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Immunology

Functional and structural characterization of P40, a mouse glycoprotein with T-cell growth factor activity

(amino acid sequence/lymphokine/helper T cells)

CATHERINE UYTTENHOVE*, RICHARD J. SIMPSON†, AND JACQUES VAN SNICK*

*Ludwig Institute for Cancer Research, Brussels Branch, B-1200, Brussels, Belgium; and †Joint Protein Structure Laboratory, Melbourne Branch, Ludwig Institute for Cancer Research and Walter and Eliza Hall Institute of Medical Research, P.O. Royal Melbourne Hospital, Parkville, 3050 Victoria, Australia

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ABSTRACT **Antigen-independent cell lines were derived from mouse helper T-cell clones by culture in autologous supernatant obtained after stimulation with concanavalin A. A factor, termed P40, supporting the growth of these lines was purified and characterized as a basic 32- to 39-kDa single-chain glycoprotein functionally distinct from previously identified T-cell growth factors and apparently unrelated structurally to any known protein. Of a number of cell lines, only helper T cells responded to P40, and this response was not mediated by either interleukin 2 or interleukin 4.**

In recent years, it has become increasingly clear that interleukin 2 (IL-2) is not the only factor controlling T-cell growth. Indeed, several cytokines, including IL-4 (1, 2), granulocyte/macrophage colony-stimulating factor (3, 4), and, in a human system, the combination of IL-1 and IL-6 (5), have now been shown to induce IL-2-independent T-cell proliferations. The regulation of T-cell growth thus seems to be more complex than originally thought, although IL-2 undoubtedly remains the most potent and broadly active T-cell growth factor.

Several years ago, we established a collection of helper T-cell lines from lymph nodes of antigen-primed mice by using the procedure described by Corradin *et al.* (6). These cell lines were initiated by culture in the presence of antigen and were subsequently maintained, without addition of exogenous growth factors, by regular feeding with antigen and irradiated splenic antigen-presenting cells. Most of these cells produce large amounts of IL-3, IL-4, IL-5, and IL-6 but no IL-2 and thus belong to the TH_2 type defined by Mosmann *et al.* (7).

Recently, we observed that two clones derived from these cells proliferated in response to their own conditioned medium in the absence of antigen and feeder cells. As this growth factor activity was not inhibited by antibodies that block the action of IL-2 and IL-4, we sought to identify it more precisely.

We describe here the biological and structural characteristics of this T-cell growth factor and show that it is distinct from known proteins.

MATERIALS AND METHODS

Medium. Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 0.55 mM L-arginine, 0.24 mM L-asparagine, and 1.25 mM L-glutamine was used for most cell lines except for 7TD1 and BCL1, which were grown in Iscove's medium.

T-Cell Clones and Lines. Helper T-cell lines were established and maintained in the absence of exogenous growth factors as described (8). Lines TUC2 and TUC7 were derived from C57BL/6 mice immunized with keyhole limpet hemocyanin. Line TUC5 was obtained from the same strain of mice but after immunization with human transferrin. TUC13 is an allospecific BALB/c anti-C57BL/6 line. Individual clones were derived from these lines by limiting dilution in the presence of 10% medium conditioned by rat spleen cells stimulated with concanavalin A (Con A) and are denoted TUCx.y (where x stands for the number of the line and y stands for the number of the clone). These clones were subsequently expanded and maintained without exogenous growth factors like the parental cell lines. Cytolytic T-cell clones of DBA/2 origin directed against syngeneic P815 mastocytoma were maintained with 50% mixed lymphocyte culture medium as described (9). For use in growth factor assays, the T cells were separated from feeder cells by centrifugation over a layer of Lymphoprep (Nycomed AS, Oslo), washed, and incubated at 5×10^4 cells per well. Proliferations were measured on day 3 after a 6-hr pulse with [*methyl*-^{3}H]thymidine (0.5 μCi per well; 1 Ci = 37 GBq).

Preparation of Helper T-Cell Supernatants (SNs). TUC2.15 and TUC7.51 cells, obtained from cultures stimulated 2 weeks earlier with antigen and feeder cells, were adjusted to 2×10^6 cells per ml and incubated for 2–3 days in medium containing 0.5% fetal calf serum and Con A (5 μg/ml). SNs were collected by centrifugation at 10,000 × *g* for 20 min. When used for culture, crude SNs, were supplemented with 0.1 M methyl α-D-mannoside.

TS1 Growth Factor Assay. TS1, a factor-dependent cell line derived from TUC2.15, was cultured in 1% (vol/vol) TUC2.15 SN. Before use in the growth factor assay, the cells were washed free of SN and cultured at a density of 3×10^3 cells per well in 200 μl with serial dilutions of samples to be tested. After 3 days, cell growth was measured by colorimetric determination of hexosaminidase levels according to Landegren (10). The dilution giving half-maximal absorbance at 405 nm was arbitrarily assigned 1 unit of activity per ml.

Other Cell Lines. CTLL-2 (11) was grown with 100 units of human recombinant IL-2 per ml, DA-1 (12), Ea3.15 (13), and FDC-P1 (14) were grown with 10% WEHI-3 SN as a source of IL-3, and 7TD1 was grown with a 1:500 dilution of TUC2.15 SN as a source of IL-6 (8). Assays using these cell lines were carried out as described for the TS1 line and proliferations were measured either by hexosaminidase determinations or by thymidine incorporation. *In vivo* passaged BCL1 cells (15) were frozen in aliquots and thawed just before use. Proliferation of BCL1 was measured by thymidine incorporation in 7-day-old cultures seeded with 10^4 cells per well.

Cytokines and Growth Factors. Purified natural human IL-1β (16), recombinant human IL-2 (17), and purified murine IL-3 (18) were given by J. Van Damme (Rega Institute, Leuven, Belgium), W. Fiers (State University of Ghent, Ghent, Belgium), and J. Ihle (National Cancer Institute, Frederick, MD), respectively. Human recombinant granulocyte colony-stimulating factor and mouse recombinant gran-

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Abbreviations: IL, interleukin; SN, supernatant.

ulocyte/macrophage colony-stimulating factor were given by J. DeLamarter (Biogen, Geneva) (19). Platelet-derived growth factor (20) was a gift of C. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). Epidermal growth factor was purchased from Boehringer Mannheim. Mouse IL-4, IL-5, and IL-6 were purified as described (8, 21).

Antibodies. Anti-IL-4 antibody 11B11 (22) and anti-IL-2 receptor antibody 5A2 (23) were gifts of W. E. Paul (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) and J. Thèze (Institut Pasteur, Paris), respectively.

Purification of TS1 Growth Factor. Adsorption to silicic acid and gel filtration were performed as described (8). Active fractions from the gel filtration column were pooled, concentrated by ultrafiltration on an Amicon YM-10 membrane in the presence of a 1:10,000 (vol/vol) dilution of Tween 20, and transferred to 1 M Na_2SO_4 buffered to pH 7.0 with 0.1 M sodium phosphate before injection onto a TSK-phenyl column (LKB, Bromma, Sweden) equilibrated in the same buffer. After a 10-min wash in the starting buffer, elution was carried out at 0.6 ml/min with a linear gradient of a 1:1 mixture of sodium phosphate buffer (0.1 M, pH 7.0) and ethylene glycol. Active fractions were further fractionated on a Mono Q column (Pharmacia) equilibrated in 20 mM ethanolamine hydrochloride, pH 9.5, 20 mM NaCl, and 1:10,000 (vol/vol) Tween 20. The column was developed at 0.8 ml/min with a 30-min linear gradient of NaCl (8 mM/min). Pooled active fractions were concentrated and adjusted to contain 0.05% trifluoroacetic acid before injection on a C_1 25-nm pore-size TSK TMS-250 HPLC column (LKB). The column was developed for the first 10 min with a linear gradient from 0 to 35% acetonitrile in 0.05% trifluoroacetic acid, which was followed by a shallow 35–36% gradient for the next 60 min. Flow rate was adjusted to 0.8 ml/min; 1-min fractions were collected in Eppendorf tubes containing 10 μl of 1 M NH_4HCO_3 and 5 μl of Tween 20 (1% in water) and lyophilized. Total protein was measured fluorometrically with benzoxanthene following Neuhoff *et al.* (24). The purity of the final product was assessed by $NaDodSO_4$/PAGE in 12% acrylamide gels. Isoelectric focusing was performed with an LKB vertical gel apparatus. Material was recovered from gels by overnight incubation in 130 mM NaCl containing Tween 20 [1:10,000 (vol/vol)] and 10 mM sodium phosphate (pH 7.0). Affinity chromatography on lentil lectin-Sepharose was done following the procedure described by the manufacturer (Pharmacia).

Amino Acid Sequence Analysis. Automated amino acid sequence analysis was performed with an Applied Biosystems sequencer (model 477A) equipped with an on-line phenylthiohydanthoin amino acid analyzer (model 120A). *In situ* cyanogen bromide cleavage of P40 ($\approx$10 μg) was performed on the glass fiber sample disk of the gas-phase sequencer according to a procedure described elsewhere (25). Sequence comparisons were made with the following data bases: Protein Sequence Database of the Protein Identification Resource, National Biomedical Research Foundation (Release 15.0, December 1987); Swiss-Prot Protein Sequence Data Bank version 5 (September 1987, compiled by A. Bairoch, University of Geneva, Medical Biochemistry Department, 1211 Geneva 4, Switzerland); G.B. trans Protein Data Base Release 1.0 (August 1987) compiled from GENBANK Release 50.0 by J. Coventry, Walter and Eliza Hall Institute of Medical Research, Parkville 3050, Australia; and PG trans Protein Data Base Release 38.0 (December 1985), GENBANK, Institut Pasteur, Paris.

RESULTS

Detection of an Unusual T-Cell Growth Factor Activity. TUC2.15 is a C57BL/6 helper T-cell line that requires antigen and antigen-presenting cells for long-term growth *in vitro*. In an attempt to grow these cells without feeders and antigen, we supplemented the culture medium with 10% autologous SN obtained after stimulation with Con A. We found that this SN was able to induce cell proliferation without further requirement for antigen or feeder cells. This growth factor activity could not be inhibited by either anti-IL-4 or anti-IL-2 receptor antibodies (Table 1), indicating that it was mediated neither directly nor indirectly by these molecules.

Table 1. Proliferation of TUC2.15 helper T cells induced by autologous SN: independence from IL-2 and IL-4

	Thymidine incorporation,* kcpm		
Antibodies added	IL-2	IL-4	TUC2.15 SN
None	152	18	37
Anti-IL-2 receptor	4	16	32
Anti-IL-4	156	<1	33

TUC2.15 helper T cells (5×10^4 per well) were incubated for 3 days with IL-2 (100 units/ml), IL-4 (100 units/ml), or TUC2.15 SN [1% (vol/vol)] in the presence of anti-IL-2 receptor antibody 5A2 (30 μg/ml) or anti-IL-4 antibody 11B11 (10 μg/ml).

*Measured on day 3. Thymidine incorporation in the absence of growth factors was <1 kcpm.

In addition to its activity in short-term proliferations, the SN also readily stimulated the development of permanent antigen-independent cell lines, which could be maintained by subcultivation every 3–4 days in medium supplemented with 1% SN (Fig. 1). Attempts similarly to derive antigen-independent cell lines with IL-2 were unsuccessful. A second helper T-cell clone, TUC7.51, also gave rise to an antigen-independent cell line upon culture in autologous SN. The factors active on the two cell lines were apparently identical, since TUC7.51 SN supported the growth of TUC2.15 cells and vice versa (data not shown).

TS1, one of the factor-dependent cell lines derived from TUC2.15, was selected for further identification of the growth factor. This choice was based on the observation that TS1 grew quickly, with a doubling time of 11 hr, and responded to very small concentrations of SN, half-maximal proliferation being obtained at dilutions between 1:100,000 and 1:10,000 (vol/vol). To determine the specificity of the TS1 assay, we incubated the cells with a variety of purified growth factors or crude SN and found that only IL-4 and TUC2.15 SN supported TS1 growth (Table 2). As anti-IL-4 antibodies failed to inhibit the effect of TUC2.15 SN, these results implied that we were dealing with a different T-cell growth factor.

Purification of the T-Cell Growth Factor Active on TS1. Large batches of T-cell SN were produced by stimulating TUC2.15 and TUC7.51 cells with Con A (see *Materials and Methods*). The active material was concentrated by adsorp-

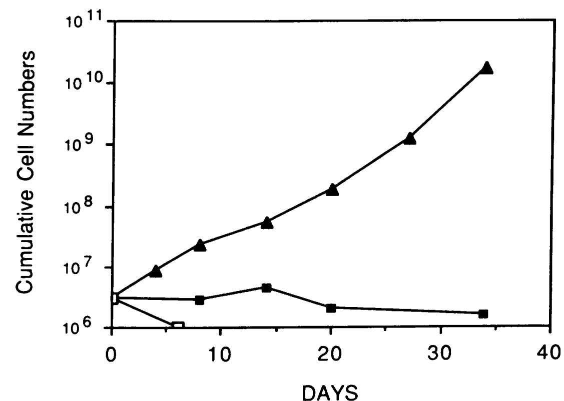

FIG. 1. Long-term antigen-independent T-cell growth induced by helper T-cell SN. TUC2.15 cells were grown without feeder cells and antigen in normal medium (□), in medium supplemented with IL-2 (20 units/ml, ■), or with TUC2.15 SN [5% (vol/vol), ▲].

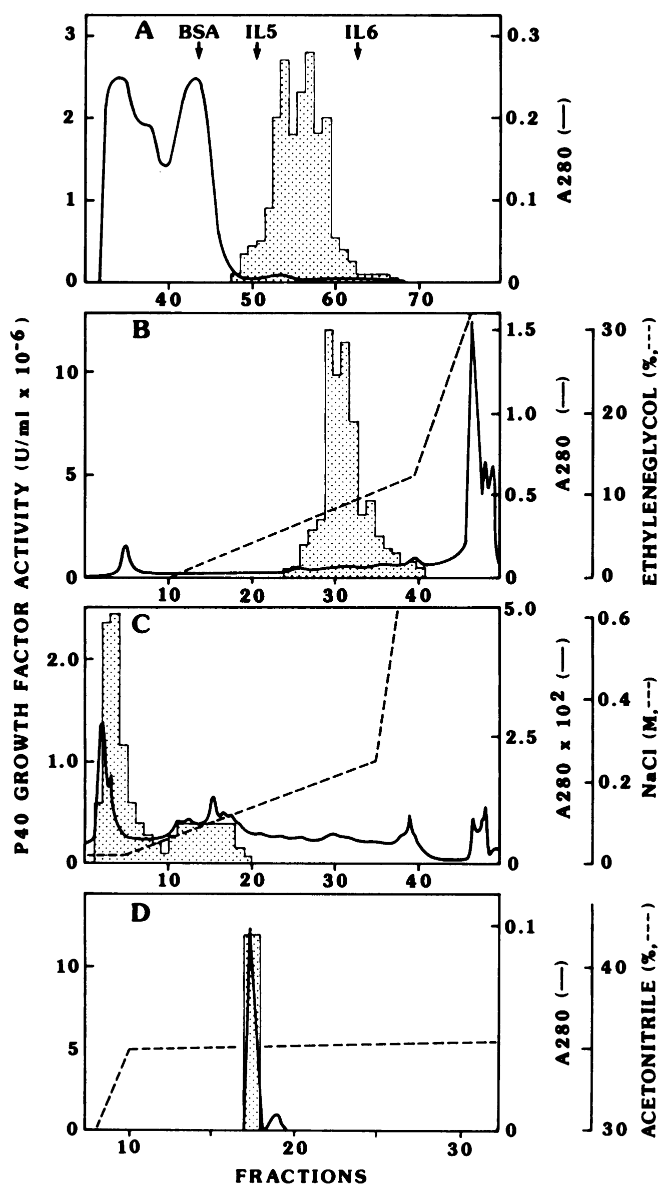

FIG. 2. Purification of TS1 growth factor (P40). TUC7.51 SN was fractionated sequentially on an Ultrogel Ac A54 gel filtration column (*A*), a TSK-phenyl hydrophobic interaction column (*B*), a Mono Q anion-exchange column (*C*), and a C_1 reversed-phase column (*D*). The shaded area represents growth factor activity. Molecular mass standards shown in *A* are bovine serum albumin (BSA, 67 kDa), natural IL-5 (45 kDa), and recombinant mouse IL-6 (22 kDa).

tion to silicic acid and applied to an Ultrogel AcA 54 (LKB) gel filtration column. The major growth-promoting activity, which was destroyed by trypsin, eluted as a symmetrical peak in the 30- to 40-kDa region (Fig. 2*A*) and was therefore designated P40. Subsequent experiments were carried out with TUC7.51 SN because the concentrations of P40 were higher in this material.

Preliminary characterization of the growth factor indicated that it had a pI ≈10 and was glycosylated, 60% of the activity being retained on a lentil lectin column. Based in part on this information, the following purification protocol was adopted. Active fractions from the gel filtration step were further separated by hydrophobic interaction chromatography on a TSK-phenyl column (Fig. 2*B*) followed by passage through a Mono Q anion-exchange column equilibrated at pH 9.5. At this elevated pH, most contaminants were retained on the column, whereas P40 eluted mainly in the flow-through fractions, as expected from its high pI (Fig. 2*C*). Final purification was achieved by reversed-phase chromatography on a C_1 column equilibrated with 0.05% trifluoroacetic acid. P40 was recovered in a single peak eluting at an acetonitrile concentration of 35% (Fig. 2*D*). At the end of this purification, P40 stimulated half-maximal growth of TS1 at a concentration of ≈5 pg/ml (Fig. 3), which corresponded to a 2000-fold purification. On average, the overall yield ranged from 5% to 10%.

The purified protein was very heterogeneous with a molecular mass of 32–39 kDa in $NaDodSO_4$/PAGE under reducing (Fig. 4) and nonreducing conditions. Biological activity could be recovered from the corresponding fractions of a nonreduced gel, but exposure to $NaDodSO_4$ and 2-mercaptoethanol destroyed most of the activity.

Amino Acid Sequence Analysis of P40. Edman degradation of P40 (≈250 pmol) yielded no N-terminal sequence. The sequence analysis was aborted and P40 (immobilized on the Polybrene-treated sample disk of the sequencer) was acyl-

Table 2. Growth of TS1 in response to various cytokines

Factor	Dose or dilution	Cell growth (A_{405})
TUC2.15 SN	1:12,500	1.96
IL-1β	100 units/ml	0
IL-2	100 units/ml	0
IL-3	100 units/ml	0.01
IL-4	100 units/ml	1.36
IL-5	100 units/ml	0
IL-6	20 ng/ml	0
GM-CSF	10 ng/ml	0
G-CSF	4 ng/ml	0
M-CSF (crude)	1:4	0.02
EGF	50 ng/ml	0
PDGF	4 μg/ml	0.02

TS1 cells were incubated for 3 days in the presence of various factors or SN. All reagents were tested over a 100-fold range, but results are given for the highest dose only. None of the factors that scored negatively at the highest dose had any effect at lower doses. Cell growth was measured by colorimetric determination of hexosaminidase levels. Absorbance of cultures incubated without growth factors ranged from 0.10 to 0.15 and was subtracted. GM-CSF, granulocyte/macrophage colony-stimulating factor; G-CSF, granulocyte CSF, M-CSF, macrophage CSF; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.

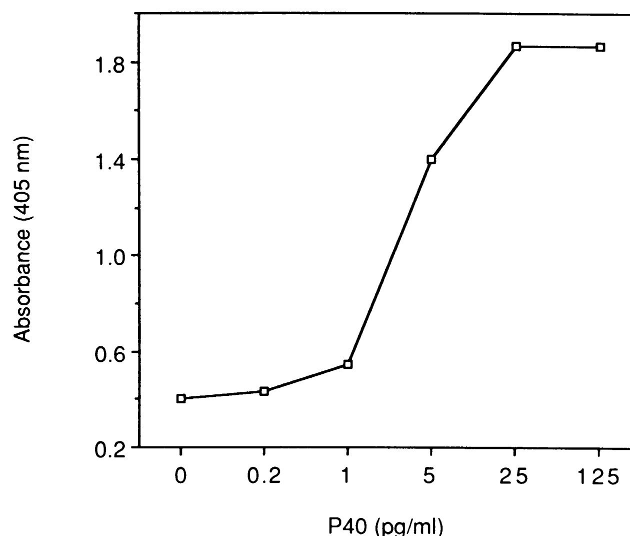

FIG. 3. Growth factor activity of purified P40. TS1 cells (3 × 10^3 cells per well) were cultivated in the presence of increasing doses of purified P40. After 3 days, cell numbers were evaluated by measuring hexosaminidase levels.

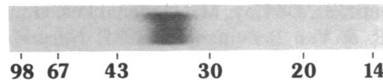

FIG. 4. Silver-stained NaDodSO$_4$/PAGE of purified P40. The sample was run under reducing conditions. Molecular masses of standards are given in kDa.

ated (26) and then subjected to *in situ* cyanogen bromide treatment (25). Sequence analysis was then continued and yielded the following major amino acid sequence (110 pmol): NH$_2$-Ala-Gly-Asn-Thr-Leu-Ser-Phe-Leu-Lys-Ser-Leu-Leu-Gly-Thr-Phe-Gln-Lys-Thr-Glu.

This internal sequence, which has been confirmed by sequence analysis of tryptic and chymotryptic peptides (unpublished data), showed no significant similarity with that of other proteins stored in the data bases listed in *Materials and Methods*.

Biological Activity of Purified P40. Purified P40, at concentrations up to 20 ng/ml, did not support the proliferation of either IL-3-dependent myeloid cell lines (FDCP-1, Ea3.15, and DA-1), IL-5-dependent B-cell lymphoma BCL1, or IL-6-dependent B-cell hybridoma 7TD1. Unlike IL-2, and to some extent IL-4, it also failed to stimulate any of six cytolytic T-cell clones tested (Table 3). By contrast, strong proliferations were observed with some but not all helper T-cell lines. IL-2-producing (TH$_1$ type, TUC7.33) and IL-4-producing (TH$_2$ type, TUC2.15) clones were found among the responders. A significant correlation, illustrated in Table 3 for clone TUC7.51, was observed between the time spent in culture and the responses to P40 and IL-4.

DISCUSSION

We have identified a protein, P40, capable of supporting long-term IL-2- and IL-4-independent growth of certain helper T-cell lines in the absence of antigen. P40 is functionally distinct from all known interleukins and colony-stimulating factors. Its specificity for helper T-cell lines, however, resembles that of a growth factor activity detected by Ogata *et al.* (27) in the SN of a thymic stroma cell line. It will be important, when this factor is purified, to examine its structural relationship to P40.

Table 3. Comparison of the T-cell growth factor activities of IL-2, IL-4, and purified P40

T-cell lines and clones	Thymidine incorporation ratio		
	IL-2	IL-4	P40
Cytolytic T cells			
CTLL-2	445	0.6	0.8
P35:10	683	24	1.2
P35:48	303	4	1.8
P91:6	195	2	1.6
P1:5	993	12	1.2
P1:204-8	630	9	1.2
Helper T cells			
TUC2	311	2	14
TUC2.15	1806	813	240
TUC5	263	20	13
TUC5.37	253	4	0.8
TUC7.33	115	276	235
TUC7.51*	1179	27	17
TUC7.51†	1345	634	199
TUC13.1	116	51	1.7

Cells were incubated with or without the indicated growth factors and thymidine incorporation was measured on day 3. Factor dosage was as follows: 100 units/ml for IL-2 and IL-4 and 1000–10,000 units/ml for P40. The results are shown as ratios of radioactivity incorporated with and without factors.
*One-month-old culture.
†One-year-old culture.

P40 was purified from SN of lectin-stimulated mouse helper T-cell lines to a specific activity of $\approx 10^8$ units/mg and characterized as a basic (pI $\approx$ 10) single-chain protein with a molecular mass of 32–39 kDa. Two observations indicate that P40 is a glycoprotein: (*i*) its heterogeneous migration pattern in NaDodSO$_4$/PAGE and (*ii*) its binding to lentil lectin, which points to the presence of N-linked carbohydrate side chains. Consistent with this observation, a number of potential N-glycosylation sites (Asn-Xaa-Thr motif) have been identified in the protein sequence determination (unpublished data). Moreover, additional evidence for extensive glycosylation of the molecule was obtained in preliminary experiments with N-Glycanase treatment, which reduced the molecular mass of P40 to $\approx$15 kDa. P40 is a stable molecule whose biological activity is not altered after exposure to NaDodSO$_4$, acid pH, or acetonitrile. By contrast, its activity is destroyed by 2-mercaptoethanol, which suggests that intramolecular disulfide bridges play an important role in maintaining appropriate folding of the molecule. No N-terminal amino acid sequence could be obtained from the purified protein, suggesting the presence of a blocked N terminus. However, P40 could be distinguished from known proteins on the basis of an internal sequence obtained after cyanogen bromide treatment. This sequence as well as additional internal sequences obtained from P40-derived peptides (unpublished data) showed no significant homology to any previously sequenced protein.

The observation that P40 was purified from the SN of helper T-cell lines and supported the growth of helper T cells suggests that it may act as an autocrine growth factor during the stimulation of these cells with antigen. Formal proof of this hypothesis will, however, require the demonstration that anti-P40 antibodies can inhibit this response. Moreover, we cannot at present completely exclude that small numbers of contaminating accessory cells, which are required for maintenance and optimal stimulation of our cultures, may contribute to the elaboration of the protein. However, there is no doubt that T cells can make P40, since the protein was recently detected in SN of a T-cell lymphoma and of T-cell hybridomas derived from TUC7.51.

The relationship of P40 with other T-cell growth factors remains to be clarified. Obviously, its activity differs from that of IL-2 in many ways: P40 was completely inactive on cytolytic T-cell clones, and, conversely, IL-2 failed to support long-term antigen-independent growth of helper T-cell lines. In contrast with these differences, a correlation was observed between the sensitivity of helper T-cell lines to P40 and IL-4, suggesting that T-cell activation by these two molecules is similarly regulated. The range of activities of IL-4, which also stimulates the growth of a variety of IL-3-dependent cell lines (28) and of cytolytic T cells (29), is, however, much broader than that of P40, indicating that the functional overlap between the two factors is only partial.

The specificity of P40 for certain helper T-cell lines is very intriguing. If confirmed with freshly isolated cells, it would suggest the existence of a growth-stimulatory mechanism restricted to a fraction of the helper T-cell subset. However, the increased response to P40 observed for a given clone with increasing time spent in culture supports another interpretation—namely, cells responsive to P40 would simply be in a different activation state. Further experiments will be required to resolve these issues.

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