four previously known mouse members (Brown *et al.*,

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                C10     MRNSKTAISFFILVAVLGSQA|GLIQEMEKEDRRYNPPIIHQGFQDTSSDCCFSY ATQIPCKR
MIP-1           MIP-1a                       MKVSTTALAVLLCTMTLCNQVFS|APYGADTPTACCFSY SRKIP RQ
Family          MIP-1b                       MKLCVSALSLLLLVAAFCAPGFS|APMGSDPPTSCCFSYTSRQLH RS
                TCA3                         MKPTAMALMCLLLAAVW IQDVDS|KSMLTVSNSCCLNTLKKELP LK
                JE                           MQVPVMLLGLLFTVAG|WSIHVLA|QPDAVNAPLTCCYSFTSKMIP MS

MIP-2           C7                              MNPSAAVIFCLILLGLSGTQG|IPLARTVRCNCIHIDDGPVRMRA
Family          MIG                             MKSAVLFLLGIIFLEQCGVRG|TLVIRNARCSCISTSRGTIHYKS
                KC                           MIPATRSLLCAALLLLATS|RLATGAPIANELRCQCLQTMAG IHLKN

                C10     FI YYFP TSGGCIK PGIIFISRRGTQVCADPSDRRVQRCLSTLKQGP RSGNKVIA
MIP-1           MIP-1a  FIVEYFE TSSLCSQ PGVIFLTKRNRQICADSKETWVQEYITDLELIA
Family          MIP-1b  FVMDYYE TSSLCSK PAVVFLTKRGRQICANPSEPWVTEYMSDLELN
                TCA3    FIQCYRKMGSS CPDPPAVVFRLNKGRESCASTNKTWVQNHLKKVNPC
                JE      RLESYKRITSSRCPK EAVVFVTKLKREVCADPKKEWVQTYIKNLDRNQMRSEPTTLFKT..

MIP-2           C7      IGKLEIIPASLSCPRVEIIATMKKNDEQRCLNPES   KT IKNLMKAFSQKRSKRAP
Family          MIG     LKDLKQFAPSPNCNKTEIIATLKNGD QTCLDPDSANVKKLMKEWEKKINQKKKQKRG
                KC      IQSLKVLPSGPHCTQTEVIATLKNG REACLDPEAPLVQKIVQKMLKGVPK
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Figure 3. **Alignment of C10 with members of the MIP-1 and MIP-2 families.** Amino acid sequences were aligned by introduction of a small number of single-residue gaps (except for one 3-residue gap in C7), partially adapted from Kawahara and Deuel (1989). The JE sequence is truncated. Residues that occur more than once at a given position are in boldface; when there are two such residues at the same position, one is in boldface and the second is underlined. The canonical cysteine residues are boxed (see text). Arrowheads indicate residues completely conserved within at least one of the two families. Predicted signal peptide cleavage sites are indicated by vertical bars. References for the sequences are in the legend to Figure 4.

1989; Miller *et al.,* 1989; Rollins *et al.,* 1989; Wolpe and Cerami, 1989; Zipfel *et al.,* 1989a,b). C10 shows a similar degree of sequence similarity to the human as to the mouse MIP-1 members (Figure 4). Figure 4 also shows that the mouse genes correspond fairly well to their assigned human homologues with respect to the degree of similarity to C10. This suggests that C10 may be more closely related to MIP-1α and MIP-1β than to the other MIP-1 family members. Hydropathicity plots of these molecules also suggest this relationship (Figure 5).

C10 is expressed in several hematopoietic lineages

A variety of murine hematopoietic and nonhematopoietic cell lines were screened for C10 expression (Figure 6). Low levels of expression (12–50 times less than induced bone marrow) were observed in two immature myeloid cell lines: DA3 and interleukin-3-supported 32D cl3 (Figure 6D, lane 2, and 6B, lane 2). However, when 32D cl3 cells were induced to differentiate to neutrophils by switching the cultures from interleukin-3 to granulocyte colony-stimulating factor (G-CSF), C10 expression was strongly elevated (Figure 6B, lane 3). C10 was also well expressed in the macrophage cell line P388D$_1$ (Figure 6A, lane 2) and in the adherent cells (predominantly macrophages) produced in GM-CSF-supported bone marrow cultures (Figure 1A, lane A). Two other macrophage cell lines, BAC1-2F5 and RAW264.7, showed no expression (Figures 1A and 6A, lane 3). The C10 message was also expressed in an interleukin-2 (IL2)-dependent T-cell line (see Figure 7). However, no mRNA was detected in spleen, thymus, EL4 (Figure 1A), or MOPC plasmacytoma cells (Figure 6D, lane 5). Finally, C10 was absent in Friend erythroleukemia cells, BALB/c-3T3 fibroblasts, CHO cells, PC12 cells, and C6 glioma cells (Figure 6, A, C, and D). Thus C10 expression has so far been observed only in a subset of myeloid and lymphoid cells. Strong expression can occur within both the neutrophil and macrophage lineages, and induction is associated with stimuli that promote myeloid differentiation in two different systems (bone marrow and 32D cells).

Pattern of regulation of C10 is distinct from that of other members of the MIP-1 family

Members of the MIP-1 family have been reported to respond to similar kinds of activating stimuli in host defense cells. For example, all of these genes have been shown to be expressed in response to lectin or antigen stimulation of T-lymphocytes, and at least three of them are stimulated in macrophages treated with lipopolysaccharide (LPS) (Kaczmarek *et al.,* 1985;

Mouse MIP-1 Family

	MIP-1a	MIP-1b	TCA3	JE
C10	40 (54)	36 (54)	24 (33)	27 (41)

Mouse MIP-2 Family

	KC	C7	MIG
C10	18 (23)	14 (17)	17 (19)

Human MIP-1 Family

	LD78	Act2	I309	MCP	RANTES
C10	49 (61)	38 (54)	32 (41)	27 (35)	31 (50)

Figure 4. **Comparison of C10 with members of the MIP-1 and MIP-2 families.** The percent identity of the predicted mature peptide sequences, aligned as in Figure 5, is indicated. For each comparison, only residue positions that are nonempty for both sequences are scored. Values in parentheses are obtained by allowing conservative amino acid substitutions. The putative human homologues of the mouse MIP-1 family members are placed beneath their respective mouse counterparts. The average score for identity of C10 with the mouse MIP-1 members is 32%; with the human members, 35%. References for the sequences are as follows: MIP-1α (Davatelis *et al.*, 1988); MIP-1β (Sherry *et al.*, 1988); TCA3 (Burd *et al.*, 1987) (note: a differentially spliced TCA3 message encoding an alternate form of the protein has been described [Brown *et al.*, 1989]); JE (Kawahara and Deuel, 1989); C7 (Ohmori and Hamilton, 1990); KC (Oquendo *et al.*, 1989); MIG (Farber, 1990); LD78 (Obaru *et al.*, 1986); Act-2 (Lipes *et al.*, 1988); I309 (Miller *et al.*, 1989); MCP (Rollins *et al.*, 1989); and RANTES (Schall *et al.*, 1988).

Introna *et al.*, 1987; Davatelis *et al.*, 1988; Brown *et al.*, 1989; Miller *et al.*, 1989; Zipfel *et al.*, 1989a). Some differences have also been detected. For example, TCA3 has not been found in macrophages, and differences have been observed in specific features of the responses in T-cells (Wilson *et al.*, 1988; Miller *et al.*, 1989). MIP-1α and MIP-1β, however, appear so far to have coregulated expression (Miller *et al.*, 1989). In view of the similarity between C10 and these two MIP-1 proteins (Figures 4 and 5), we considered the possibility that all three molecules would be similarly regulated. In Figure 7, we compared the expression of C10 and MIP-1α on stimulation of either macrophages or L2 cells. Both genes showed constitutive expression in P388D$_1$ cells (Figure 7A, lane 2). However, in RAW264.7 cells, MIP-1α was strongly and rapidly induced by LPS, as reported previously (Davatelis *et al.*, 1988), whereas C10 mRNA, in a comparably exposed autoradiograph, was not evident (Figure 7A, lanes 3–6). A long exposure did reveal faint expression of C10 (<3% of the level seen in GM-CSF-stimulated bone marrow) after exposure to LPS (not shown). Increasing the concentration of LPS to 1.0 μg/ml did not augment C10 production (data not shown). In addition, MIP-1α was strongly expressed in the CSF-1-dependent macrophage cell line, BAC12F5 (not shown), whereas C10 was absent (Figure 1A).

Even more distinct regulation of these two genes was seen in L2 T-lymphocytes (Figure 7B). L2 cells, removed from antigen and growth factors for 1 d before subculture, showed a low level of C10 mRNA (Figure 7B, lane 2). MIP-1α was only detected on long exposure (not shown). Neither message was affected by mitogenic stimulation with IL2 (Figure 7B, lanes 3–6). However, when the cells were stimulated with a combination of IL2 and the activating

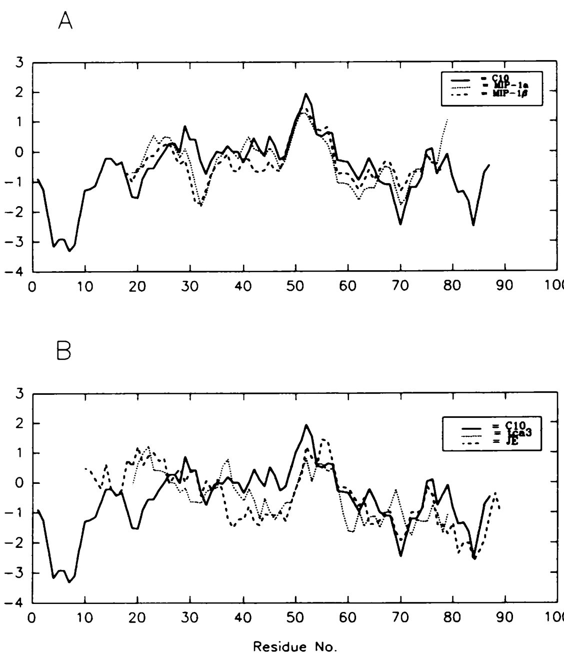

Figure 5. **Hydropathic profiles of C10 and MIP-1 family members.** C10 is compared with either MIP-1α and MIP-1β (A) or with TCA3 and JE (B). The predicted mature polypeptide sequences were aligned by the use of the third conserved cysteine residue (see Figure 5). Values for the first four residues at each terminus are not included. The JE sequence has been truncated. Hydropathy values are according to Kyte and Doolittle (1982), with a window of nine.

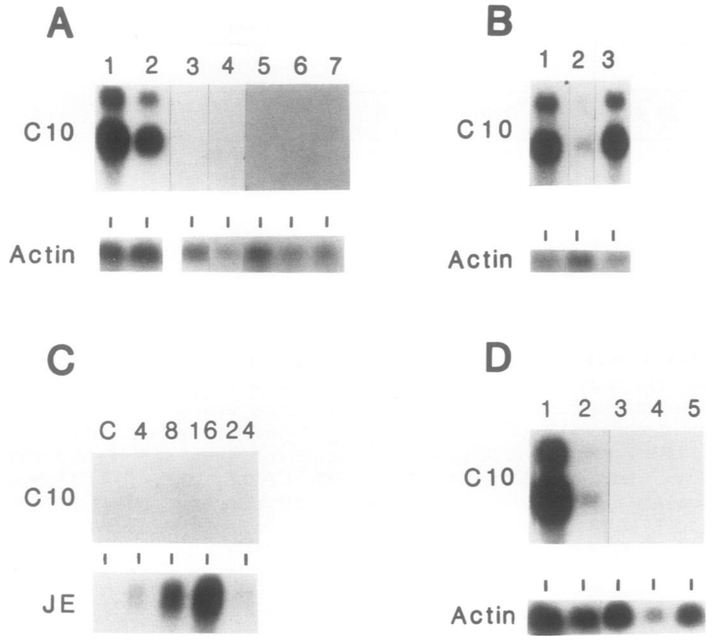

Figure 6. **Cell type-specific expression of C10.** Four Northern blots of total RNA (10 μg/lane) from various cell lines were hybridized sequentially to C10 and either β-actin or JE probes. Lanes 1 of A, B, and D contain RNA from bone marrow cultured for 5 d in GM-CSF (Figure 3). (A) Lane 2, P388D$_1$; lane 3, RAW264.7; lane 4, Chinese hamster ovary; lane 5, rat C6 glioma; lane 6, PC12, untreated; lane 7, PC12, treated for 7 d with nerve growth factor. (B) Lane 2, 32D cl 3 cells, grown in interleukin-3; lane 3, 32D cl 3 cells, switched to G-CSF for 6 d. (C) BALB/c 3T3 cells harvested at confluence (C) or after replating with fresh FCS for the indicated number of hours. (D) Lane 2, DA3; lane 3, Friend erythroleukemia cells, uninduced; lane 4, Friend cells, induced to differentiate with 1.8% dimethylsulfoxide for 4 d; lane 5, MOPC plasmacytoma cells.

agent concanavalin A (ConA), MIP-1α was strongly stimulated, whereas the level of C10 mRNA actually declined (Figure 7B, lane 7). This marked downregulation of C10 mRNA was reproducible and occurred within several hours of exposure to ConA (data not shown). We have also failed to observe C10 expression in ConA-stimulated splenocytes (data not shown). Thus,

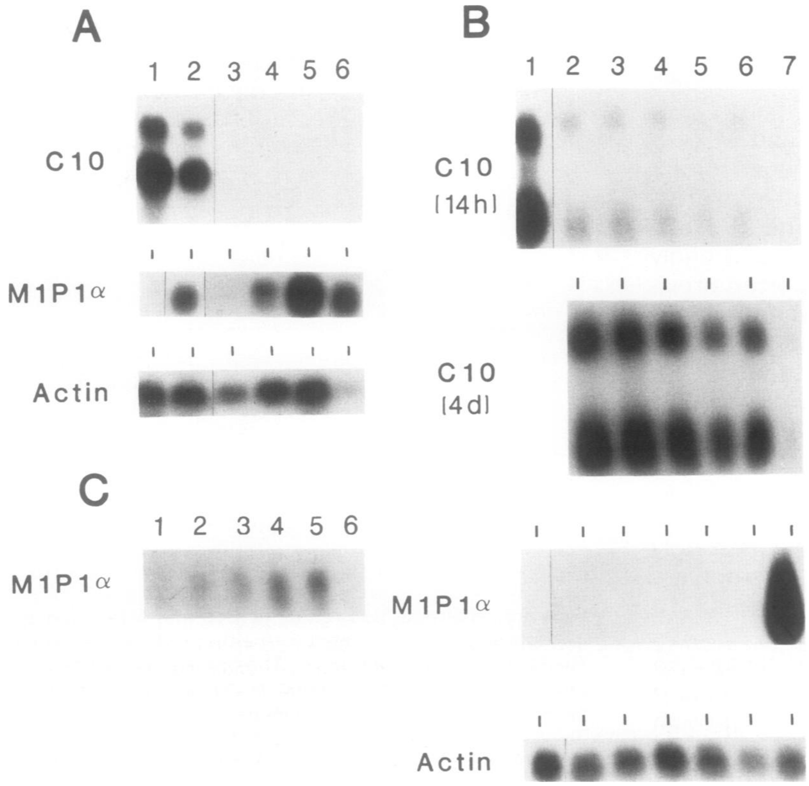

Figure 7. **C10 and MIP-1α are differentially expressed.** Northern blots were hybridized to the indicated probes. Lanes 1 of A and B contain RNA from bone marrow cultured for 5 d in GM-CSF. (A) Lane 2, P388D$_1$ cells; lane 3, RAW264.7 cells, untreated; lanes 4–6, RAW264.7 cells, treated with 0.1 μg/ml LPS for 1, 4, or 25 h. (B) Lane 2, resting L2 cells; lanes 3–6, resting L2 cells treated with 100 U/ml IL2 for 1, 4, 24, or 50 h; lane 7, resting L2 cells treated with 100 U/ml IL2 + 10 μg/ml ConA for 24 h. The upper two panels of B are two different exposures of the filter after hybridization to the C10 probe (upper, 14 h; lower, 4 d). (C) A filter from the experiment shown in Fig. 1B was hybridized to the MIP-1α probe. Lanes 1–5: bone marrow cultures were kept in GM-CSF for 0, 1, 2, 3, or 5 d. Lane 6: EL4 cells.

whereas MIP-1α was expressed in every macrophage cell line examined and responded dramatically to activating stimuli in both macrophages and T-cells, C10 was expressed in only certain macrophage lines and showed either negative or weak positive regulation in response to the stimuli tested. Conversely, MIP-1α, unlike C10, did not show an acute response to GM-CSF in bone marrow cultures, although a gradual increase over several days was apparent, possibly because of the accumulation of macrophages in these cultures (Figure 7C; in some experiments stronger expression was observed at late time points [not shown]). The TCA3 gene, as previously reported (Wilson *et al.*, 1988), was expressed in ConA-stimulated T-cells (data not shown). Furthermore, unlike JE, C10 was not expressed in serum-stimulated fibroblasts (Figure 6C). The expression pattern of this gene is therefore distinct from each of the other murine members of the MIP-1 family.

Discussion

The MIP-1 family products have been shown to be expressed in response to inflammatory and immunologic stimuli and, in several cases, to perform functions expected of inflammatory mediators (Wolpe and Cerami, 1989; Graham *et al.*, 1990; Schall *et al.*, 1990). To our knowledge, C10 is the first member shown to be stimulated in the context of hemopoietic stimulation. Furthermore, C10 was not induced by the activating stimuli (serum, lectin, or LPS) to which the other members are susceptible. It is thus possible that the function of C10 is related more to hemopoietic development than to activation. Indeed, the MIP-1 protein, to which C10 shows the greatest sequence similarity, has been shown to function in vitro as a hemopoietic regulator: MIP-1 enhances the myelopoietic activity of GM-CSF, and MIP-1α can inhibit the proliferation of multipotential precursor cells (Broxmeyer *et al.*, 1989; Graham *et al.*, 1990). However, a large variety of activating stimuli and cell systems have yet to be examined for C10 expression. To date, we have failed to observe more than a very weak response in RAW264.7 cells to agents including interleukins-1, -2, and -6; gamma-interferon; and GM-CSF (data not shown).

A further major problem for gleaning functional possibilities from the expression studies using G-CSF and GM-CSF is that these two cytokines, which induce C10, can both act not only as myelopoietic factors but also as activating agents for mature granulocytes, monocytes, and macrophages (Yuo *et al.*, 1989; Arai *et al.*, 1990). As an activator, GM-CSF has been shown to stimulate the expression of a number of genes in the monocyte/macrophage lineage, including those encoding Ia antigen and the cytokines G-CSF, macrophage colony-stimulating factor, and tumor necrosis factor-α (Cannistra *et al.*, 1987; Oster *et al.*, 1989; Willman *et al.*, 1989). In granulocytes, GM-CSF can induce the messages for interleukin-1 (Lindemann *et al.*, 1988) and interleukin-6 (Cicco *et al.*, 1990). G-CSF can also induce cytokine gene expression in mature granulocytes (Shirafuji *et al.*, 1990).

A striking finding in our expression study was the observation of a sharp decline ($\sim$10-fold) in the level of C10 mRNA on treatment of the L2 T-cell line with a combination of IL2 and ConA (Figure 7). We do not know if this reflects a transcriptional shutoff of the C10 gene, a destabilization of the message, or both. The decline is not related to mitogenic stimulation per se, because treatment with IL2 alone is adequate for mitogenesis (Reed *et al.*, 1985; and data not shown) but does not alter C10 expression. It is intriguing that the message for a human member of the MIP-1 family, designated RANTES, has also been reported to decrease sharply after antigenic stimulation of a CTL cell line (Schall *et al.*, 1988). RANTES has recently been shown to encode a factor chemotactic for monocytes and for a subset of T-cells (Schall *et al.*, 1990). C10, however, does not appear to be the RANTES homologue (Figure 5). Functional studies of the polypeptides encoded by C10 and the other members of the family will be required to determine the significance of their expression patterns.

Materials and methods

Cell culture

Bone marrow was expressed from the femurs of 5- to 12-wk-old female CBA/J mice (Jackson Laboratories, Bar Harbor, ME), suspended in 3-(*N*-morpholino)propanesulfonic acid (MOPS)-buffered Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) (Moscinski and Prystowsky, 1990), and centrifuged over Ficoll/Hypaque (Pharmacia LKB, Piscataway, NJ) for partial enrichment of immature myeloid cells as described (Jaffe *et al.*, 1988). The final Ficoll/Hypaque interface was diluted fourfold with bone marrow medium (alpha medium [Flow Laboratories, McLean, VA] containing 10% heat-inactivated fetal calf serum [FCS] [Hyclone, Logan, UT] 50 μM β-mercaptoethanol, and other supplements as described [Jaffe *et al.*, 1988]); pelleted at 1600 $\times$ *g* for 10 min; washed once in this medium; and resuspended for culture in the same medium containing 10 ng/ml recombinant murine GM-CSF, kindly provided by Immunex (Seattle, WA). Before GM-CSF addition, a portion of the cells were lysed for RNA isolation ("day 0" RNA). All steps between marrow expression and culture were at room temperature. Cultures were incubated for various periods in 10-cm Petri dishes (Falcon, Lincoln Park, NJ) (20 ml/dish; 3.3×10^5 cells/ml initially), at 37°C in a humidified atmosphere with 5% CO_2. Activity of GM-CSF was verified by observation of proliferation and of myeloid colony formation in these cultures (not shown). For the generation of adherent monolayer cells, cultures were initiated at 2.5×10^5 cells/ml and, after 3 d, 80% of the medium was replaced. Washed monolayers were harvested for RNA at day 9. BAC1-2F5, a factor-dependent murine macrophage cell line (Morgan *et al.*, 1987), was obtained from Dr. E.R. Stanley (Albert Einstein College of Medicine, Bronx, NY) and grown, starved, and restimulated with partially purified colony stimulating factor-1 as described (Orlofsky and Stanley, 1987). The mouse macrophage tumor cell line RAW264.7 was obtained from Dr. K.R. Manogue (Rockefeller University, New York, NY) and grown in RPMI 1640 (GIBCO) with 10% FCS. The mouse macrophage tumor cell line P388D$_1$ (interleukin-1 high-producing subclone) and the mouse plasmacytoma cell line MOPC-31C were obtained from the American Type Culture Collection (Rockville, MD) and grown as recommended by the supplier. Friend erythroleukemia cells were obtained from Dr. P. Curtis (Wistar Institute, Philadelphia, PA) and were stimulated to differentiate by treatment with 1.8% dimethylsulfoxide for 5 d. The IL2-dependent T_H1-cell line L2 was maintained and stimulated as described (Sabath *et al.*, 1990). Mitogenic stimulation with murine recombinant IL2 was verified by flow cytometric analysis of DNA content (not shown). The murine T-lymphoma cell line EL4 was maintained as described (Moscinski and Prystowsky, 1990). The factor-dependent cell line DA3, described as a primitive myeloid line (Ihle and Keller, 1985), was obtained from Dr. J. Ihle (St. Jude Children's Research Hospital, Memphis, TN). It was grown in RPMI 1640 with 10% FCS and 5% WEHI-3B-conditioned medium. CHO cells were grown in MOPS-buffered Dulbecco's modified Eagle's medium (Moscinski and Prystowsky, 1990).

RNA isolation and Northern blot analysis

Nonadherent cultured bone marrow cells and cell lines grown in suspension were pelleted, washed once in phosphate-buffered saline (PBS), suspended in a small volume of PBS and lysed by addition of guanidinium isothiocyanate lysis buffer (Maniatis *et al.*, 1982), to which 0.1% antifoam A (Sigma, St. Louis, MO) was added. Adherent cells were rinsed with PBS and directly lysed in this buffer. Mouse tissues were minced in lysis buffer and homogenized with a Dounce homogenizer. Lysates were pelleted through 1.5 ml of 5.7 M CsCl, 25 mM sodium acetate (pH 5.2) and processed essentially as described (Maniatis *et al.*, 1982). Samples of total cellular RNA prepared from the diploid, interleukin-3-dependent cell line 32D cl 3 were a kind gift of Brent Kreider (Wistar Institute). The cells had been switched for varying periods of time from interleukin-3 to G-CSF, which induces terminal granulocytic differentiation in this line (Valtieri *et al.*, 1987). Total cellular RNAs from rat C6 glioma cells and from rat pheochromocytoma cells (PC12) exposed for either 0 or 7 d to nerve growth factor were the kind gifts of Dr. Jeffrey Cohen (University of Pennsylvania, Philadelphia, PA). Total cellular RNAs (10 μg/lane) were separated on 1.3% agarose gels containing 2.3% formaldehyde in 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA. Ribosomal bands were photographed to verify equal loading and integrity. RNA was transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL) and fixed by calibrated UV irradiation (Stratalinker; Stratagene, La Jolla, CA). A similarly prepared filter containing RNA from quiescent or stimulated BALB/c-3T3 fibroblasts was provided by Dr. Xiaoxia Gai in our laboratory. Confluent monolayers had been stimulated by trypsinization and splitting at a 1:2 ratio in fresh medium containing 10% FCS. Filters were prehybridized at 42°C for 3–6 h in 50% deionized formamide, 5× SSC (Maniatis *et al.*, 1982), 10 mM $NaPO_4$, 5 mM EDTA, 5× Denhardt's solution (Maniatis *et al.*, 1982), 0.5% sodium dodecyl sulfate, 10 μg/ml polyadenylic acid, and 50 μg/ml denatured salmon sperm DNA (Sigma). This was then replaced by hybridization buffer, which was identical except that it contained 2× Denhardt's, 10% dextran sulfate, and 2×10^5–1×10^6 cpm/ml of ^{32}P-labeled DNA probe. Most probes were prepared by purifying plasmid cDNA inserts in low melting point agarose (FMC, Rockland, ME) and extending random primers with either Klenow fragment (Feinberg and Vogelstein, 1983) or a T7 polymerase-based kit (Stratagene) in the presence of ^{32}P-dCTP (Amersham). A cDNA clone for β-actin was isolated during the cDNA library screening described in this work and was labeled as a whole plasmid using a nick translation kit (Bethesda Research Laboratories, Gaithersburg, MD) and ^{32}P-dCTP. The probes for MIP-1α and TCA3 were prepared by labeling oligonucleotides derived from their 3′ untranslated regions. Oppositely oriented, gel-purified 60mers, with a 20-nt overlap, were annealed and mutually extended with Klenow fragment and ^{32}P-dCTP to produce labeled 100mer probes essentially as described (Ausubel *et al.*, 1987). The 100mers, chosen to be 55% C + G, consisted of nt 391-490 of either the TCA3 or MIP-1α published sequences (Burd *et al.*, 1987; Davatelis *et al.*, 1988). After hybridization for 16–40 h, filters were washed with 2× SSC and 0.5% sodium dodecyl sulfate, first at RT and then at 60°C for 2 h. Filters were exposed to X-ray film with two intensifying screens at −80°C. Filters were stripped of probe at 75°C for 3 h as described (Thomas, 1980). Molecular weights were estimated with the use of the ribosomal RNAs as markers. Quantitation of labeled bands was performed by exposure to a phosphor screen followed by laser scanning (PhosphorImager; Molecular Dynamics, Sunnyvale, CA).

DNA and predicted protein sequence analysis

The cDNA library from GM-CSF-stimulated bone marrow has been previously described (Moscinski and Prystowsky, 1990). Briefly, mRNA was isolated after 3 d of culture and cDNAs inserted in lambdaZAP (Stratagene). Plaques (25 000) were differentially screened for presence in bone marrow and low or no expression in the EL4 cell line (Moscinski and Prystowsky, 1990). LambdaZAP phage isolates that had survived two rounds of differential screening were used to generate pBluescript-based plasmids according to the instructions of the lambdaZAP supplier (Stratagene). Plasmids were grown in JM109 cells and purified by twice banding in CsCl essentially as described (Maniatis *et al.*, 1982). Inserts were partially sequenced (150–300 nt) from each end using a Sequenase 1.0 kit (United States Biochemical, Cleveland, OH) and T3 and T7 primers. The complete sequence of the C10 cDNA was obtained by the use of a succession of synthetic oligonucleotides as primers. Sequences were compared with databases using the FASTDB program (Intelligenetics, Mountain View, CA). Hydropathy plots were generated using the values of Kyte and Doolittle (1982), with a window of nine.

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