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COOH-Terminal–Modified Interleukin-3 Is Retained Intracellularly and Stimulates Autocrine Growth

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Autocrine growth due to dysregulated growth factor production may have a role in the development of neoplasia. Whether autocrine growth is stimulated by growth factor secretion in an autocrine loop or by intracellular binding of the growth factor to a receptor has been unclear. The carboxyl-terminus coding sequence for murine interleukin-3 (IL-3) was extended with an oligonucleotide encoding a four–amino acid endoplasmic reticulum retention signal. IL-3–dependent hematopoietic cells became growth factor–independent when the modified IL-3 gene was introduced by retroviral gene transfer, despite lack of secretion of the modified IL-3. Hence autocrine growth can occur as a result of the intracellular action of a growth factor and this mechanism may be important in neoplastic and normal cells.

AUTOCRINE GROWTH, EXHIBITED BY cells that both produce and respond to a growth factor, may play a role in the development of neoplasia (*1*). Release from normal growth restraints by amplified or dysregulated mitogenic signals may give autocrine cell populations a selective growth advantage. Dysregulated expression of hematopoietic growth factor genes in leukemic blasts carrying receptors for those factors has been documented in some naturally occurring human leukemias (*2*). Retroviral transfer of dysregulated growth factor genes into growth factor–dependent hematopoietic cell lines leads to factor independence and makes these lines tumorigenic in vivo (*3, 4*).

A number of models have been proposed to explain the mechanism of autocrine stimulation. In the classical model, an autocrine growth factor is secreted before binding to its receptor on the same or a neighboring cell. Autocrine cell populations exhibiting features consistent with this model have been described (*2, 5*). Such features include additive stimulation of growth by exogenous growth factor, density-dependent growth characteristics, and inhibition of proliferation by neutralizing antibodies specific for the growth factor. However, we and others have investigated systems in which the amount of factor secreted by various autocrine clones does not influence growth rate; furthermore, growth is independent of cell density, and neutralizing antibodies do not prevent proliferation (*4, 6, 7*). These findings are compatible with a model in which binding of a growth factor to its receptor occurs within the cell. Recent observations of cells transformed by platelet-derived growth factor (PDGF) or v-*sis* lends credence to this possibility, since investigators localized activated ligand-receptor complexes internally (*8*).

We proposed to test the hypothesis that the interaction between a growth factor and its receptor can occur intracellularly in autocrine cell populations by preventing secretion and enhancing intracellular accumulation of the hematopoietic growth factor interleukin-3 (IL-3). We reported earlier that deletion of the signal sequence of the IL-3 gene leads to cytoplasmic accumulation of IL-3 but does not establish factor-independent growth (*7*). Presumably, free cytoplasmic IL-3 does not have access to its receptor. We have now tried to retain IL-3 within the endoplasmic reticulum or Golgi apparatus, where binding to a receptor is at least theoretically possible (*9*). Recent observations by Pelham and his colleague (*10*) suggested an appropriate strategy. They reported that the four–amino acid COOH-terminus sequence Lys-Asp-Glu-Leu (KDEL) is common to several proteins that normally reside within the endoplasmic reticulum and found this sequence necessary and sufficient for retention.

We used recombinant DNA technology to create a modified IL-3 protein with the KDEL COOH-terminus extension. We replaced the stop codon of a murine IL-3 cDNA with an oligonucleotide encoding the KDEL sequence and a new stop codon (Fig. 1). This modified IL-3 gene was inserted into a retroviral vector with a neomycin resistance gene to be used for selection. In our initial construct (SPL-KIL3), IL-3 mRNA was transcribed from the retroviral long terminal repeat (LTR) via cryptic splicing. These constructs were transfected into the ψ-2 retroviral packaging cell line (*11*), and high titer, virus-producing ψ-2 clones were used to infect IL-3–dependent murine 32D cells (*12*).

These 32D cells were exposed to ψ-2 supernatants and plated in methylcellulose in the presence of neomycin and IL-3. *neo*^{r} clones arose at a frequency of one per 10^3 cells plated, and six clones were isolated and characterized. A previously isolated and described 32D clone containing the unmodified IL-3 gene in an otherwise identical vector was used for comparison (*7, 13*). On DNA hybridization analysis, all clones exhibited the expected unrearranged provirus and had unique integration sites (*14*). RNA hybridization analysis revealed transcripts of the expected sizes, and the KDEL modification in the IL-3 coding sequence did not affect the level of the specific spliced IL-3 transcript, as compared to the clone with the unmodified sequence (*14*).

All six clones grew in liquid culture without the addition of exogenous IL-3. Growth curves of a representative clone (SPL-KIL3-2) with and without WEHI3B-conditioned media as a source of IL-3 activity are shown in Fig. 2B. The curves are superimposable,

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and the doubling time of this clone (12.5 ± 0.9 hours) was identical to that of the clone containing the unmodified IL-3 provirus. Plating efficiency in methylcellulose down to a concentration of five cells per milliliter was linear for this clone and not affected by exogenous IL-3 (*14*).

Conditioned media collected from exponentially growing cultures of each clone were assayed for secreted IL-3 bioactivity on factor-dependent 32D cells. All six clones secreted less material with IL-3 bioactivity than the clone containing the unmodified IL-3 provirus. Two clones that secreted only 2% as much bioactive material were studied in detail. The dilution of conditioned media at which 50% maximal stimulation of the target 32D cells occurred is shown in Fig. 3A. We next asked whether decreased IL-3 secretion by these cells stemmed from intracellular retention owing to the KDEL modification or simply from decreased overall production of IL-3 by these particular clones. Membrane-free cell lysates were prepared from the same bulk cultures of the clones used to produce conditioned media. These lysates were bioassayed on factor-dependent 32D cells and the results are shown in Fig. 3B. There was four to six times more intracellular bioactive IL-3 found within the two KDEL-modified clones than in the control clone containing the unmodified IL-3 provirus. These results were also corroborated by immunoassay (*15*).

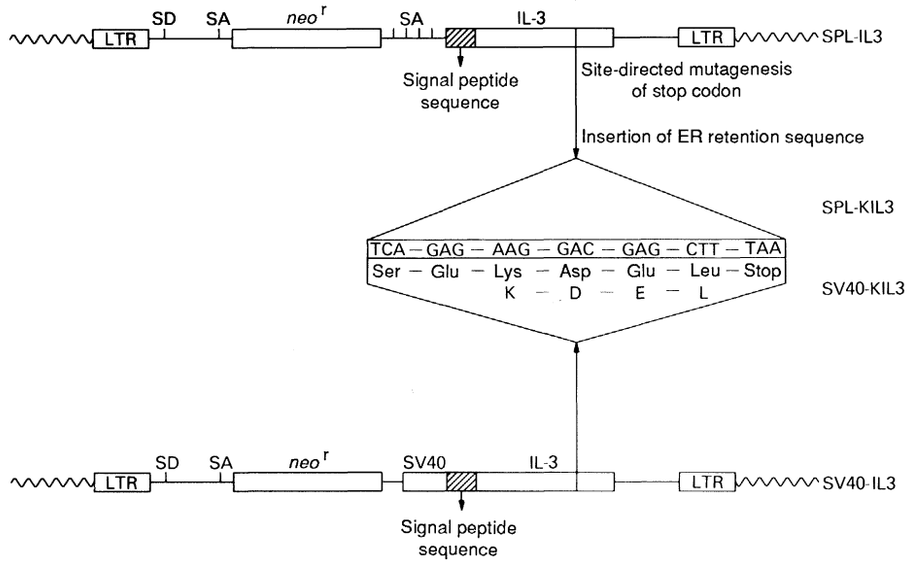

Fig. 1. Retroviral vector construction. The base XM5 vector containing Moloney murine leukemia virus LTRs, ψ-2 packaging signal, neomycin resistance gene, and splice donor (SD) and splice acceptor (SA) sites has been described (*20*). The construction of SPL-IL3 and SV40-IL3, containing the murine IL-3 cDNA transcribed from the LTR via alternative splicing or from the SV40 early region promoter, respectively, has also been reported (*3, 7, 13*). The Pst I–Xho I fragment of the murine IL-3 cDNA was subcloned into Bluescribe (Stratagene), and site-directed mutagenesis was used to destroy the stop codon and replace it with a new Hpa I restriction site (*7*). An oligonucleotide encoding a six–amino acid sequence including K-D-E-L and a new stop codon was inserted, and the modification was confirmed by DNA sequencing (*21*). This modified IL-3 fragment was subcloned back into the full murine cDNA sequence preceded either by SV40 splice-acceptor sites or the SV40 early region promoter, and this larger fragment was inserted into the XM5 retroviral vector at the Xho I cloning site.

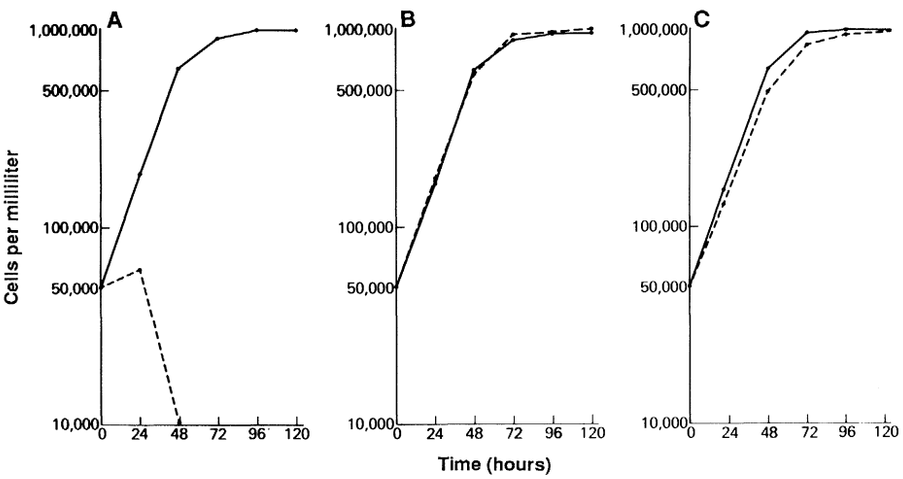

Fig. 2. Growth curves of (**A**) parental 32D cells, (**B**) clone SPL-KIL3-2, and (**C**) clone SV40-KIL3-3, grown in RPMI, 10% fetal calf serum, and glutamine (30 mg/dl), with and without the addition of 10% WEHI3B-conditioned medium as a source of exogenous IL-3. (——) With WEHI3B-conditioned medium; (- - - -) without WEHI3B-conditioned medium. These results were confirmed in three separate experiments.

Previous work has shown that the KDEL retention system is saturable, with secretion of KDEL-modified proteins if synthesis is high (*16*). Our system likewise appeared to saturate, allowing enough secretion of IL-3 for maximal stimulation extracellularly, even in the clones with greatly reduced secretory rates. Thus these clones were not useful for proving our hypothesis that an intracellular autocrine mechanism exists. To try to circumvent this problem, we made a second construct, using the SV40 early promoter instead of the LTR (Fig. 1). When coupled to an unmodified IL-3 gene, this promoter leads to much lower levels of secretion from 32D cells (*7*).

Neomycin-resistant 32D cell clones exposed to ψ-2 supernatants containing the SV40-KIL3 virus arose at a frequency of five per 10^5 cells plated. Four clones were isolated and characterized. On DNA blots, all exhibited the unrearranged provirus. Each clone had a unique insertion site and all were shown to produce the expected mRNA species by RNA blot (*14*). All four clones grew independently of added growth factor but had doubling times in the absence of exogenous IL-3 that were slightly slower than in its presence. Growth curves with and without WEHI-conditioned medium for clone SV40-KIL3-3 are shown in Fig. 2C. With WEHI-conditioned medium, its mean doubling time, calculated in log phase between 24 and 48 hours, was 13.2 ± 0.8 hours, and without WEHI-conditioned medium its doubling time was 14.4 ± 1.0 hours. Plating efficiency of this clone in methylcellulose was linear and insensitive to exogenous IL-3 down to a density of five cells per milliliter (*14*).

Despite exhibiting autonomous growth, three of the four clones secreted no detectable bioactive IL-3 and the fourth secreted a very low level (Fig. 3A). The 32D bioassay used is sensitive down to IL-3 concentrations of 5 to 10 pg/ml (*17*). The same assay for clone SV40-IL3, containing the unmodified IL-3 provirus, is shown for comparison. Cell lysates of three of the clones were

prepared and assayed as described above. The clone (SV40-KIL3-2) that secreted minimally had levels of IL-3 in its lysate comparable to those of the SPL-KIL3 clones (Fig. 3B), and the nonsecreting clones had lower levels, but still three to six times as great as the level found in SV40-IL-3, the clone containing the unmodified IL-3 provirus. Immunoassay on lysates from these clones corroborated the bioassay data (*15*).

For further confirmation that these clones could grow autonomously without secretion of IL-3, we performed antibody inhibition studies with the IL-3–neutralizing monoclonal antibody 8F8 (*18*). Low concentrations of this antibody prevented the proliferation of 32D cells in 10% WEHI3B-conditioned media (Fig. 4). To confirm that this antibody also neutralizes KDEL-modified IL-3, we tested a cell lysate from clone SPL-KIL3-2, which was shown (Fig. 3B) to have the highest intracellular concentration of bioactive IL-3. The 8F8 antibody did inhibit stimulation of factor-dependent 32D cells by this lysate, with a dose response consistent with the relative concentration of IL-3 in this lysate versus that in WEHI-conditioned media. All four SV40-KIL3 clones grew without inhibition by the antibody, even at an antibody concentration of 150 μg/ml, which was more than ten times as great as the antibody concentration necessary to inhibit proliferation of the parental 32D cells in 10% WEHI3B-conditioned media. Representative results for two of the clones are shown in Fig. 4. As previously reported (*7*), the SV40-IL3 clone showed minimal inhibition only at high antibody concentrations.

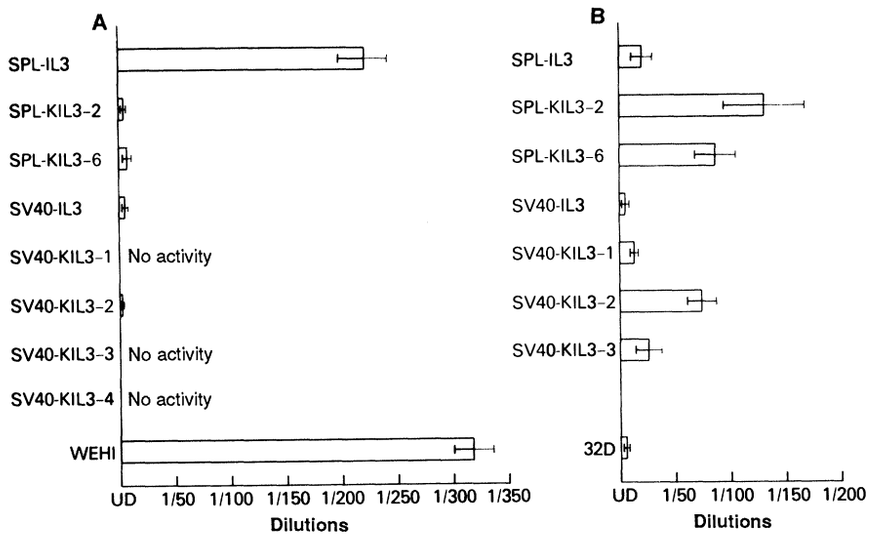

Fig. 3. (**A**) Assay of IL-3 bioactivity in conditioned medium from factor-independent 32D clones seeded at a density of 5×10^4 cells per milliliter and collected 48 hours later at a density of 8×10^5 cells per milliter. After filtering, we assayed samples of conditioned medium for IL-3 bioactivity by applying serial dilutions, beginning with undiluted (UD) samples, to 5×10^3 factor-dependent 32D cells in 96-well plates, and by counting viable cells 48 hours later. The dilution at which 50% maximal stimulation occurred for each sample is shown. For each clone, three or four separate collections and bioassays were done, and the mean and standard deviation for each clone are plotted. WEHI3B-conditioned medium was assayed in each experiment as a positive control. (**B**) Cell lysates were prepared from each clone and parental 32D cells (*7*) with the same bulk cultures used to obtain conditioned medium. Cells were washed four times with saline, brought to a concentration of 5×10^7 cells per milliliter, and lysed with four cycles of freezing and thawing. They were centrifuged at 70,000*g* in a Beckman Vti50 rotor for 1 hour, and membrane-free supernatants were collected. Total protein concentrations were checked on each sample by a spectrophotometric assay (Bio-Rad). Bioassay of each lysate was carried out as described above and repeated for a total of three separate lysate procurement procedures from each clone. The mean and standard deviation of the dilution producing 50% maximal stimulation of target 32D cells is shown.

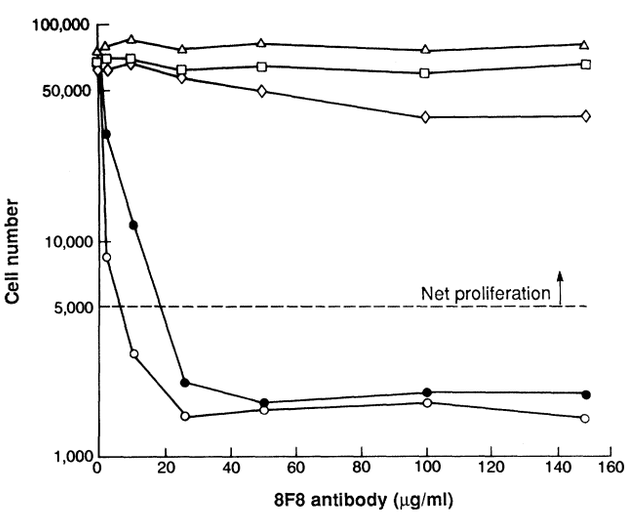

Fig. 4. Antibody inhibition by murine IL-3–neutralizing antibody 8F8 (*17*). Total antibody concentration was kept constant at 150 μg/ml for each point by addition of an isotype-matched monoclonal antibody to β-galactosidase. Washed cells (5×10^3) were separated into aliquots in a total volume of 150 μl for each point, and viable cells were counted 48 hours later. Results were confirmed in three separate experiments. (●) 32D cells plus 10% WEHI3B-conditioned medium; (○) 32D cells plus 20% SPL-KIL3 cell lysate (procurement described in Fig. 3B); (◇) clone SV40-IL3; (□) clone SV40-KIL3-1; (△) clone SV40-KIL3-2.

Tumorigenicity assays in BALB/c *nu/nu* (nude) mice were performed for 32D clones SV40-IL3, SV40-KIL3-1, and SV40-KIL3-2; 5×10^6 cells were injected subcutaneously into each mouse. All three clones produced palpable tumors with a latency of 10 to 12 weeks in all five of the mice receiving injections. None of the five mice given injections of the parental 32D cells developed tumors by 15 weeks. Thus tumorigenicity in vivo does not appear to depend on secretion of IL-3.

Our study indicates that autocrine growth stimulation can result from intracellular action of IL-3 and suggests that signal transduction may also occur without requirement for surface display. 32D cell clones containing the KDEL-modified IL-3 gene were able to grow without exogenous growth factor and with no detectable secretion of IL-3 and could not be inhibited with neutralizing antibody. Presumably the high concentrations of IL-3 that occurred in an intracellular compartment with access to receptor binding stimulated maximal or near maximal proliferation. Nonsecretory autocrine stimulation could help to explain the proliferative advantage neoplastic clones have over surrounding normal cells that do not actually produce their own growth factors. Further studies with the KDEL-modified IL-3 retrovirus may help clarify the role of autocrine versus paracrine or endocrine

The frequency of spontaneous factor-independent mutants in hematopoietic cell lines has previously been reported as no more than two to three per 10^7 cells (*19*). Six clones infected with an identical retrovirus carrying a *neo*^{r} gene but no IL-3 gene were not factor independent.

effects resulting from dysregulated growth factor production in vivo.

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Transformation by v-*sis* Occurs by an Internal Autoactivation Mechanism

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Transformation by the v-*sis* oncogene appears to require an interaction of its protein product, p28$^{v\text{-}sis}$, with the receptor for the platelet-derived growth factor (PDGF). However, this interaction may not occur at the cell surface as predicted by the autocrine hypothesis because phenotypic transformation was not reversed by incubation of SSV-NRK cells with antisera to PDGF and because morphological transformation did not occur when nontransformed NRK cells were cultured continuously with p28$^{v\text{-}sis}$. A mutant of the wild-type v-*sis* gene was constructed that encodes a v-*sis* protein targeted for retention within the endoplasmic reticulum and Golgi. NRK cells expressing the mutant v-*sis* gene did not secrete any detectable v-*sis* protein but were as fully transformed as wild-type v-*sis* transfectants. The results support a mechanism of transformation by v-*sis* in which internal activation of the PDGF receptor occurs before expression of either p28$^{v\text{-}sis}$ or the PDGF receptor at the cell surface.

THE ONCOGENE OF THE SIMIAN SARcoma virus (SSV), v-*sis*, encodes a polypeptide that is 92% homologous to the nonglycosylated B chain of PDGF, a potent mitogen and chemoattractant for cells of mesenchymal origin (*1, 2*). According to the autocrine hypothesis (*3*), unregulated growth of transformed cells occurs through the secretion of a growth factor and its subsequent productive interaction with its own cell surface receptor. Evidence for an autocrine mechanism in SSV stimulation of cell growth was that SSV-transformed NIH 3T3 and NRK cells secrete a potent PDGF-like mitogen that interacted with the PDGF receptor (*2, 4*) and that [^{3}H]thymidine incorporation into DNA was reduced by 50% when SSV-NRK cells were grown in the presence of antisera to PDGF (*4*). However, the autocrine hypothesis was inadequate to fully explain the mechanism by which SSV induced transformation because addition of high concentrations of antiserum to PDGF to the medium of SSV-transformed cells did not reverse the transformed phenotype, and high levels of exogenous p28$^{v\text{-}sis}$ continuously added to the medium of nontransformed NRK cells did not induce morphological transformation. Furthermore, the SSV-transformed marmoset fibroblast cell line SSV-NP1 did not secrete detectable levels of growth factor, nor was the growth of SSV-NP1 cells inhibited by antisera to PDGF. These results led to the hypothesis that the mechanism of transformation by the v-*sis* gene product may involve activation of the PDGF receptor through an internal activation mechanism before the receptor is expressed at the cell surface (*4, 5*).

A COOH-terminal amino acid sequence causes the retention of soluble proteins within the endoplasmic reticulum and Golgi apparatus (*6*). This six–amino acid sequence Ser-Glu-Lys-Asp-Glu-Leu (SEKDEL) is specific, as a change in the last two amino acids Ser-Glu-Lys-Asp-Ala-Ser (SEKDAS) is sufficient to allow proteins to be secreted. We therefore cloned a synthetic DNA oligomer that encoded the SEKDEL retention sequence at the 3′ end of a truncated but fully active v-*sis* gene. NRK fibroblasts transfected with the v-*sis* gene containing the SEKDEL retention sequence (v-*sis* SEKDEL) were compared to cells transfected with constructs that secreted p28$^{v\text{-}sis}$ for their transforming capabilities.

Clones pSEB3, pSEB12, and pSEB34 (Fig. 1) (*7*), as well as the vector pSVFVXM, were integrated into the host chromosome at nearly identical rates, as determined by the number of G418-resistant colonies per microgram of DNA transfected (*8*). Although the pSVFVXM plasmid, which lacked the v-*sis* gene, was unable to induce foci of NRK cells, each of these plasmids that contained a v-*sis* gene construct readily and equally induced foci (Fig. 2). Similarly, cells that were transfected with each of the v-*sis* constructs had increased saturation densities at confluence, formed colonies in soft agarose, and established tumors in nude mice (Table 1). Thus, the v-*sis* SEKDEL mutant is fully transforming. RNA blot analyses and mitogenic assays of cell lysates established that all NRK cell lines containing the v-*sis* gene transcribed the v-*sis* gene at high levels, and each gene expressed a protein product that was mitogenically active (Table 1). The v-*sis* SEKDEL protein was also fully active when analyzed in fractions rich in endoplasmic reticulum and Golgi; nearly three times as much growth-promoting activity was observed per milligram of protein as was detected in preparations from v-*sis* SEKDAS. These results establish that the v-*sis* SEKDEL protein is an active mitogen and is highly enriched in

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