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30. For Northern blot analysis, 10 μg of polyadenylated RNA [resuspended in 20 m*M* 3-[*N*-morpholino] propanesulfonic acid (MOPS), *p*H 7.0), 5 m*M* sodium acetate, 0.5 m*M* EDTA, 6% formaldehyde, and 50% formamide] was subjected to electrophoresis in a 1.0% agarose gel and 40 m*M* MOPS, 10 m*M* sodium acetate, 1 m*M* EDTA, and 6% formaldehyde. RNA was transfered to nitrocellulose (Schleicher & Schuell, Keene, NH) and hybridized with the ^{32}P-labeled, random-primed, 671-bp cDNA fragment.
31. For nuclease protection analysis, the general procedures previously described were used as a guide [J. Favaloro, J. Treisman, R. Kamen, *Methods Enzymol.* **65**, 718 (1980); J. E. Krause, J. D. Cremins, M. S. Carter, E. R. Brown, M. R. MacDonald, *ibid.* **168**, 634 (1989)]. A cDNA fragment encoding nucleotides +637 to +1224 of the SPR cDNA was inserted into the Hinc II site of pBluescript, and a ^{32}P-labeled antisense cRNA was transcribed from the T7 polymerase promoter. A[^{32}P]uridine triphosphate (UTP)–labeled cRNA fragment of 696 bases (200,000 cpm; specific activity, 2.37×10^8 cpm/μg) was produced, which was annealed with 25 μg of total RNA (prepared by the guanidine thiocyanate method [J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979)]) from various tissues for 12 hours in 80% formamide containing 1 m*M* EDTA, 400 m*M* NaCl, and 40 m*M* 1,4-piperazinediethanesulfonic acid (*p*H 6.4). Nonannealed nucleic acids were digested with 300 U of S1 nuclease in a buffer containing 280 m*M* NaCl, 4.5 m*M* $ZnSO_4$, and 30 m*M* sodium acetate (*p*H 4.4). Products were analyzed by electrophoresis in a 6% polyacrylamide gel containing 7*M* urea, followed by autoradiography. The amount of specifically hybridized SPR mRNA (the 588-base species) was quantitated by densitometry with a model 620 Bio-Rad video densitometer (Richmand, CA).
32. Y. Yokota *et al.*, *J. Biol. Chem.* **264**, 17649 (1989).
33. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E. Glu; F, Phe; G, Gly; H, His; I. Ile; K. Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
34. We thank Y. Takeda for preparation of the ^{125}I-labeled Tyr^8-SP and helpful discussions, P. Dykema for genomic library screening, R. Hynes for the rat genomic library, and B. Cullen for the plasmid expression vector pBC12BI. Supported in part by the Division of Biology and Biomedical Sciences, Washington University; NIH grant NS21937; and the Pew Memorial Trust. J.E.K. is a Pew Scholar in the Biomedical Sciences.

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Ligand-Induced Transformation by a Noninternalizing Epidermal Growth Factor Receptor

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Identification of a mutant epidermal growth factor (EGF) receptor that does not undergo downregulation has provided a genetic probe to investigate the role of internalization in ligand-induced mitogenesis. Contact-inhibited cells expressing this internalization-defective receptor exhibited a normal mitogenic response at significantly lower ligand concentrations than did cells expressing wild-type receptors. A transformed phenotype and anchorage-independent growth were observed at ligand concentrations that failed to elicit these responses in cells expressing wild-type receptors. These findings imply that activation of the protein tyrosine kinase activity at the cell membrane is sufficient for the growth-enhancing effects of EGF. Thus, downregulation can serve as an attenuation mechanism, without which transformation ensues.

ACTIVATION OF THE EGF RECEPTOR initiates a cascade of cellular events (*1, 2*). On binding ligand, the intrinsic tyrosine kinase is triggered, and this is immediately followed by a rise in cytosolic-free calcium concentration and receptor internalization, which results in receptor degradation. Specific gene transcription is stimulated within minutes. Hours later, DNA synthesis and cell division occur. Mutational analysis has shown that the tyrosine kinase activity is necessary for all subsequent receptor actions, including internalization (*3*). The relation between these early events and the mitogenic response has been unclear. This has led to suggestions that receptor or ligand internalization, or both, may be required for DNA synthesis to occur (*4*). Cells expressing a membrane-bound EGF receptor ligand, the precursor of transforming growth factor α (pro–TGF-α), were capable of causing some of the immediate responses in neighboring cells (*5*), but effects on DNA synthesis are still unknown.

An EGF receptor that failed to undergo ligand-dependent endocytosis would allow determination of the function of internalization in initiating cell growth. Serial truncations of the COOH-terminus of the EGF receptor identified a region that couples ligand binding to receptor internalization and degradation (*6*). The COOH-terminal 213 amino acids of the 1186–amino acid EGF receptor contain the inhibitory domain (*7*) and the calpain hinge (*8*), as well as the newly defined internalization region (amino acids 973 to 1022). A receptor mutant truncated at amino acid 973 (c'973) failed to downregulate but had a competent kinase, with binding of ligand leading to increased transcription (*6*). Because this mutant was expressed in a transformed cell and at supraphysiologic levels, the effects on cell growth and morphology could not be evaluated.

Expression of the internalization-defective mutant on the surface of a nontransformed cell line that does not express endogenous EGF receptors, the NIH 3T3–derived NR6 cells (*9*), allowed delineation of the link between endocytosis and cell growth. NR6 cell lines that presented either the wild-type (WT) or the truncated (c'973) receptor were derived. Cells were transfect-

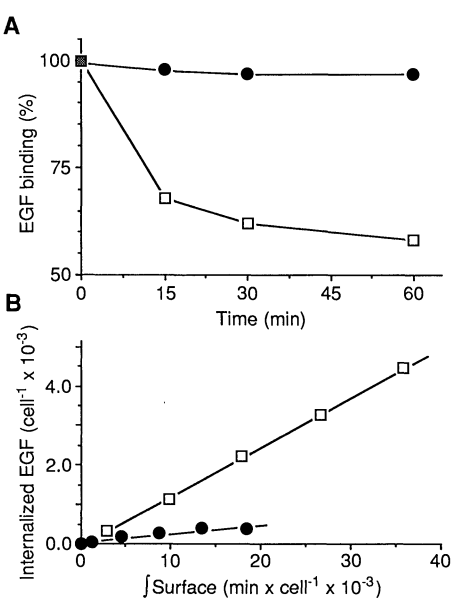

Fig. 1. Ligand-induced downregulation (**A**) and internalization (**B**) of EGF receptors. (A) NR6 cells expressing either the WT (□) or truncated c'973 (●) EGF receptors were treated with 50 n*M* EGF for the indicated times at 37°C. Cell surface ligand was removed by the acid-stripping procedure, and ^{125}I-labeled EGF binding was measured (*21*). Results are the means of triplicates, which differed by <5%. (B) Endocytic rates for NR6 cells expressing the WT (□) or c'973(●) EGF receptors. The plot was derived according to previously published methods (*22*). The internalization incubations were performed at 37°C for 5 min. The failure of the c'973 receptor to internalize was confirmed by immunofluorescent studies (*23*).

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ed with a Moloney murine leukemia virus vector containing the receptor constructs in the *gag* position and the gene encoding neomycin phosphotransferase in the *env* position (*10*). Stable, polyclonal lines consisting of more than 20 colonies each were established in Dulbecco's minimum essential medium (DMEM), containing 10% fetal bovine serum and the neomycin analog G418 (400 μg/ml; Gibco). Scatchard analyses of both receptors (WT and c'973) revealed equivalent dissociation constants (K_d) of ~0.5 n*M*, with both cell lines binding ~100,000 EGF molecules per cell. A second series of polyclonal isolates presented only about 10,000 receptors per cell. In addition, a kinase-negative, internalization-competent receptor that was truncated at amino acid 1022 (c'1022K−) (*7*) and a receptor clone with a frameshift mutation at codon 295 (FS) were expressed in NR6 cells. The cells containing the c'1022K− mutant presented about 10,000 ligand binding sites whereas, as expected, specific ligand binding was not detected with the FS mutant. The FS mutant provided a control for selection procedures that were used to isolate the NR6 cell lines, and the c'1022K− receptor accounts for ligand and receptor internalization in the absence of kinase. These lines constituted a genetic system in which EGF receptor number and properties are physiologic.

The c'973 receptors, expressed in NR6 cells, failed to internalize, whereas the WT receptors underwent downregulation (Fig. 1A). Even at 120 min, cells expressing the truncated receptor presented 98% of the basal level of receptors (*11*). It remained possible, however, that rapid recycling of the truncated receptor was masking ligand-induced internalization. Endocytosis of labeled EGF was therefore evaluated (Fig. 1B). The endocytic rate constant of the WT receptor (K_e = 0.13 min^{-1}) was consistent with that seen for EGF receptors on normal human fibroblasts (*12*). The rate calculated for the c'973 receptor was indistinguishable from background membrane turnover (K_e = 0.02 min^{-1}). Thus, the mutant receptor does not downregulate because it fails to internalize.

Examination of cells presenting either WT or c'973 receptors revealed EGF-dependent increases in cell density, an effect seen at both high and low levels of receptor expression. EGF-dependent growth was not observed with cells expressing either the c'1022K− or FS receptors. If receptor downregulation and degradation abrogate signaling, cells expressing the noninternalizing c'973 receptor would be expected to be more sensitive than WT receptors at low concentrations of ligand. Dose-response

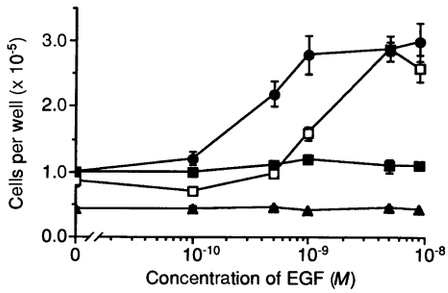

Fig. 2. Ligand-induced mitogenesis. NR6 cells expressing the various EGF receptor constructs were plated in 20-mm-diameter wells. The cells were carried in MEMα, containing 2% fetal bovine serum and various concentrations of EGF. Cells (40,000) were seeded, and after 9 days were counted. Values represent means ± S.D. of triplicate wells (when not shown, error bars are smaller than the symbols). A representative experiment is shown, which was reproduced in two independent trials. NR6 cells expressed (●), the c'973 EGF receptor; (□), the WT receptors; (■), the c'1022K− receptors; and (▲), the FS receptors.

studies revealed that the internalization-defective mutant induced mitogenesis at an EGF concentration approximately one-eighth that required to stimulate growth through the WT receptor (Fig. 2).

Cells expressing very high numbers of EGF receptors (*13*) and those exposed to constant, high concentrations of ligand become transformed (*1, 14*). Therefore, it might be expected that cells in which the mitogenic signal is not attenuated would exhibit the transformed phenotype even at low ligand concentrations. NR6 cells expressing c'973 receptors developed a transformed phenotype when grown in the presence of low concentrations of EGF (Fig. 3). The cells became refractile and overgrew the monolayer. Cells expressing WT receptors did not form the dense foci characteristic of transformed cells; however, at higher EGF concentrations (100 n*M*) they overgrew the monolayer (*11*). The isolates that carried the FS and c'1022K− mutants were indistinguishable in the presence or absence of EGF (*11*). The polyclonal cells did not clone in soft agar in the absence of EGF (in 5% serum), nor in low serum (0.5%) even with EGF present (7 n*M* EGF) (*15*). However, with adequate serum (5%), the receptor internalization–defective cells demonstrated an increased sensitivity to low concentrations of ligand; 70% of the cells gave rise to macroscopic colonies within 21 days at 7 n*M* EGF and 12% at 0.7 n*M*. Cells containing the WT receptor were approximately one-tenth as responsive, with only 6.5 and 0.8% proliferating at the higher and lower ligand concentrations, respectively. The c'1022K− and FS cells failed to grow into colonies under any of the conditions.

Thus, the noninternalizing EGF receptor is capable of eliciting ligand-induced mitogenesis and transformation. As mitogenesis is a late action requiring a certain threshold of EGF-receptor activation (*1, 13*), failure to remove activated receptors would be reflected as a shift to the left in dose-response curves. The cells carrying the internalization-defective receptor differed from cells expressing WT receptors mainly in their increased sensitivity to low concentrations

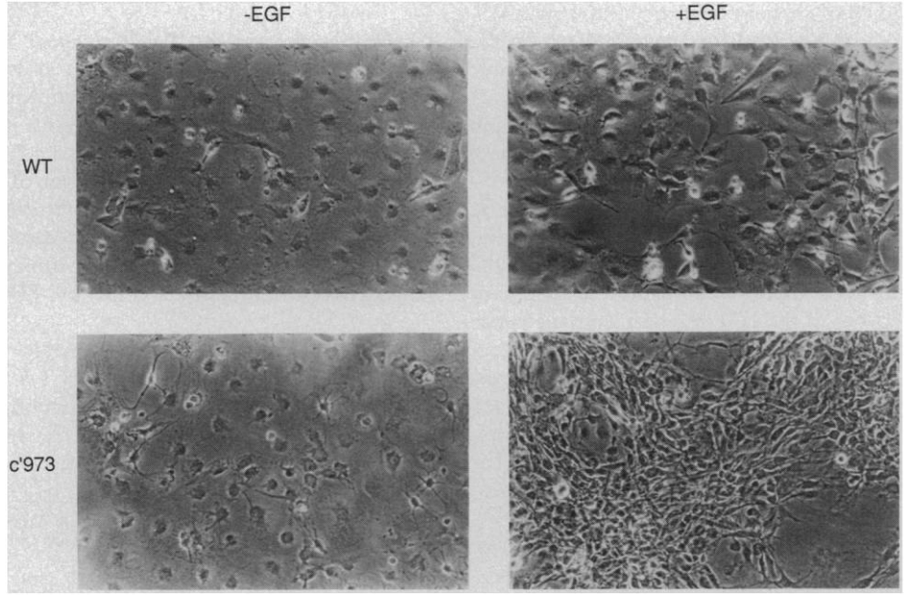

Fig. 3. Morphologic transformation of EGF-treated cells. NR6 cells expressing 100,000 WT or c'973 receptors per cell were grown as described in Fig. 2. Morphologic changes were noted at 1 n*M* EGF for cells expressing the c'973 receptor but required 10 n*M* in the case of cells expressing the WT receptor. The cells shown were grown at 1 n*M* EGF. Optical magnification was ×400.

of ligand, though the receptor dissociation constants were indistinguishable. It seems highly unlikely that the mitogenic response is due to residual receptor internalization and intracellular targeting beneath our level of detection given the increased sensitivity of cells carrying low numbers of this mutant to subsaturating ligand concentrations. Although, theoretically, any receptor mutation could alter the kinase specificity, analysis with antibodies to phosphotyrosine did not reveal significant alterations in substrate phosphorylation with the c'973 receptor (*11*). In addition, previous studies suggest that an altered kinase activity of the c'973 mutant, if any, is not biologically relevant (*6*).

Removal of the entire region COOH-terminal to the kinase domain, a 213–amino acid truncation, resulted in an enzyme not subject to the normal mechanisms of attenuation. Previous reports have suggested that the COOH-terminal sequences of the EGF receptor facilitate the mitogenic effects of receptor activation (*16*). Elimination of the autophosphorylation sites present in the terminal 69 amino acids does not result in enhanced growth. This contrasts with other tyrosine kinases in which removal of these sites results in activation (*17*). The explanation for this difference appears to be that in addition to the autocatalytic substrates, which function as competitive inhibitors (*18*), the EGF receptor tail contains a second element responsible for quenching receptor signaling, an internalization domain (*6*). A receptor lacking both control elements would be expected to maintain ligand-induced signaling. Cells presenting such a receptor should be more sensitive than WT cells at low levels of ligand and exhibit the transformed phenotype. Our results imply that the activation of the tyrosine kinase (*3*) at the plasma membrane is sufficient for cell division. As a consequence the membrane-bound forms of the pro-EGF (*19*) and pro–TGF-α (*20*) should be capable of eliciting cell growth in neighboring receptor-bearing cells. Internalization and degradation of the receptor appear to abrogate the long-term actions of the EGF receptor. In the absence of this attenuation mechanism, low concentrations of ligand would result in uncontrolled proliferation; that is, the transformed phenotype. It may be speculated that mutations disabling the endocytic pathway for growth factor receptors would result in neoplastic transformation.

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15. One thousand NR6 cells presenting the various receptors were suspended in 0.35% low–melting point agarose (FMC Corp., Rockland, ME) in DMEM containing different concentrations of fetal bovine serum and EGF. Every 5 days the cells were overlayed with 0.7% agarose in medium of similar composition. Macroscopic colonies were noted after 13 days; these increased in size, but not number, until 21 days, at which point the experiment was terminated. Each condition was assessed in two independent plates, with the ranges being <10% of the values. Cloning efficiencies of <0.2%, the spontaneous background of the NR6 cells, were considered negative.
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22. Cells were plated on fibronectin-coated plates and grown in minimum essential medium α (MEMα) plus 10% dialyzed fetal bovine serum. After 48 hours the medium was replaced by MEMα plus bovine serum albumin (1 mg/ml), and the cells were incubated overnight. The specific internalization rates of the EGF receptors were determined as described [H. S. Wiley, *J. Cell Biol.* **107**, 801 (1988)]. The ^{125}I-labeled EGF concentration was 0.5 n*M* at a specific activity of 250,000 cpm/ng. Nonspecific binding was determined at all time points in the presence of 1 μ*M* unlabeled EGF and was <2% of total binding.
23. Internalization of the WT and truncated receptors was investigated by immunofluorescence. Cells were treated with 100 n*M* EGF for 30 min at 37°C and then fixed. The receptors were visualized by a monoclonal antibody to the EGF receptor [J. R. Glenney *et al.*, *Cell* **52**, 675 (1988)]. Cells expressing the truncated c'973 receptors were identical before and after the EGF treatment, whereas those expressing the WT receptors displayed a relocation of the receptors to the intracellular compartment.

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Requirement for Cotolerogenic Gene Products in the Clonal Deletion of I-E Reactive T Cells

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T cells that express the T cell receptor V_β5.2 domain react with the class II major histocompatibility complex (MHC) molecule I-E, and V_β5.2$^+$ T cells are deleted in mouse strains that express I-E glycoproteins. By examination of genetically defined recombinant inbred (RI) mouse strains, it was found that the deletion was dependent on the expression of I-E and one of a limited number of non-MHC gene products (cotolerogens). The gene encoding one of these cotolerogens maps to chromosome 12 and is linked to the endogenous provirus Mtv-9. These observations suggest that the I-E–mediated and minor lymphocyte-stimulating antigen (Mls)–mediated deletions of αβ T cells from the repertoire are similar; both require the expression of a class II MHC glycoprotein and a second non-MHC gene product.

THE MATURATION OF T LYMPHOcytes in the thymus involves two selective steps, the positive selection of self-MHC–restricted T cells and the negative selection (deletion) of self-reactive T cells (*1*). Several murine T cell receptor (TCR) V_β gene segments (V_β5.1, V_β5.2, V_β11, and V_β17) encode reactivity with the class II MHC antigen I-E, and mice expressing I-E delete the majority of these T cells from the peripheral repertoire (*2–6*). However, it is not known whether the expression of additional non-MHC genes is required for the deletion of I-E–reactive T cells. We have previously described the variation in V_β11 expression among the B6 × DBA/2 (B × D) RI strains (*4*). Although the genetic background has a role in determining the

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