CLONING AND EXPRESSION OF A cDNA FOR THE HUMAN HOMOLOG OF MOUSE T CELL AND MAST CELL GROWTH FACTOR P40

Jean-Christophe Renauld,[1] Aline Goethals,[2] Frédéric Houssiau,[1]
Emiel Van Roost,[2] Jacques Van Snick[2]

A cDNA encoding the human homolog of mouse T-cell and mast cell growth factor P40 was derived from peripheral blood mononuclear cells (PBMC) stimulated with phytohemagglutinin and phorbol myristate acetate. Sequence analysis of the cDNA predicted a precursor protein of 144 amino acids including a signal peptide of 18 residues, a structure identical with that of mouse P40. The homology between the mouse and human proteins is 55% with a perfect conservation of the 10 cysteine residues present in the mature polypeptide. Expression of the cDNA for human P40 in a baculovirus vector yielded a protein capable of enhancing in vitro survival of human T cell lines.

P40 is a lymphokine that we originally identified in the mouse as a 30 to 40 kDa T-cell–derived factor capable of supporting the growth of certain helper T cell clones (Uytlenhove et al., 1988). It consists of a protein core of 15 kDa associated with considerable amounts of N-linked sugars and characterized by an elevated isoelectric point and a high cysteine content (10 residues out of 126) (Simpson et al., 1989). Analysis of cDNA clones derived from mouse helper T cells indicated that P40 mRNA encodes a precursor of 144 amino acids including a typical signal peptide of 18 residues (Van Snick et al., 1989). P40 shows no significant sequence homology with previously identified proteins.

Our original observations suggested that P40 was a growth factor with an unusually narrow specificity. It strongly stimulated the growth of some helper T cell lines but had no effect on many others. Moreover, it failed to stimulate cytolytic T-cell clones or fresh T cells (Schmitt et al., 1989) and was completely inactive on a variety of promiscuous factor-dependent cell lines (Uyttenhove et al., 1988). Recently, however, the spectrum of activities of P40 was broadened by the observation that it enhanced the response of certain mast cell lines to interleuken-3 (IL-3), an activity previously ascribed to a distinct factor termed MEA (Hültner et al., 1989; Moeller et al., 1989). This factor now appears to be identical to P40 (Hültner et al., manuscript in preparation). This activity on mast cells suggests that, like other cytokines, P40 may be more pleiotropic than originally thought, and that other target cells remain to be discovered.

In order to pursue our analysis of the biological role of this cytokine, we attempted to identify its human counterpart. Here, we describe the cloning and expression of cDNA encoding the human homolog of mouse P40 and its expression in a baculovirus vector. We demonstrate that recombinant human P40 enhances the in vitro survival of long-term T-cell lines.

RESULTS AND DISCUSSION

Identification of Human P40 cDNA Clones

Low stringency screening of a human genomic DNA library with mouse P40 cDNA yielded a positive phage clone (λH40.3a1) from which restriction fragments were isolated and sequenced. Analysis of these fragments identified a 900 bp HindIII fragment that showed a region of high homology with the 3′ region of the murine cDNA, indicating that λH40.3a1 contained the gene of a human P40 homolog. The sequencing of this genomic clone will be described elsewhere.

Probing of Northern blots with the 0.9-kb HindIII genomic fragment demonstrated that human peripheral mononuclear cells (PBMC) stimulated for 24 h with phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) expressed high levels of the corresponding mRNA. Hence, these cells were selected for the construc-

[1]Unit of Experimental Medicine, International Institute of Cellular and Molecular Pathology, Université Catholique de Louvain, Belgium, and [2]Ludwig Institute for Cancer Research, Brussels Branch, Belgium.
Reprint requests should be addressed to Dr. Jean-Christophe Renauld, Unité de Médecine Expérimentale, UCL 7430, 74 avenue Hippocrate, B1200 Brussels, Belgium.

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tion of a cDNA library in phage λgt10. After screening of about 50,000 plaques with the 0.9-kb HindIII probe, two positive clones, termed λcH40.2 and λcH40.4, were identified. The EcoRI insert cH40.2 was subcloned into an M13 vector but turned out to be incomplete upon sequencing. The insert of λcH40.4 could not be retrieved by EcoRI digestion, probably as a result of incorrect reconstruction of the 3′ EcoRI site. However, a 489-bp EcoRI–HindIII fragment could be isolated, which was found to contain a sequence corresponding to the complete coding sequence of the murine cDNA, flanked by putative 5′ and 3′ untranslated regions of 11 and 46 nucleotides, respectively (Fig. 1). The 3′ untranslated region of this fragment stops 100 base pairs before the presumptive polyadenylation signal as indicated by sequencing of λcH40.2. Four copies of the sequence ATTTA, which is characteristic of transiently expressed genes (Shaw and Kamen, 1986), are present between the end of the coding sequence and the presumptive polyadenylation site.

Clone cH40.4 has an open reading frame of 432 nucleotides specifying a polypeptide of 144 amino acids, which includes, by homology with the mouse protein, a presumptive signal peptide of 18 residues (Fig. 1). The predicted molecular mass of the unglycosylated mature protein is 14,110 D. As for mouse P40, the presence of four potential N-linked glycosylation sites makes it likely that the molecular mass of the native protein is much higher. The homology with mouse P40 is 69% at the nucleotide level and 55% at the protein level. It is noteworthy that the 10 cysteines in the mature protein are perfectly conserved, but human P40 has an additional cysteine in the presumptive signal peptide (Fig. 2). No other homologies were detected between human P40 and previously sequenced proteins.

The size of the RNA for human P40 was determined in Northern blots of poly(A)$^+$ RNA isolated from PBMC stimulated with PHA (0.5%) and PMA (100 ng/ml) for 24 h. P40 cDNA hybridized with a band of ≈700 bp (Fig. 3), a size comparable to that reported for the mouse P40 message (Van Snick et al., 1989). No message was observed in freshly isolated unstimulated PBMC.

Expression of Human P40 cDNA in Insect Cells

Recombinant human P40 was expressed in *Spodoptera frugiperda* Sf9 cells using a recombinant baculovirus containing the 489-bp EcoRI–HindIII fragment isolated from λcH40.4. This expression system was chosen because it allows high level expression of mouse P40 (C. Druez et al, manuscript in preparation). As shown in Fig. 4, the recombinant protein had a M_r of ≈23 kDa, a value that is similar to that of mouse P40 produced in the same system (unpublished observations). For the mouse protein, we have found that N-glycanase treatment reduced this M_r to the predicted 14 kDa (C. Druez et al.). It is therefore likely that the M_r of the human recombinant protein also reflects the presence of N-linked sugars.

We have originally identified mouse P40 by its capacity to induce long-term proliferation of certain murine helper T-cell lines (Uyttenhove et al., 1988). However, subsequent experiments have shown that a more frequent effect of P40 is to increase cell survival without inducing cell proliferation (C. Uyttenhove, unpublished results). Therefore, we tested the biological activity of the recombinant human protein by examining its capacity to enhance the survival of a number of human T-cell lines that had been maintained in culture in the presence of PHA, irradiated PBMC as feeders, and IL-4. Prolonged survival was observed with 2 out of 10 lines tested, demonstrating the functional integrity of recombinant human P40 expressed in insect cells (illustrated in Fig. 4). So far, we have failed, however, to obtain permanent P40-dependent cell lines similar to those that were derived in the mouse system. It is of interest that mouse P40 also enhanced the survival of the human T-cell lines, whereas human P40 was apparently not active on mouse P40-dependent cell lines (data not shown).

Conclusions

Mouse P40 was originally identified as a potent growth factor for some mouse helper T-cell lines. More recently, it was shown to enhance the effect of IL-3 on the growth of mast cells (Hültner et al., manuscript in

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          -18                                                                   1
          MET LEU LEU ALA MET VAL LEU THR SER ALA LEU LEU LEU CYS SER VAL ALA GLY GLN GLY CYS PRO THR LEU ALA
CCGCTGTCAAG ATG CTT CTG GCC ATG GTC CTT ACC TCT GCC CTG CTC CTG TGC TCC GTG GCA GGC CAG GGG TGT CCA ACC TTG GCG   86
      10                                    20                                    30
GLY ILE LEU ASP ILE ASN PHE LEU ILE ASN LYS MET GLN GLU ASP PRO ALA SER LYS CYS HIS CYS SER ALA ASN VAL THR SER
GGG ATC CTG GAC ATC AAC TTC CTC ATC AAC AAG ATG CAG GAA GAT CCA GCT TCC AAG TGC CAC TGC AGT GCT AAT GTG ACC AGT  170
            40                                    50                                    60
CYS LEU CYS LEU GLY ILE PRO SER ASP ASN CYS THR ARG PRO CYS PHE SER GLU ARG LEU SER GLN MET THR ASN THR THR MET
TGT CTC TGT TTG GGC ATT CCC TCT GAC AAC TGC ACC AGA CCA TGC TTC AGT GAG AGA CTG TCT CAG ATG ACC AAT ACC ACC ATG  254
                  70                                    80                                    90
GLN THR ARG TYR PRO LEU ILE PHE SER ARG VAL LYS LYS SER VAL GLU VAL LEU LYS ASN ASN LYS CYS PRO TYR PHE SER CYS
CAA ACA AGA TAC CCA CTG ATT TTC AGT CGG GTG AAA AAA TCA GTT GAA GTA CTA AAG AAC AAC AAG TGT CCA TAT TTT TCC TGT  338
                        100                                   110
GLU GLN PRO CYS ASN GLN THR THR ALA GLY ASN ALA LEU THR PHE LEU LYS SER LEU LEU GLU ILE PHE GLN LYS GLU LYS MET
GAA CAG CCA TGC AAC CAA ACC ACG GCA GGC AAC GCG CTG ACA TTT CTG AAG AGT CTT CTG GAA ATT TTC CAG AAA GAA AAG ATG  422
120                     126
ARG GLY MET ARG GLY LYS ILE
AGA GGG ATG AGA GGC AAG ATATGAAGATGAAATATTATTTATCCTATTTATTAAATTTAAAAAGCTTTCTCTTTAAGTTGCTACAATTTAAAAATCAAGTAAGCT  527

ACTCTAAATCAGTATCAGTTGTGATTATTTGTTTAACATTGTATGTCTTTATTTTGAATAAAT                                                  591
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Figure 1. cDNA and predicted protein sequence of human P40.

Amino acid number 1 corresponds to the N-terminal residue of mature mouse p40. N-glycosylation sites and presumptive polyadenylation signal are underlined.

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        -10          1         10         20
h MLLAMVLTSALLLCSVAGQGCPTLAGILDINFLINKMQED
m --VTYI-A-V--FS--L--R-S-TW--R-T-YL-ENLKD-

        30         40         50         60
h PASKCHCSANVTSCLCLGIPSDNCTRPCFSERLSQMTNTT
m -P---S--G---------SV-T-D--T--YR-G-L-L--A-

        70         80         90        100
h MQTRYPLIFSRVKKSVEVLKNNKCPYFSCEQPCNQTTAGN
m QKS-LLPV-H---RI------IT--S---K-----M---

       110        120
h ALTFLKSLLEIFQKEKMRGMRGKI
m T-S------GT---TE-QRQKSRP
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Figure 2. Alignment of predicted protein sequences of human (h) and murine (m) P40.

Conserved cysteine residues are underlined.

preparation), suggesting that its range of activities may be much broader than anticipated from the original findings. The cloning of human P40 and the expression of the recombinant human protein presented here should make it possible to investigate systematically the activity of this cytokine on human cells and to evaluate its potential involvement in disease.

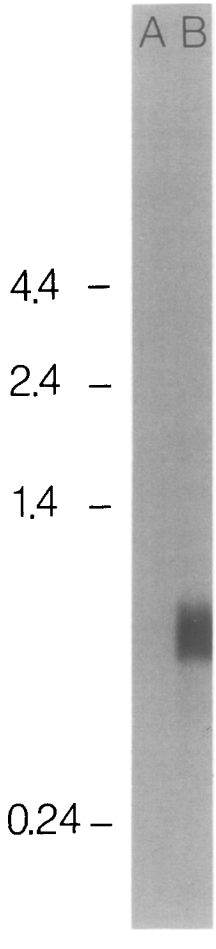

Figure 3. Northern blot analysis of human P40 mRNA.

Poly(A)$^+$ RNA (10 μg) from resting (**A**) or PHA-PMA stimulated PBMC (**B**) was hybridized with a ^{32}P-labeled P40 cDNA insert. Size of standards is indicated in kilobases.

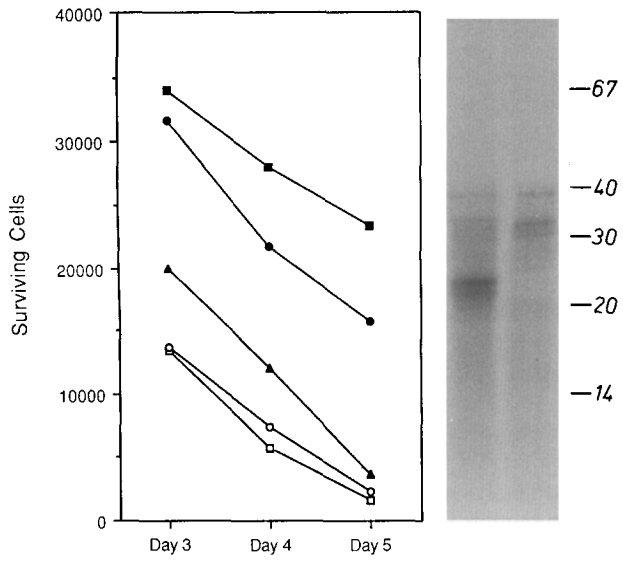

Figure 4. Expression and biological activity of recombinant human P40.

Left panel shows survival curves of a human T-cell line (EL) incubated in medium without factors (open squares □), or in medium supplemented with saturating concentrations of semipurified baculovirus-derived human P40 (closed circles ●), with a control preparation derived from cells infected with wild-type baculovirus (open circles ○), with purified mouse recombinant P40 (closed squares ■) or with human IL-4 (closed triangles ▲). All cultures were seeded with 50,000 cells on day 0. Right panel shows SDS-polyacrylamide gel electrophoresis of ^{35}S-labeled semipurified recombinant human P40 (left lane) and of control preparation (right lane).

MATERIAL AND METHODS

Construction and Screening of DNA Libraries

A human genomic library was made using high molecular weight DNA isolated from the EBV-immortalized lymphoblastoid cell line CESS. This DNA was partially digested with Sau3AI and fragments of appropriate size obtained by centrifugation on a NaCl gradient were cloned in the BamHI site of phage λGEM11 (Promega, Madison, WI). The library was screened with ^{32}P-labeled mouse P40 cDNA under low stringency (two washes at 55 °C in 30 mM NaCl, 3 mM sodium citrate pH 7, and 0.1% SDS).

A human cDNA library was constructed in λgt10 using poly(A)$^+$ RNA from human peripheral blood mononuclear cells (PBMC) stimulated for 24 with PHA-P (30 μg/ml) and PMA (100 ng/ml). This library was screened with a 0.9-kb HindIII fragment derived from the human genomic P40 clone. Filters were washed at 65 °C with 30 mM NaCl, 3 mM sodium citrate and 0.1% SDS.

Northern Blot Analysis

Poly(A)$^+$ RNA from PBMC stimulated for 24 h with PHA-P (30 μg/ml) and PMA (100 ng/ml) and from fresh PBMC was electrophoresed in the presence of formaldehyde and transferred to nitrocellulose (Davis et al., 1986). Filters were hybridized with the ^{32}P-labeled P40 cDNA cH40.4 and washed as described (Van Snick et al., 1988).

Expression of Human P40 cDNA

A 489-bp EcoRI–HindIII fragment of λcH40.4 was subcloned into the BamHI site located in the 5′ end of the polyhedrin gene in plasmid pVL941 (Luchow and Summers, 1989). This construct was transfected along with DNA of *Autographa californica* nuclear polyhedrosis virus into *Spodoptera frugiperda* Sf9 cells, and recombinant baculovirus clones were isolated by limiting dilution and dot hybridization as described by Summers and Smith (1988). Expression of the recombinant protein was verified in infected cells incubated with ^{35}S-labeled methionine. A P40-enriched fraction was obtained by adsorption of cationic proteins on sulfopropyl-Sepharose after dilution with 5 vol of 33 mM sodium acetate pH 5. Adsorbed proteins were eluted with 0.9 M NaCl, 0.1M Tris-HCl pH 8, and Tween-20 (10^{-4} vol/vol), precipitated with 10% trichloroacetic acid, and analyzed by SDS-polyacrylamide gel electrophoresis. The recombinant protein used in the T-cell survival assay was semipurified by adsorption to and elution from sulfopropyl-Sepharose under similar conditions and dialyzed against phosphate buffered saline before use. Expression and purification of mouse P40 in the same system will be described elsewhere.

DNA Sequencing

Sequences were performed by the dideoxynucleotide procedure after subcloning the cDNA in a M13 vector. Homology searches were conducted in the nucleic acid data base Genbank (release June 1989) using the FASTN program (Lipman and Pearson, 1985).

Cellular Preparations

Peripheral blood mononuclear cells were prepared by Lymphoprep (Nycomed AS, Oslo, Norway) density gradient centrifugation. Stimulations were performed for 24 h in RPMI 1640 medium supplemented with 10% fetal calf serum. Long-term T-cell lines were derived from PBMC of patients with hemachromatosis by repeated weekly stimulation with irradiated (3000 rads) allogeneic PBMC, PHA-P (30 μg/ml) and recombinant human IL-4 (25 U/ml).

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NOTE ADDED IN PROOF

The human homolog of mouse P40 was recently identified by expression cloning of a growth factor for a megakaryoblastic leukemia (Yang et al., 1989). It was proposed to rename the protein interleukin-9.

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