Signal Transduction Through the EGF Receptor Transfected in IL-3–Dependent Hematopoietic Cells

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An expression vector for the epidermal growth factor (EGF) receptor was introduced into the 32D myeloid cell line, which is devoid of EGF receptors and absolutely dependent on interleukin-3 (IL-3) for its proliferation and survival. Expression of the EGF receptor conferred the ability to utilize EGF for transduction of a mitogenic signal. When the transfected cells were propagated in EGF, they exhibited a more mature myeloid phenotype than was observed under conditions of IL-3–directed growth. Moreover, exposure to EGF led to a rapid stimulation of phosphoinositide metabolism, while IL-3 had no detectable effect on phosphoinositide turnover either in control or EGF receptor–transfected 32D cells. Although the transfected cells exhibited high levels of functional EGF receptors, they remained nontumorigenic. In contrast, transfection of v-*erb*B, an amino-terminal truncated form of the EGF receptor with constitutive tyrosine kinase activity, not only abrogated the IL-3 growth factor requirement of 32D cells, but caused them to become tumorigenic in nude mice. These results show that a naïve hematopoietic cell expresses all of the intracellular components of the EGF-signaling pathway necessary to evoke a mitogenic response and sustain continuous proliferation.

SIGNAL TRANSDUCTION MEDIATED BY growth factor interaction with specific membrane receptors is of critical importance in the regulation of normal cell growth and differentiation. Moreover, increasing evidence indicates that aberrations in these pathways are important in the neoplastic process (*1*). The molecular cloning of growth factor receptor genes (*2, 3*) has made it feasible to investigate the ability of specific receptors to couple with intracellular components needed to evoke a functional response in foreign host cells. In the present study, we sought to determine the effects of introduction of the epidermal growth factor (EGF) receptor or v-*erb*B genes into a nontumorigenic hematopoietic cell line, designated 32D (*4*), which lacks EGF receptors and is normally dependent on interleukin-3 (IL-3) (*5*) for proliferation and survival.

Eukaryotic expression vectors (*6*) were engineered for both the human EGF receptor complementary DNA (LTR/EGFR) (*2*) and the v-*erb*B oncogene (LTR/v-*erb*B) (*7*). These vectors contain the transcriptional initiation sequences of the Moloney murine leukemia virus long terminal repeat, along with the *Ecogpt* selectable marker, which confers resistance to mycophenolic acid (*8*). Cells (32D) were transfected with either the LTR/EGFR or the LTR/v-*erb*B DNAs by electroporation (*9*) and selected for *Ecogpt* expression by their ability to grow in IL-3–containing medium supplemented with mycophenolic acid. After 2 to 3 weeks in the selective medium, viable lines, designated 32D-EGFR and 32D–v-*erb*B, emerged from the vector-transfected cultures, while no cells survived in mock-transfected control plates. Radioimmunoprecipitation analysis revealed that the 32D-EGFR transfectant synthesized a 170-kD protein that was specifically precipitated by antiserum to EGF receptor peptide, RK-2 (*10*). This EGF receptor–specific protein was indistinguishable in size from the EGF receptor expressed in human cell lines (*11*). The 32D–v-*erb*B transfectant expressed a 68- to 74-kD protein similar to the v-*erb*B gene product synthesized by the avian erythroblastosis virus (AEV)–transformed avian erythroblast line, 6C2 (*12*). No EGF receptor or v-*erb*B–related proteins were detected in the control 32D line.

To determine whether the EGF receptor was expressed on the surface, we measured the capacity of 32D-EGFR to bind ^{125}I-labeled EGF under saturating concentrations. The IL-3–dependent 32D line demonstrated no significant binding when compared to the murine NR6 line, which is devoid of EGF receptors (*13*) (Fig. 1). The 32D–v-*erb*B line also showed no evidence of EGF binding under these conditions. These findings were consistent with the fact the v-*erb*B–transforming gene product l the amino-terminal binding domain of EGF receptor (*2, 14*). In contrast, bin of ^{125}I-labeled EGF by 32D-EGFR cells almost as high as that of A431 cells, w have approximately 3×10^6 EGF recep per cell (*15*).

The parental 32D line was unrespon to the addition of fetal calf serum (F alone or in the presence of EGF but sho a marked increase in [^{3}H]thymidine in poration in response to 50 units of IL-3 milliliter and serum (Table 1). In cont the 32D-EGFR line responded dramati to addition of EGF. Dose-response exp ments revealed a half-maximal stimula by EGF at approximately 25 pg/ml (The presence of functional EGF recep did not impair the responsiveness of 32D-EGFR cells to IL-3, as evidenced their ability to incorporate [^{3}H]thymidi levels equivalent to those of the par 32D line after treatment with IL-3. In trast, the 32D–v-*erb*B transfectant requ only serum to elicit a maximal prolifer response. The addition of exogenous I or EGF did not significantly enhanc growth. Therefore, it appeared that exp sion of v-*erb*B, the oncogenic counterpa the avian EGF receptor, was able to a

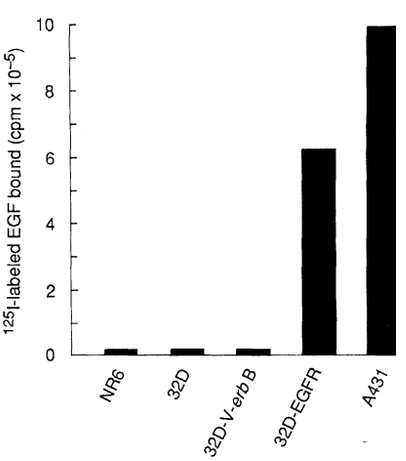

Fig. 1. Binding of ^{125}I-labeled EGF to 32D 32D transfectants in comparison to NR6 A431 cells. The binding of ^{125}I-labeled EGF assayed as described (*27*). Adherent cells (A and NR6) were plated (5×10^5 cells per we day before assay, and cells from a sample were counted at the time of the assay. Equiv numbers of nonadherent 32D cells and tran tants were plated under serum-free conditio minutes before assay. Cells were washed incubated for 60 minutes in ^{125}I-labeled (200 ng/ml) (Collaborative Research). Free was removed by washing three times, cells lysed in 0.5*M* NaOH, and measured with counter. The extent of nonspecific binding measured by incubating cells in the presen 100-fold excess of unlabeled EGF and these ues were subtracted from bound counts. Dat the mean of duplicate samples.

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gate the IL-3 requirement of these cells. Similar findings have been reported for oncogenes including v-*abl*, v-*fms*, and v-*myc* (*17*).

We next investigated the effects of EGF receptor or v-*erb*B expression by 32D cells on the ability of the cells to proliferate indefinitely. The parental 32D line remained viable only in the presence of IL-3 and serum. When these cells were transferred to medium containing only serum or serum and EGF, their viability declined rapidly, and no live cells were present after 48 hours. When 32D-EGFR cells were transferred to medium lacking IL-3 but supplemented with EGF and serum, they required somewhat higher levels of EGF to sustain continuous growth than were necessary to induce DNA synthesis. Although the addition of EGF at 1 ng/ml greatly increased the viability of 32D-EGFR, the cells required at least 10 ng of EGF per milliliter for continued proliferation (Fig. 2). In fact, cell growth remained slow during the first few weeks after switching from IL-3 to EGF supplementation. After this adaptation period, 32D-EGFR cells could be cultured continuously in EGF-containing medium but remained EGF-dependent. In contrast, when the 32D–v-*erb*B transfectant was transferred to medium containing only FCS, no lag period was observed. The cells continued to proliferate indefinitely with no change in doubling time or saturation density. These results as well as the ability of 32D–v-*erb*B cells to readily form colonies in soft agar in the absence of IL-3 (Table 1) confirmed that transfection of v-*erb*B into 32D cells completely abrogated their IL-3 dependence.

IL-3–dependent 32D cells can be induced to terminal granulocytic and monocytic differentiation when IL-3 is removed and granulocyte–colony-stimulating factor (G-CSF) is added to the culture (*18*). When 32D-EGFR cells were propagated in EGF, there was an increase in the presence of lactoferrin-positive and chloroacetate esterase–positive granulocytes, as well as lysozyme and nonspecific esterase–positive monocytes (Table 2). The extent of granulocytic differentiation in the presence of EGF was much less than that reported for G-CSF–induced differentiation of 32D cells (*18*). These results suggest that while IL-3 induces a proliferative signal in these cells and G-CSF primarily triggers a differentiation program, EGF appeared to modulate proliferation coupled with a shift in the differentiation state of the cells toward more mature myeloid phenotypes.

To analyze the malignant potential of 32D and transfected lines, each was inoculated subcutaneously into ten female nude mice (5×10^6 cells per mouse). Neither the parental 32D or 32D-EGFR lines yielded any detectable tumors within the 2-month observation period. In contrast, 32D–v-*erb*B cells produced large tumors at the site of inoculation in all mice within 2 weeks. IL-3–independent cell lines derived from such tumors expressed v-*erb*B–encoded glycoproteins and had the male karyotype of the donor cells (*19*). These results confirmed that a bypass of the pathway of IL-3–induced proliferation by v-*erb*B was associated with conversion of the cells to the malignant phenotype.

One signaling pathway implicated in growth control involves the hydrolysis of inositol lipids (*20*). EGF has been reported to stimulate phosphoinositide turnover in A431 cells and Swiss 3T3 cells (*21*). In an attempt to determine whether IL-3 and EGF induced similar or different intracellular responses, we measured phosphoinositide turnover in 32D-EGFR cells after treatment with EGF of IL-3. The production of [^{32}P]phosphatidic acid in response to IL-3 was not increased over control unstimulated values (Fig. 3A). In contrast, EGF induced phosphatidic acid formation to a level threefold that of the control. Moreover, the EGF-induced formation of inositol phosphates and phosphatidic acid, metabolites of inositol lipid hydrolysis, was extremely rapid (Fig. 3B). Inositol 1,4,5-trisphosphate, separated by the high-performance liquid chromatography method of Irvine *et al.* (*22*), reached its peak 15 seconds after EGF addition and returned to control levels within 5 minutes, and inositol 1,3,4-trisphosphate levels remained elevated over this period (*23*). Thus, the effects of IL-3 and EGF on phosphoinositide turnover could be readily distinguished in 32D-EGFR cells. The steady-state levels of diacylglycerol and inositol phosphates in the 32D–v-*erb*B transfectant were also increased over that of 32D (*23*), indicating that the v-*erb*B gene product constitutively enhanced inositol phos-

Table 1. Mitogenic response and cloning efficiency of 32D transfectants. The cell proliferation assay was performed on cells cultured in Iscove's modified Dulbecco's medium (IMD) supplemented with 15% fetal calf serum (FCS) and IL-3 (50 U/ml). Cells were washed twice and plated (3×10^4 cells per milliter) in IMD with 15% FCS (Gibco), IL-3 (50 U/ml) (Genzyme), or EGF (100 ng/ml) (Collaborative Research) for 24 hours. Five microcuries of [^{3}H]thymidine ([^{3}H]TdR) (New England Nuclear) was then added to each well. Incubation was continued for 24 hours, and [^{3}H]TdR incorporation was measured. Results are expressed as a stimulation index (SI): cpm [^{3}H]TdR incorporation with treatment/cpm [^{3}H]TdR incorporation of 32D with serum. Data are the mean of duplicate samples. The cloning efficiency was established by plating cells at various concentrations in 5 ml of IMD supplemented with 15% FCS and 0.48% sea plaque agarose (marine colloids). IL-3 (50 U/ml) or EGF (100 ng/ml) was included when specified. Plates were incubated at 37°C in 5% CO_2. Visible colonies were scored at 12 days after plating.

Cell line	Treatment	SI of [^{3}H]TdR incorporation	% Cloning efficiency
32D	IL-3 + serum	234.5	35.5
	EGF + serum	1.0	<0.1
	Serum	1.0	<0.1
32D–v-*erb*B	IL-3 + serum	225.5	28.0
	EGF + serum	231.0	30.5
	Serum	228.0	28.5
32D-EGFR	IL-3 + serum	215.5	27.5
	EGF + serum	185.5	25.0
	Serum	1.0	<0.1

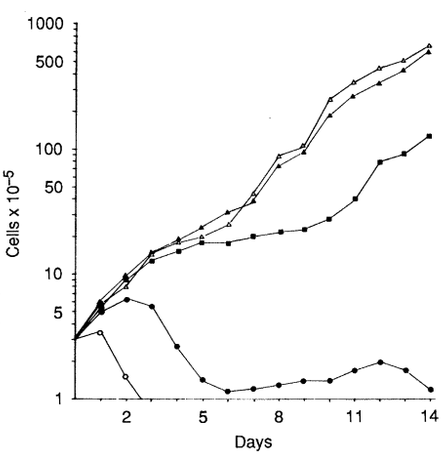

Fig. 2. Growth curve of 32D-EGFR in different growth factors. IDM medium containing 15% FCS (○); 15% (FCS and EGF) (1 ng/ml) (●); 15% FCS and EGF (10 ng/ml) (■); 15% FCS and IL-3 (50 U/ml) (▲); or 15% FCS, IL-3 (50 U/ml), and EGF (10 ng/ml) (△). 32D-EGFR were seeded at 3×10^5 cells per milliliter. Cells were transferred at a 1:10 ratio every 2 days. Cell number was determined daily after testing for cell viability by trypan blue exclusion.

pholipid metabolism in these cells.

Our present studies show that introduction of the EGF receptor gene into 32D cells allows them to proliferate in response to EGF. These results establish that components of the EGF-signaling pathway distal to the receptor and necessary for transmitting intracellular signals must be available in these cells. In other studies, the c-*fms* protooncogene, which is thought to encode the CSF-1 receptor (*24*), has been introduced to NIH 3T3 cells, which lack functional CSF-1 receptors (*25*). In this system, addition of CSF-1 induced the transformed phenotype, implying that the foreign CSF-1 receptor is also able to couple with an intracellular signaling pathway constitutively present in NIH 3T3 cells. Further studies will be required to determine whether the intracellular components of all growth factor pathways are present independent of cell type. If so, the major determinants controlling cell proliferation must be at the level of growth factor receptor expression and ligand availability.

Table 2. Effect of EGF or IL-3 on the differentiation status of 32D-EGFR cells. Cytochemical analysis was performed on 32D-EGFR cells continuously grown in medium containing EGF (10 ng/ml) or IL-3 (50 U/ml) according to published procedures (*18*). Values indicate the percent of positive cells for all markers analyzed except lysozyme, which was analyzed by the lysoplate technique (*26*) and is indicated as micrograms of lysozyme produced per 10^6 cells in 24 hours.

Cytochemical marker	Differentiation of 32D-EGFR cells	
	EGF	IL-3
Myeloperoxidase	54	15
Chloroacetate esterase	35	7
Lactoferrin	12	0
Nonspecific esterase	22	9
Lysozyme	2.3	0.2

Proliferation and maturation of hematopoietic cells is a complex but orderly process involving growth factor–controlled programs that lead to self-renewal or terminal differentiation. After introduction of the EGF receptor into an immature hematopoietic cell, the cells showed evidence of maturation as well as the ability to proliferate in response to EGF. The phenotype of the 32D-EGFR cells grown in the presence of EGF corresponded to that of intermediate myeloid progenitor cells and displayed early markers of either granulocytes or macrophages. The response to EGF was associated with a rapid increase in phosphoinositide metabolism not observed with IL-3, which induced cell growth in the absence of differentiation. By introduction of a variety of receptor genes into a common target cell, it may now be possible to correlate specific alterations induced in second messenger systems with particular differentiation programs.

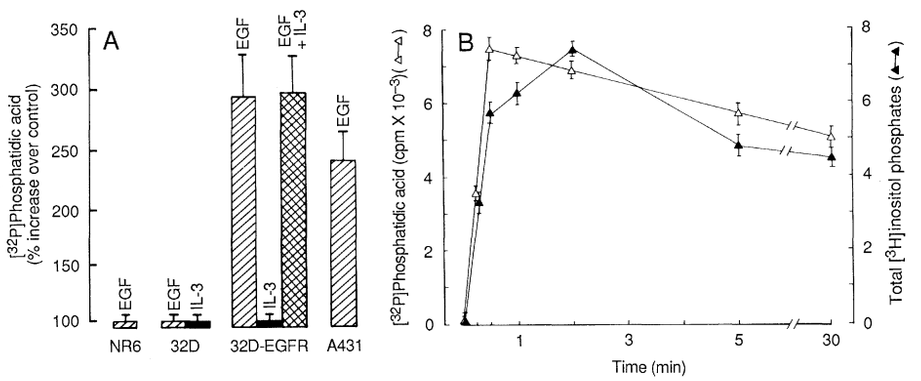

Fig. 3. (**A**) Effect of EGF on phosphatidic acid formation in different cell lines. Monolayers of serum-starved cells in 35-mm dishes, prelabeled with 10 μCi per dish of [^{32}P]orthophosphate (New England Nuclear) as previously described (*28*), were incubated with EGF (100 ng/ml), IL-3 (500 U/ml), or EGF (100 ng/ml) and IL-3 (500 U/ml) for 10 minutes at 37°C. The reaction was stopped and [^{32}P]phosphatidic acid was analyzed as described (*28*). [^{32}P]phosphatidic acid formation was normalized for the amount of radioactivity remaining at the origin of the thin-layer chromatography plate, which corresponds to total phospholipids. Results are expressed as percent increases in comparison with control (unstimulated) values and are the means ± SEM of three experiments, each performed in triplicate. (**B**) Time course of EGF-induced phosphatidic acid and inositol phosphate formation in 32D-EGFR cells. Monolayers of serum-starved 32D-EGFR cells in 35-mm dishes, prelabeled with 10 μCi of *myo*-[2-^{3}H]inositol per dish (10 to 20 Ci/mmol, Amersham International) as described (*29*) or with 10 μCi of [^{32}P]orthophosphate per dish, were incubated with EGF (100 ng/ml) in the presence of 10 m*M* lithium chloride. The reaction was stopped at different time intervals and [^{32}P]phosphatidic acid and [^{3}H]inositol phosphates were analyzed as described (*28, 30*). The formation of phosphatidic acid induced by EGF (△) is expressed as the radioactivity (cpm) (means ± SEM, $n = 3$) after subtraction of basal (unstimulated) values. Basal values of a representative experiment were: 5972 ± 315 cpm (means ± SEM of triplicate samples). The accumulation of [^{3}H]inositol phosphate induced by EGF (▲) is expressed as the radioactivity in "total inositol phosphates" fraction per 10^5 cpm in total inositol phospholipids (means ± SEM; $n = 3$) after subtraction of basal (unstimulated) values. The radioactivity recovered in "total inositol phosphates" fraction in unstimulated samples was: 2120 ± 185 cpm (means ± SEM of triplicate samples).

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Replacements of Pro^{86} in Phage T4 Lysozyme Extend an α-Helix But Do Not Alter Protein Stability

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To investigate the relation between protein stability and the predicted stabilities of individual secondary structural elements, residue Pro^{86} in an α-helix in phage T4 lysozyme was replaced by ten different amino acids. The x-ray crystal structures of seven of the mutant lysozymes were determined at high resolution. In each case, replacement of the proline resulted in the formation of an extended α-helix. This involves a large conformational change in residues 81 to 83 and smaller shifts that extend 20 angstroms across the protein surface. Unexpectedly, all ten amino acid substitutions marginally reduce protein thermostability. This insensitivity of stability to the amino acid at position 86 is not simply explained by statistical and thermodynamic criteria for helical propensity. The observed conformational changes illustrate a general mechanism by which proteins can tolerate mutations.

A MAJOR PROBLEM IN UNDERSTANDING the physical basis of protein stability is to quantitate the contributions made by specific interactions observed in the folded structure (*1, 2*). One aspect of this general problem is to determine how the amino acid sequence establishes the stability of α-helices and other secondary structures (*3–10*). The stabilizing contribution of each secondary structural element might then be understood as a product of its intrinsic stability and its affinity for the remainder of the protein (*5*). To systematically investigate the relation between protein thermostability and the predicted stability of individual secondary structural elements, we replaced a proline in a helix in phage T4 lysozyme with ten other amino acids.

A degenerate oligonucleotide mixture was used to simultaneously create several amino acid substitutions at position 86 (*6, 11*). A mutagenic 22-base primer specifying all four nucleotides (N) in the first two positions and thymine (T) in the third position of the CCG codon for Pro^{86} was used in the two-primer protocol of Zoller and Smith (*12*). This combination of sequences, NNT, encodes 15 different amino acids including proline. By virtue of the mismatch in the third position of codon 86 (T ≠ G), mutants could be distinguished from wild-type (WT) clones by differential hybridization to the ^{32}P-labeled mutagenic oligonucleotide mixture (*12*). Of the 14 possible amino acid substitutions, 10 specified by the primer were identified by DNA sequencing (*13*), namely Ser, Thr, Cys, Ala, Gly, Leu, Ile, His, Arg, and Asp.

The mutant lysozyme genes were subcloned into the expression vector pHSe5 and the mutant proteins were purified to homogeneity by ion exchange chromatography on CM-Sephadex. The protein samples each produced a single band on an SDS-polyacrylamide gel stained with silver (*14, 15*).

The effect of each substitution at Pro^{86} on the thermodynamic stability of the protein was assessed by monitoring the circular dichroism at 223 nm as a function of temperature (*16*). The changes in melting temperature, T_m, are listed in Table 1. The stability of the enzyme is surprisingly insensitive to these mutations. With the exception of Pro^{86} → Asp, which is almost indistinguishable from wild-type T4 lysozyme, all of the mutant proteins are marginally destabilized. Using the relation $g_0 = -T\Delta S$ (*17*), the reductions in stability were estimated to be less than 0.5 kcal/mol at 42°C.

The stabilities of the mutant proteins do not rank according to the helix propensity, P_α (*3*), or the helix-coil stability constant, s (*4*), of the amino acid at position 86 (Table 1). In addition, the observed changes in stability are smaller in magnitude than the reported changes due to mutations in helices of λ repressor (*7*), staphylococcal nuclease (*8*), and in other helices of T4 lysozyme (*6*).

The substitutions at position 86 cause more obvious changes in enzymatic activity (*18*) than in protein stability (Table 1). At *p*H 6.8, the protein containing Arg^{86} has one-fifth the activity of the wild type enzyme and the protein with Asp^{86} is 10% more active than the wild type. These results suggest that the catalytic efficiency is sensitive to the charge of residue 86, despite the fact that this site is ~24 Å from the scissile bond.

Mutant lysozymes were crystallized under conditions similar to those for the wild-type enzyme and equilibrated with *p*H 6.7 mother liquor prior to data collection (*19*). Variants with Gly, Ser, Cys, Leu, Asp, Arg, and His at position 86 were used to obtain x-ray data to 1.7 to 1.9 Å resolution by oscillation photography (*20*) (Table 2). Electron densi-

Table 1. Properties of wild-type (Pro^{86}) and mutant T4 lysozymes. T_m is the temperature at the midpoint of the reversible thermal denaturation transition measured by the method in (*16*). The protein concentration was 30 μg/ml. P_α is the normalized frequency of occurrence in α-helices from (*3*), and s is the helix propagation constant at 20°C from (*4*).

Residue 86	T_m (1°C)			Relative specific activity*	Average *B*-value of residue 86 side chain (Å^2)	P_α	s
	*p*H 2	*p*H 4	*p*H 6				
Pro	42	63	66	1.0	20.8	0.57	0.66
Ala	40			0.5		1.42	1.07
Arg	40	62	63	0.2	49.6	0.98	1.03
Asp	42	63	65	1.1	38.4	1.01	0.68
Cys	41			0.7	35.4	0.70	0.92
Gly	40	62		0.7		0.57	0.59
His	40		62	0.7	67.5	1.00	0.69
Ile	40			0.7		1.08	1.14
Leu	40			0.5	41.2	1.21	1.14
Ser	41			0.7	27.5	0.77	0.76
Thr	41			0.8		0.83	0.82

*Activity was measured as the relative amount of enzyme required to give a cleared area with a radius of 7.0 mm in the lysoplate assay of Becktel and Baase (*18*). Each plate contained 0.65 g of purified *Escherichia coli* peptidoglycan in 5% glycerol, 120 m*M* potassium chloride, 20 m*M* phosphate, *p*H 6.8. The assay was carried out for 24 hours at 5°C.

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