Short paper

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Mast cell growth-enhancing activity (MEA) is structurally related and functionally identical to the novel mouse T cell growth factor P40/TCGFIII (interleukin 9)*

We have previously shown that certain bone marrow-derived mast cell (BMMC) lines proliferate in response to a mast cell growth-enhancing activity (MEA) that is distinct from interleukin (IL) 3 and IL 4. Here we provide evidence that MEA is identical with the recently cloned mouse T cell growth factor P40. The evidence is as follows: (a) recombinant P40 displayed all the biological activities ascribed to MEA: it supported the growth of MEA-sensitive BMMC lines, it induced IL 6 secretion by these cells, and it enhanced survival of primary mast cell cultures; (b) highly purified MEA stimulated the growth of P40-dependent cell lines; (c) a rabbit monospecific antiserum directed against P40 specifically inhibited the action of MEA on BMMC; (d) specific binding sites for P40 were detected on BMMC and (e) MEA competed with P40 for binding to P40-dependent T cells, indicating that the two molecules interact with the same receptor. These observations further extend the range of biological activities ascribed to P40 and warrant its proposed designation as IL 9.

1 Introduction

BM-derived mast cells (BMMC) which are phenotypically and functionally related to mucosal mast cells *in vivo* [1], can be maintained *in vitro* in a state of factor-dependent growth for several weeks [1]. Originally, IL 3 was identified as the growth factor required by these cells [2], but subsequently, it appeared that IL 4 also played an important role in their development by providing a co-mitogenic signal [3, 4]. IL 4 proved essential when connectieve tissue-type mast cells are grown under clonal conditions [5].

Recently we described a novel mast cell growth-enhancing activity (MEA) derived from pokeweed mitogen-stimulated mouse spleen cell conditioned medium (SCM; [6, 7]). Initially MEA has been characterized as a factor synergyzing with IL 3 to enhance the proliferation of permanent BMMC lines in the presence of saturating levels of IL 3 [6, 7]. Only later did we find that it also acts in the absence and independently of IL 3 and IL 4 [8]. In addition to its growth-promoting activity MEA effectively stimulated IL 6 production in a factor-dependent BMMC line and in an autonomous malignant subline [9]. A similar activity has been detected in, and purified from, the SN of a murine IL 2-dependent, Mls^a-specific T cell line (MLS4.2; [10]).

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Abbreviations: BMMC: Bone marrow-derived mast cell **MEA:** Mast cell growth-enhancing activity **SCM:** Spleen cell-conditioned medium

The same cell line was shown by Schmitt et al. to secrete a new T cell growth factor capable of stimulating the proliferation of a $CD4^+$ T cell clone. This factor, termed TCGFIII, was found to be identical to P40, a recently cloned cytokine that displays the same type of biological activity [11–13].

In the present report we provide evidence that MEA is functionally identical with P40/TCGFIII. This finding further extends the spectrum of activities of this protein, whose human homologue was recently shown to act not only on T cells [14] but also on cells of the myeloid lineage [15]. We therefore support the proposal that this new cytokine be renamed IL 9.

2 Materials and methods

2.1 Mast cell cultures and cell lines

The generation in a LD microculture system of homogeneous populations of mouse BMMC (≥ 99% Alcian Blue positive) has been described [16]. In the present study a number of established cell lines were used which have been described in the literature: L138.8A, a factor-dependent IL 3-/IL 4-/MEA-responsive BMMC line [6, 16]; TS1, a P40-responsive mouse T cell line [12]; ST2/K9.4a2, a TCGFIII-responsive $CD4^+$ T cell clone [11]; MLS4.2, a murine IL 2-dependent, Mls^a-specific T cell line producing MEA and TCGFIII upon activation [10, 11]; TUC7.51, a P40-producing T_h cell clone [12] and 7TD1, an IL 6-dependent mouse hybridoma cell line [17].

2.2 Cytokines and antibodies

Purified mouse rIL 3 and rIL 4 were commercially available from Genzyme (Boston, MA). Murine rIL 4 was also kindly provided by W. Müller (Institut für Genetik, Köln, FRG).

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Murine rIL 2 was a gift from R. Palacios (Basel Institute for Immunology, Basel, Switzerland). Anti-IL 4 and anti-IL 2 antibodies were derived from 11B11 cells [18] and S4B6.1 cells [19] obtained from W. E. Paul (National Institutes of Health, Bethesda, MD) and T. Mosmann (DNAX, Palo Alto, CA), respectively. Partially purified MEA was derived from SCM as reported recently [7]. Highly purified MEA was derived from MLS4.2-CM. Details of the MEA purification protocol including partial amino acid sequencing will be published elsewhere [10]. Purification of TCGFIII has been shown recently [11]. The production and purification of rP40 expressed in a baculovirus vector will be described elsewhere (C. Druez, in preparation). Rabbit antiserum was prepared against native P40 purified to homogeneity [12].

2.3 Cytokine assays

MEA was quantitated in cultures of L138.8A cells stimulated with serial dilutions of MEA in the presence of saturating IL 3 levels using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [20] as described [6]. P40 activity was measured using the P40-dependent TS1 line [12]. TCGFIII activity was assessed with the TCGFIII indicator cell line ST2/K9.4a2 [11]. IL 6 activity was measured using the IL 6-dependent hybridoma cell line 7TD1 [17]. Details of the various cytokine assays have been presented in the referenced literature.

2.4 Radioiodination of P40 and binding studies

rP40 was iodinated in a two-step procedure using iodogen (Pierce, Rockford, IL)-coated tubes to oxidize $Na^{125}I$ and transferring the oxidized material to the protein solution. By this technique, sp. act. of 100 000 cpm/ng were regularly achieved without significant loss in biological activity. Binding was performed in DMEM containing 1% BSA and 10 mM NaN_3 following the procedure described in Coulie et al. [21].

3 Results

3.1 Introductory remarks

During MEA and TCGFIII purification from conditoned medium (CM) of the alloreactive T cell line MLS4.2, both activities were co-purified in independent purification protocols (data not shown). Since partial amino acid sequencing as well as functional data revealed an identity between TCGFIII and the new T cell growth factor P40 [11, 12], our present experiments were designed to ultimately prove or disprove a structural and/or functional similarity between MEA and P40/TCGFIII.

3.2 MEA is active on P40/TCGFIII target cells

MEA partially purified from mouse SCM as well as highly purified from MLS4.2-CM was not only active on mast cells as shown previously [6–10], but also stimulated the proliferation of P40/TCGFIII-responsive mouse T cells independently of IL 2 and IL 4 (Fig. 1).

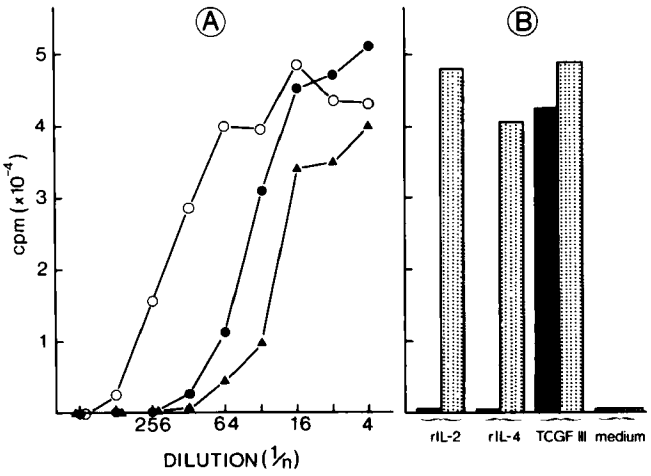

Figure 1. MEA stimulates proliferation of P40/TCGFIII-responsive T cells. (A) [^{3}H]thymidine uptake (0.1 μCi/well = 3.7 kBq/well; added for the last 18 h) in ST2/K9.4a2 cells (2.5×10^3/well; 100 μl/well) stimulated for 48 h with serial dilutions of rP40 (▲; stock: 1 ng/ml) or MEA partially purified from SCM (●; stock: 10 U/ml) or highly purified from MLS4.2-CM (○); stock: 25 U/ml). (B) Control cultures stimulated with rIL 2 (2 U/ml), rIL 4 (40 U/ml), TCGFIII (10 U/ml) or medium in the presence (■) or absence (▨) of anti-IL 2/IL 4 (ascites fluid, 1/400 each).

3.3 The biological effects of MEA are blocked by rabbit anti-P40 antiserum

A rabbit anti-P40 antiserum abolished both the proliferative and the IL 6-inducing capacity of MEA in mast cells as demonstrated in Fig. 2. Anti-P40 antiserum was unable to

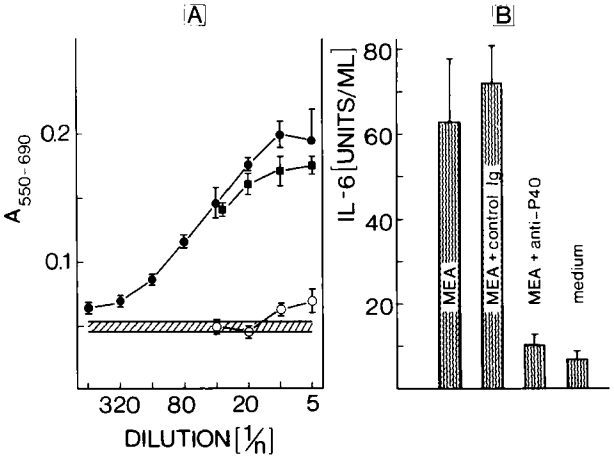

Figure 2. Anti-P40 blocks the effect of MEA in cultures of mast cells (L138.8A). (A) Triplicate cultures of L138.8A cells (1.5×10^4/ml; 100 μl/well) were stimulated with serial dilutions of highly purified MEA (stock: 50 U/ml) alone (●) or together with constant amounts of anti-P40 antibodies (○) or control Ig (■; 10 μg/ml each). Medium (▨). (B) Duplicate cultures (0.5 ml/well) of L138.8A cells (2×10^5/ml) were stimulated with MEA (20 U/ml) either alone or together with anti-P40 or control Ig (10 μg/ml each). After 24 h SN were individually tested for IL 6. Mean values ± SD ($n = 4$) are shown.

Table 1. rP40 stimulates IL6 production in L138.8A mast cells[a)]

Growth factor used[b)]		IL6 activity[c)] (U/ml)
rP40	(20 ng/ml)	66.8 ± 1.7
MEA	(40 U/ml)	64.9 ± 11.0
rIL3	(100 U/ml)	14.5 ± 2.7
rIL4	(400 U/ml)	20.8 ± 2.8
Medium		6.1 ± 1.4

a) Duplicate 24-well plate cultures (0.5 ml/well) of L138.8A mast cells (2×10^5/ml) were stimulated for 24 h with the indicated mast cell growth factors or control medium.
b) The growth factors were pre-tested within a range of concentrations. Only results obtained with saturating maximum doses, are shown. Doses of rIL3 and rIL4 refer to the definition given by Genzyme.
c) Values (mean ± SD; $n = 4$) were calculated from dose-response curves obtained with individual mast cell SN in the IL6 assay with 7TD1 hybridoma cells.

affect the IL3/IL4-induced proliferation or the IL3/IL4-stimulated IL6 production of mast cells which excludes potential nonspecific effects of the antiserum (data not shown). The serological similarity between P40 and MEA was further confirmed by the structural analysis of highly purified MEA displaying complete amino acid sequence homology in five individual tryptic peptides [10].

3.4 rP40 is active on mouse BMMC

P40/TCGFIII had been reported to be a novel growth factor for certain long-term cultured $CD4^+$ mouse T cell clones [11, 12]. As illustrated in Fig. 3, rP40 was also able to promote the proliferation of the MEA-responsive mast cell line L138.8A in the absence or presence of saturating levels of IL3. Moreover, rP40 significantly stimulated IL6 production in the factor-dependent mast cell line L138.8A (Table 1). Both the mast cell growth factor activity and the stimulation of IL6 production in mast cells are properties previously ascribed to MEA [6–10]. rP40 was not active on IL3-dependent 32Dcl.23 cells [22], IL4-responsive F4/4K.6 T_h cells [6] or IL6-dependent 7TD1 cells [17]. MEA as well as rP40 were also able to synergistically enhance the

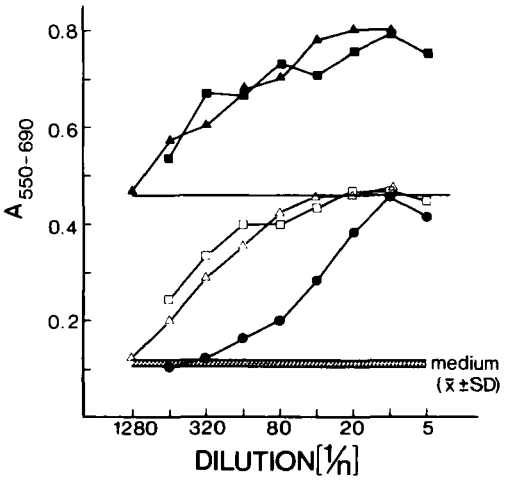

Figure 3. rP40 stimulates the proliferation of MEA-sensitive mast cell line L138.8A. Microcultures (100 µl/well) of L138.8A cells (1.5×10^4/ml) were stimulated with serial dilutions of purified growth factors [IL3 (●); rP40 (○); MEA (△); rP40 + const. IL3 (■) and MEA + const. IL3 (▲); dilutions 1/5 corresponding to 20 ng/ml rP40, 50 U/ml MEA and 40 U/ml IL3]. MEA and rP40 were also tested in the presence of a saturating amount of IL3 (20 U/ml). After 3 days the MTT assay was performed.

proliferation of primary BMMC (2–4 weeks in culture) in the presence of IL3. In these primary cultures, however, no proliferative activity could be demonstrated with MEA or P40 alone (data not shown). Instead, rP40 significantly prolonged the survival of primary BMMC in the absence of other growth factors resulting in an increase of the half-life of these cells from 12.5 h ± 1.8 h to 24.6 h ± 3.4 h.

3.5 Specific binding sites for P40 are present on MEA-responsive mast cells and MEA competes for the binding of P40 on T cells

The existence of specific binding sites for P40 was demonstrated on a factor-dependent and on an autonomous BMMC line. The factor-dependent cell line L138.8A expressed ≈2000 receptors, *i.e.* approximately the same number as the P40-dependent cell line TS1 C3. Moreover, a weak but significant binding corresponding to ≈100 sites/cell was measured on primary BMMC (Table 2).

Table 2. Expression of P40 receptors on mast cells and inhibition of P40-binding by MEA[a)]

Cells	MEA (U/ml)	Specific binding (molecules/cell)
TS1.C3 (P40-dependent T cells)	–	4109
	8000	214
	2000	605
	500	1818
	125	2040
	31	3776
L138.8A (factor-dependent) mast cell line	–	2208
L138.C (autonomous mast cell line)	–	331
BMMC (2 weeks old)	–	128
MlethA (fibrosarcoma)	–	< 10

a) Cells were incubated for 3 h at 4 °C with radioiodinated rP40 (0.5 nM) in the presence or absence of a 100-fold excess of unlabeled rP40. The radioactivity measured in the presence of excess cold P40 was subtracted to calculate specific binding. MEA activity was measured in the TS1 assay.

Additional evidence in support of the functional identity between P40 and MEA was obtained in competition experiments, which indicated that highly purified MEA was capable of inhibiting the binding of iodinated P40 to its receptor (Table 2). The characterization of the P40 receptor will be described in detail elsewhere (C. Druez et al., in preparation).

4 Discussion

In this report we provide structural and functional evidence that two different biological activities can be ascribed to the same regulatory protein. The one, tentatively termed MEA, acts on mouse BMMC [6–10], while the other one promotes the growth of certain murine T cell lines and was termed P40 and TCGFIII [11–13]. P40/TCGFIII is produced upon mitogenic or allogeneic activation of some permanent mouse T cell lines [11, 12] as well as by a subpopulation of $CD4^+$ primary murine T cells [11] functionally identical to T_H2 cells [19].

Highly purified MEA and rP40 were correspondingly active on both mouse mast cells (Figs. 2 and 3, Table 1) and murine T cells (Fig. 1, Table 2). These results, together with the finding that a monospecific anti-P40 antiserum was able to completely neutralize the presently known bioactivities of MEA *in vitro* (Fig. 2), strongly suggested that MEA and P40/TCGFIII were identical or at least closely related. Nevertheless, MEA and P40/TCGFIII could have been different molecules, MEA acting via the endogenous induction of P40/TCGFIII or *vice versa*. However, the demonstration of specific competition between MEA and radiolabeled P40, in cultures of P40/MEA-responsive T cells, ultimately proved that both MEA and P40 specifically interact with the same binding sites present on T cells and mast cells (Table 2). Moreover, the serological similarity between P40 and MEA (Fig. 2) was confirmed by a partial amino acid sequencing of purified MEA [10]. In primary BMMC, P40/MEA stimulated proliferation only in concert with IL3, whereas it acted as a genuine growth factor for immortalized factor-dependent mast cells ([8] and Figs. 2 and 3). At present it is not known whether the latter finding is related to the low number of P40-binding sites on primary mast cells and/or whether there is a difference in post-receptor signaling between primary mast cells and immortalized mast cell lines.

Recently, the cDNA for human P40 was identified by expression cloning [15] and by cross-hybridization with mouse P40 cDNA [14]. Surprisingly, the human recombinant protein was found not only to enhance the survival of certain human T cell lines [14], but also to stimulate the proliferation of a human megakaryoblastic leukemic cell line [15], indicating that human P40 acts on more than one cell lineage. This finding led to the proposal that the protein be renamed IL9 [15], a designation that seems further warranted in view of the additional activity demonstrated here.

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