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Aberrant Expression and Regulation of Hepatic Epidermal Growth Factor Receptor in a c-*myc* Transgenic Mouse Model

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Abstract In an attempt to elucidate the mechanism by which c-*myc* and transforming growth factor-α (TGF-α) cooperate in hepatocyte tumor development, we have analyzed signaling by the epidermal growth factor (EGF) receptor and the consequent regulation of receptor number in transgenic mice bearing the c-*myc* transgene under the control of the albumin enhancer/promoter. ^{125}I-EGF binding and Scatchard analysis indicated a single class of high affinity receptors with the total number of binding sites of $1.2 \times 10^4 \pm 600$ and $2.5 \times 10^5 \pm 1000$ sites/cell in the normal and c-*myc* hepatocytes in primary culture, respectively. After 72 h of EGF exposure in culture, the number of detectable EGF receptors on the cell surface of the c-*myc* hepatocytes was not reduced, whereas the number of EGF receptors on normal hepatocytes was reduced to 32% that of untreated hepatocytes. Nuclear run-on experiments done with nuclei isolated from intact livers demonstrated that transcription of the EGF receptor was 4.9-fold higher in c-*myc* mice. Increased levels of the transcriptional factor SP1 in the c-*myc* hepatocytes in vivo and in primary culture, suggest a mechanism for the increased transcription of the EGF receptor. c-*myc* also increases the expression of TGF-α; a consequent increase in tyrosine phosphorylation is also detected in vivo. Thus, the increased number of EGF receptors in c-*myc* expressing hepatocytes, even after prolonged exposure to EGF, or TGF-α in vivo, may allow greater triggering of the EGF receptor signaling cascade. J. Cell. Biochem. 64:651–660. © 1997 Wiley-Liss, Inc.†

Key words: TGF-α; mitogenic signal; tyrosine kinase activity; SP1; transcription

The transgenic mouse system provides a unique model for assessment of the in vivo effects of individual genes acting either alone or in combination with other genes within the organism. Previous work in our laboratory [Murakami et al., 1993] has demonstrated that liver tumor development in c-*myc* transgenic mice results following selective expression of c-*myc* in the liver directed by the albumin enhancer/promoter [Sandgren et al., 1989]. The tumor incidence in these c-*myc* transgenic mice is 50–60% at 16 months [Murakami et al., 1993; Santoni-Rugiu et al., 1996]. When the c-*myc* mouse is bred with a mouse expressing TGF-α as a transgene under the mouse metallothionein promoter [Jhappan et al., 1990], the resultant c-*myc*/TGF-α double transgenic mouse has a 100% incidence of tumors within 11 months [Murakami et al., 1993]. These results demonstrate a synergistic effect of c-*myc* and TGF-α in neoplastic development and provide a model for analysis of their cooperation in vivo.

The deregulation of c-*myc* and the amplification and overexpression of EGF receptors are often observed in both experimental and human tumors, as well as transformed cell lines [Chandar et al., 1989; Yaswen et al., 1985; Gu et al., 1986; Moroy et al., 1991; Nagy et al., 1988; Ro et al., 1988; Hunts et al., 1985; DiFiore et al., 1987]. The EGF receptor is a transmembrane glycoprotein whose carboxyl cytosolic portion is responsible for EGF mediated tyrosine kinase activity. The EGF receptor kinase domain is homologous to the viral oncogene erbB and is considered the cellular equivalent or c-erbB [DiFiore et al., 1987]. The level of EGF receptor gene expression varies in normal and transformed cell types [Xu et al., 1984]. In malignant cells, the EGF receptor gene has been observed

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to undergo both gene amplification with rearrangement as well as increased expression without gene amplification [King et al., 1985]. While overexpression of EGF receptor alone is not sufficient to transform cells in vitro, the EGF receptor can function as an oncogene as shown by the ability of normal fibroblasts expressing high levels of EGF receptor to grow in soft agar and to develop tumors in nude mice in an EGF-dependent manner [DiFiore et al., 1987; Yamamoto et al., 1983; Velu et al., 1987]. Like the constitutively active v-erbB, this finding suggests that constant signaling through the EGF receptor has transforming capabilities.

In nontransformed cells, c-*myc* expression is tightly linked to mitogenic stimuli and is a prerequisite for cell growth [Marcu et al., 1992]. Mitogenic stimulation of quiescent cells rapidly induces c-*myc* expression from nearly undetectable levels to a transient peak, followed by a return to a baseline level of expression [Waters et al., 1991]. In proliferating cells, c-*myc* is continuously expressed at various levels throughout the cell cycle [Hann et al., 1985]. Following induction of differentiation in many cell types or withdrawal of serum from untransformed fibroblasts [Rabbitts et al., 1985], c-*myc* expression is rapidly downregulated. Since growth arrest and withdrawal into the G0 resting state accompany these processes, it has been suggested that c-*myc* downregulation may be required for growth arrest and withdrawal from the cell cycle. Moreover, activation of c-*myc* in resting cells is sufficient to induce entry into the cell cycle in the absence of other mitogenic stimuli [Eilers et al., 1991]. It has been shown that deregulated expression of an exogenous c-*myc* gene in primary or established rodent fibroblasts renders them unable to exit from the cell cycle upon serum withdrawal. These cells continue to cycle and concomitantly undergo cell death [Evan et al., 1992].

To investigate the molecular basis for the cooperation of the effects of constitutive overexpression of c-*myc* and mitogenic signaling of TGF-α in the c-*myc*/TGF-α double transgenic mouse, a series of experiments were initiated examining the regulation of EGF receptor levels as well as EGF-mediated protein phosphorylation in primary hepatocytes isolated from c-*myc* transgenic mice. In this study, we demonstrate that the EGF receptor is present as a single class of high-affinity receptor in normal and c-*myc* primary hepatocytes, both with similar dissociation constants but, is increased 20-fold in number in the hepatocytes containing and expressing the c-*myc* transgene. Regulatory mechanisms governing the number of EGF receptors at the cell surface and their kinase activity in the presence of EGF were also affected by the overexpression of the c-*myc* transgene, allowing for an increased number of EGF receptors and increased tyrosine phosphorylation after prolonged exposure to its ligand EGF. However, following EGF treatment, we found no significant differences in the phosphotyrosine containing protein patterns between the normal and c-*myc* hepatocytes, which suggests that while overexpression of c-*myc* affects the number of EGF receptors, c-*myc* has negligible effects on the substrates by which activated EGF receptor tyrosine kinases propagate mitogenic signals. This further suggests that in the c-*myc*/TGF-α double transgenic mouse, the c-*myc* transgene cooperates with TGF-α by increasing and maintaining a high number of EGF receptors allowing for constant and elevated mitogenic signaling.

MATERIALS AND METHODS

Transgenic Mice

The generation of the ALB c-*myc* transgenic mice (line 166.8) was previously described [Murakami et al., 1993]. All normal and c-*myc* transgenic mice used in these studies were males aged 2–3 months.

Cell Culture and Stimulation

Hepatocytes were obtained by collagenase perfusion technique [Seglen, 1979]. Cells were plated at various densities onto collagen type I-coated tissue culture plates in Dulbecco's Modified Eagles's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 10^{-6} M dexamethasone, 2.0 mM glutamine, 0.1% ITS^{+} (Collaborative Research, Bedford, MA) and gentamycin (50 μg/ml). Cells were allowed to attach for 4 h, after which unadherent cells were removed and serum-free DMEM was added to the cultures. After 24 h, cells were stimulated with EGF (300 ng/ml) for 15 min at 37°C, unless otherwise stated. Cells were then lysed with a buffer containing 1% Triton X-100, 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 5 mM EGTA, 10 mM sodium orthovanadate, 4 mM phenylmethylsulfonylfluoride (PMSF), 20 mM sodium pyrophosphate and aprotinin (100 μg/ml), and analyzed by gel electrophoresis as described below.

SDS–PAGE and Western Blotting

Whole cells were solubilized in sample buffer [2% (w/v) SDS, 2% (w/v) 2-mercaptoethanol, 10% glycerol and bromophenol blue in 0.25 M Tris–HCl pH 6.8] and heated to 100°C for 5 min. Protein concentrations were determined with BioRad Protein Assay Kit, using bovine serum albumin (BSA) as standard. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [Lammelli, 1970] in 7.5% or 10% acrylamide gels and then transferred to nitrocellulose membranes (NOVEX) by electroblotting. Immunoblotting was performed after incubating the membrane in 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 for 1 h. Phosphotyrosine containing proteins were detected using antiphosphotyrosine monoclonal antibody (mAb) (4G10) (Upstate Biotechnology), and the EGF receptor was detected using sheep antihuman EGFR (Upstate Biotechnology, Lake Placid, NY) at a concentration of 2 µg/ml. Each primary antibody was followed by incubation with mouse IgG–horseradish peroxidase (HRP)-linked whole antibody (1 in 10,000 for 1 h) and ECL detection reagents (Amersham International, Arlington Heights, IL).

Radioiodination of EGF and ^{125}I-EGF-Binding Assay

EGF was radioiodinated by the chloramine-T method [Sambrook et al., 1989]. Cells isolated from five separate mice were plated at 20,000 cells/well (approximately 80% confluent) on 24-well plates. Samples from each mouse were analyzed in triplicate or quadruplicate for each concentration of EGF on the binding curve. Culture media was changed daily with either serum-free media or media containing 300 ng/ml of EGF. The used media was collected each day, and the amount of EGF left was quantitated by enzyme-linked immunosorbent assay (ELISA) (Oncogene Science, Cambridge, MA). Before the binding assay was performed the cells were washed once with binding buffer (Hanks' solution containing 20 mM HEPES pH 7.0) and equilibrated in the same buffer for 30 min at 4°C. Ice-cold binding buffer containing increasing concentrations of ^{125}I-EGF (0 to 1,000 pM ^{125}I-EGF) was added and the preparation was incubated at 4°C for 1 h. Tenfold excess of unlabeled EGF was used to determine nonspecific binding. Cultures were washed three times with ice-cold binding buffer, and the bound and unbound ^{125}I-EGF was measured in an LKB Rackgamma II γ-counter.

Nuclear Run-on Assay

Nuclei were isolated by cell lysis in 10 mM Tris, pH 7.5; 10 mM $MgCl_2$; 10 mM dithiothreitol (DTT); and 0.25% NP-40. Tissue was homogenized by douncing with a type B pestle (10 strokes), until cytoplasmic fraction was removed from the nuclei, as examined by phase-contrast microscopy and trypan blue staining. Nuclei were recovered by centrifugation at 500*g* for 5 min at 4°C. The nuclei were resuspended in 1 ml of 20 mM Tris, pH 8; 40% glycerol; 5 mM $MgCl_2$; 100 mM KCl; 2.5 mM DTT; and 0.1 mM EDTA and then centrifuged. The pellet was resuspended in the glycerol buffer and stored at −70°C until analyzed. Transcription reactions and isolation of the nascent RNAs were performed as previously described [Srivastava et al., 1993]. After purification, equal numbers of counts of newly transcribed RNA were hybridized to slot blots of 5 µg each of heat-denatured plasmid on Hybond N (Amersham International, Arlington Heights, IL) for 24 h at 60°C. The filters were washed two times in 2× SSC/0.1% SDS for 30 min at room temperature, and one time in 0.1× SSC/0.1% SDS at 60°C. The signals were visualized and quantified with a Molecular Dynamics phosphoimager. Slots containing 5 µg of the plasmid pBluescript were included as a negative control in each experiment and did not show any signal after hybridization and washes.

Northern Blot Analysis

Poly(A)$^+$ RNA was isolated and selected using oligo(dt) cellulose, as previously described [Bradley et al., 1988], fractionated under denaturing conditions by electrophoresis through 1.2% agarose–formaldehyde gel, transferred to nitrocellulose and hybridized with the 1.9-kb mouse c-*myc*/β-globin transgene construct [Murakami et al., 1993], or with a 0.3-kb fragment representing the rat TGF-α.

RESULTS

Expression of EGF Receptor

To examine the expression of the EGF receptor in the liver, total protein from the normal and c-*myc* transgenic mice was subjected to Western blot analysis with anti-EGF receptor antibody. Probing whole cell lysates from nor-

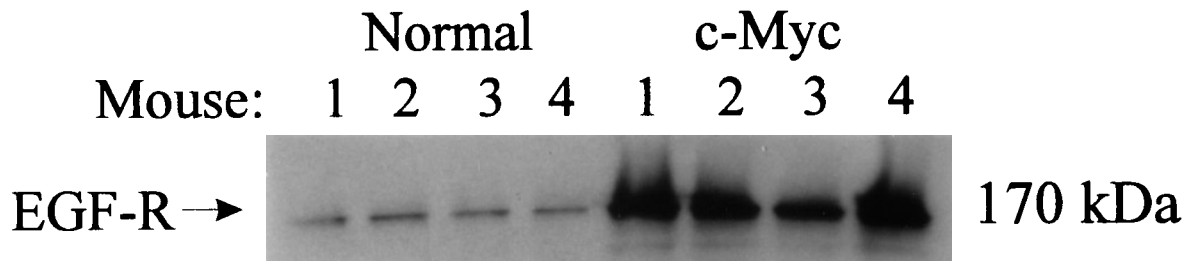

Fig. 1. Western blot analysis of the EGF receptor in normal and c-*myc* transgenic mice. Whole cell lysates (40 µg) from four different normal (normal 1–4) and four different c-*myc* transgenic (c-*myc* 1–4) mice were analyzed with the anti-EGF receptor antibody and detected by ECL.

mal and c-*myc* transgenic mice, the Western blots with anti-EGF receptor antibody showed that the c-*myc* mouse liver consistently expressed an increased level of the 170-kDa endogenous EGF receptor (Fig. 1).

To quantitate the number of EGF receptors in the normal and c-*myc* transgenic mice, we measured specific binding of ^{125}I-EGF to isolated primary hepatocytes. Saturation curves indicated an increased number of EGF receptors on the c-*myc* hepatocytes and Scatchard analysis resulted in a linear plot, indicating a single class of high-affinity binding sites (Table I). Analysis of the binding indicated that c-*myc* and normal primary hepatocytes express an EGF receptor with similar Kd values, in the range of 140–160 pM. The total number of binding sites were determined to be $1.2 \times 10^4 \pm 600$ and $2.5 \times 10^5 \pm 1{,}000$ sites/cell in normal and c-*myc* hepatocytes, respectively.

EGF-Dependent Tyrosine Phosphorylation

To examine if the increased number of EGF receptors in the c-*myc* hepatocytes affected signal transduction, efforts were directed toward optimization of the conditions for triggering and detecting autophosphorylation of the EGF receptor and other cellular phosphotyrosine substrates in c-*myc* and normal primary hepatocytes. Western blot analysis with antiphosphotyrosine antibody indicated that treatment of hepatocytes with EGF at a concentration of 30 ng/ml or 300 ng/ml for 30 min at 37°C, both induced high levels of tyrosine phosphorylation of the EGF receptor in both the normal and c-*myc* primary hepatocytes (Fig. 2). The 1D-PAGE pattern of phosphotyrosine-containing proteins observed after stimulation with EGF was very similar for both normal and c-*myc* primary hepatocytes at both concentrations. The only significant and consistent difference detectable in the phosphotyrosine patterns between the normal and c-*myc* primary hepatocytes was an increase in phosphorylated forms of the EGF receptor and of other phosphotyrosine proteins in the c-*myc* primary hepatocytes.

TABLE I. Summary of [^{125}I]EGF-Binding Assay and Scatchard Analysis*

	No. of receptors	Kd (pM)	N
Normal	$1.2 \times 10^4 \pm 600$	146 ± 23	5
c-*myc*	$2.5 \times 10^5 \pm 1{,}000$	166 ± 26	5

*Cells were plated at 20,000 cells/well in a 24-well plate. Samples for each mouse were done in triplicate or quadruplicate, and saturation curves were done with concentrations ranging from 0 to 1,000 pM of ^{125}I-EGF. Competition with a 10-fold excess of unlabeled EGF was done to calculate the amount of nonspecific binding.

Changes of EGF Number Receptor in the Presence of EGF

Prolonged exposure of normal primary hepatocytes to EGF downregulates the responsive-

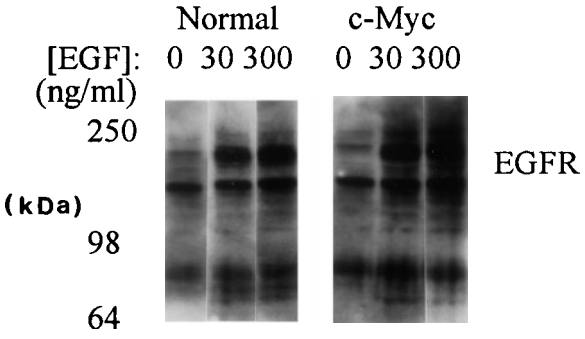

Fig. 2. Tyrosine phosphorylation of cellular proteins in isolated normal and c-*myc* transgenic primary hepatocytes: EGF dose response. Serum-starved cells were either mock-treated or treated with different concentrations of EGF for 30 min at 37°C before lysis. Whole cell lysates (40 µg) were separated by 7.5% SDS–PAGE, electroblotted to nitrocellulose, and analyzed with antiphosphotyrosine immunoblotting.

ness of the cell to EGF by decreasing the number of receptors at the cell surface. To characterize the regulation of the EGF receptor in c-*myc* primary hepatocytes, we examined changes in ^{125}I-binding after prolonged exposure to EGF. Isolated hepatocytes from normal and c-*myc* transgenic mice were treated for 24 and 72 h with 300 ng/ml EGF. As shown in Figure 3, Scatchard analysis indicated a decrease in EGF receptor number of 30% and 50% at 24 h in the normal and c-*myc* primary hepatocytes, respectively. At 72 h of EGF treatment, the number of EGF receptors in the c-*myc* hepatocytes were restored to 100% of control. In contrast, the normal hepatocytes decreased to 32% of control.

To demonstrate that the c-*myc* transgene was expressed throughout the 3-day time course, Northern blot analysis was done. Cells from three mice used in the EGF-binding assay were also plated on 150-mm culture dishes for isolation of RNA. The cells were harvested on each of the days on which the binding assay was done and mRNA was prepared. Figure 4 shows that the 2.3-kb endogenous and the 1.9-kb transgene transcripts for c-*myc* were expressed throughout the time course of the experiment in all three mice.

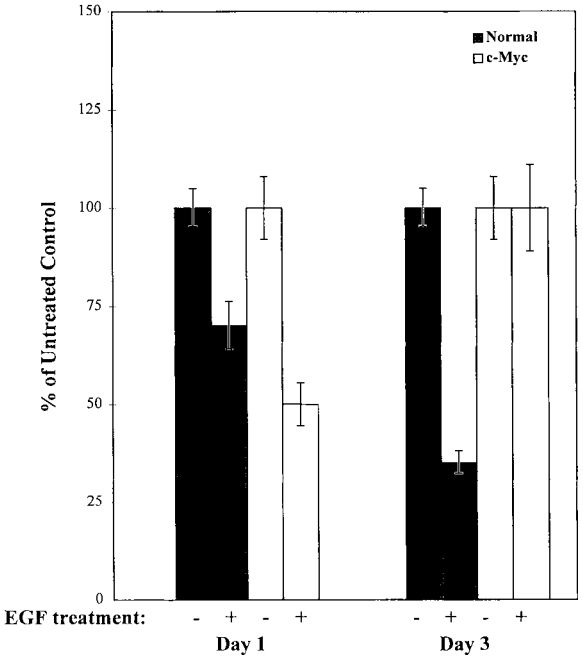

Fig. 3. Prolonged exposure to EGF: effect on receptor number. Isolated hepatocytes from normal and c-*myc* transgenic mice were either mock-treated or treated with EGF, 300 ng/ml, for 1 or 3 days. The binding of ^{125}I-EGF and Scatchard analysis was used to determine receptor number. Each bar represents a percentage of untreated control (mock-treated hepatocytes).

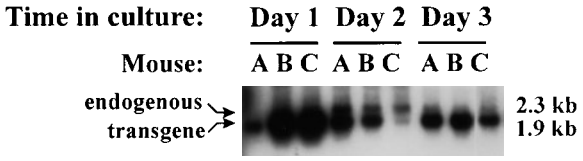

Fig. 4. Northern blot analysis of c-*myc* hepatocytes for the presence of c-*myc* transcripts. Poly(A)$^+$ RNA (5 µg/lane) was electrophoresed in a 1.2% formaldehyde gel and hybridized with ^{32}P-labeled c-*myc* fragment, as described under Experimental Procedures. The following time points represent hepatocytes from three different mice (A, B, and C) cultured in serum-free media for the indicated number of days.

Nuclear Run-on Analysis of EGF-Receptor Transcript

Northern blot analysis indicated a 2.5-fold increase of mRNA for the EGF receptor in the liver and primary hepatocytes from the c-*myc* mouse (data not shown). One mechanism for the increased and a maintained high number of EGF receptors in the c-*myc* hepatocytes can be an increase in the transcription of the EGF receptor. To investigate the role of transcription on the increased number of EGF receptors, a nuclear run-on assay was performed on nuclei isolated from the liver of normal and c-*myc* mice. Results from this assay showed that transcription rates for the EGF receptor were consistently higher in the c-*myc* mouse. The signal for the EGF receptor when compared directly was 4.9 times higher in the c-*myc* mouse (Fig. 5). However, it is evident that the transcription rate of S14 is also increased in the c-*myc* mouse. Therefore, when the signal is normalized to S14, the increase was 2.3 times higher in the c-*myc* mouse. This quantitation is consistent with Northern blot results, and suggests that an increase in the transcriptional rate for the EGF receptor in the c-*myc* mouse contributes to the increase in number of the EGF receptors.

TGF-α and Phosphotyrosine Protein Expression in Normal and c-*myc*-Expressing Liver

To examine the expression of TGF-α, a ligand for the EGF receptor, poly(A)$^+$ RNAs from the normal and c-*myc* transgenic mice were subjected to Northern blot analysis (Fig. 6). The endogenous TGF-α mRNA transcript was expressed in increased amounts in the c-*myc* transgenic mouse. To assess whether the in-

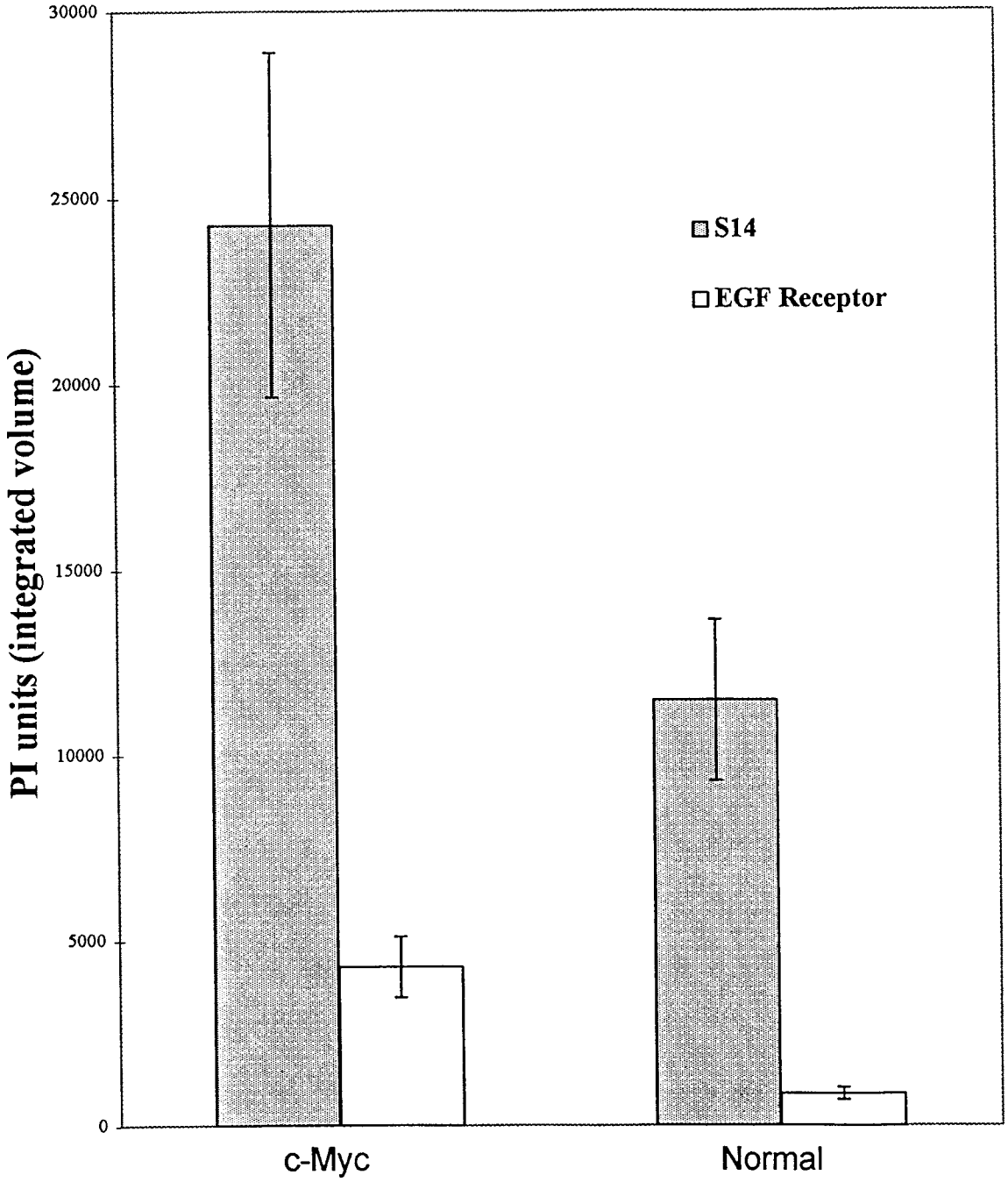

Fig. 5. Nuclear run-on analysis for EGF receptor transcript. Equal number of nuclei were incubated for run-on transcription as described under Materials and Methods. RNA was isolated and equal number of counts were used to hybridize against the filter-bound plasmids containing inserts for S14 and EGF receptor. The graph shows the average values of two separate experiments done in duplicate as determined by a phosphoimager.

creased TGF-α expression was associated with higher levels of tyrosine phosphorylation in vivo, whole cell lysates were prepared from these livers and Western blot analysis was performed with an antiphophotyrosine antibody. This analysis showed an increased expression of several phosphotyrosine-containing proteins, including the 170-kDa EGF receptor in c-*myc* hepatocytes as compared to normal hepatocytes (Fig. 7).

DISCUSSION

In the current study, we have used the c-*myc* transgenic mouse model to analyze the effect of overexpression of c-*myc* on signaling through the EGF receptor. We have demonstrated that

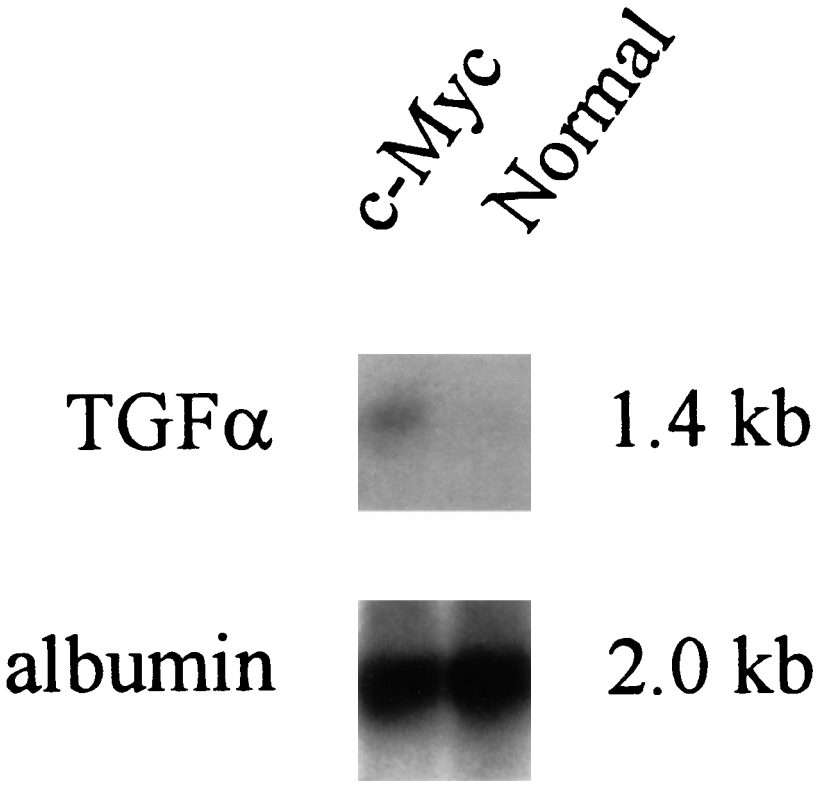

Fig. 6. Northern blot analysis of normal and c-*myc* liver for the presence of TGF-α transcripts. Poly(A)$^+$ RNA (5 μg/lane) was electrophoresed in a 1.2% formaldehyde gel and hybridized with ^{32}P-labeled TGF-α fragment, as described under Experimental Procedures. *Lane 1,* RNA isolated from c-*myc* transgenic hepatocytes; *lane 2,* RNA isolated from normal hepatocytes. The blot was stripped and reprobed with albumin to demonstrate equal loading.

expression of the c-*myc* transgene in the mouse liver modulates the expression and regulation of EGF receptor. The increased number of EGF receptors in hepatocytes from the c-*myc* mouse increases the amount of tyrosine phosphorylated EGF receptor as well as other proteins, after stimulation with EGF. In vivo, the increased expression of TGF-α and the elevated number of EGF receptors leads to constant mitogenic signaling through the EGF receptor as demonstrated by the increase in phosphotyrosine detected on the EGF receptor and several other proteins.

Analyzing the steady-state level of surface EGF receptor, using primary hepatocytes isolated from normal and c-*myc* transgenic mice, has demonstrated that the constitutive overexpression of the c-*myc* transgene (Fig. 4) can maintain a higher level of EGF receptors in culture (Table I). This increased number of EGF receptors in culture was consistent with our observation in vivo of Western blot analysis of whole liver extracts with anti-EGF receptor (Fig. 1). As can be seen in Figure 1, there was variability in the amount of EGF receptor from mouse to mouse in the c-*myc* mouse line, which was also reflected in the greater standard error in our Scatchard analysis. However, the amount of EGF receptor was consistently higher in the c-*myc* mice in all experiments. Recent evidence has indicated that increased EGF receptor number alone is not sufficient to produce cellular transformation but can produce growth in soft agar and in nude mice in an EGF-dependent manner [DiFiore et al., 1987; Velu et al., 1987; Naldini et al., 1991]. The increased expression of TGF-α in the c-*myc* mouse suggests that the endogenous ligand for the EGF receptor is present in elevated levels in vivo. Western blot analysis with antiphosphotyrosine antibody of whole cell lysates from the intact liver of c-*myc* transgenic mice further suggests that the combination of the increased number of EGF receptors and its ligand, TGF-α, also affects the state of tyrosine phosphorylation in vivo (Fig. 6). The tremendous acceleration in hepatocarcinogenesis in the c-*myc*/TGF-α double transgenic mice [Murakami et al., 1993] could then be explained by the very high expression of the TGF-α transgene constantly triggering a mitogenic signal

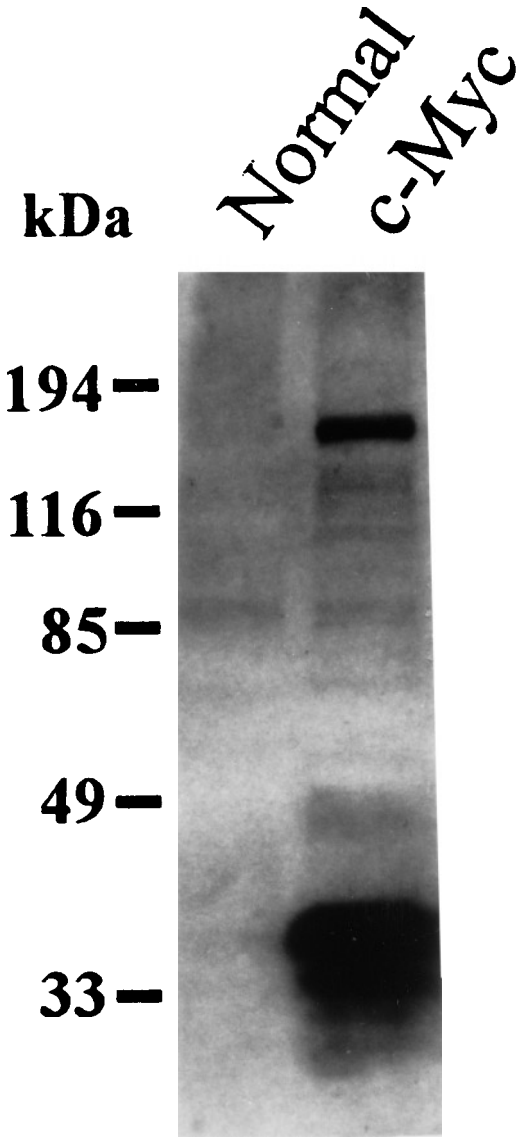

Fig. 7. Western blot analysis of liver whole cell extract with antiphosphotyrosine antibody. Whole cell lysates (40 μg) were separated by 10% SDS–PAGE, electroblotted to nitrocellulose and analyzed with antiphosphotyrosine immunoblotting.

through the EGF receptor. The increase in tyrosine phosphorylation of the EGF receptor and other proteins (Fig. 7) and the higher mitotic index [Santoni-Rugiu et al., 1996] in the livers of the c-*myc*/TGF-α mice supports this hypothesis.

Western blot analysis and ^{125}I-EGF-binding assays has been done in our laboratory to quantitate the number of EGF receptors in the TGF-α transgenic and c-*myc*/TGF-α double transgenic mice. Results from these experiments demonstrate that the amount of EGF receptor in the TGF-α transgenic mouse is much less than that of the normal wild-type control, while the number of receptors in the c-*myc*/TGF-α double transgenic is the same as wild-type control (Woitach and Conner, unpublished observation). This suggests that overexpression of TGF-α alone downregulates the amount of EGF receptor, while in the c-*myc*/TGF-α, overexpression of c-*myc* counteracts the downregulating effect of TGF-α.

Although there is a 20-fold increase in the total number of high-affinity binding sites in the c-*myc* hepatocytes, the dissociation constant (Kd) of the EGF receptor for EGF, as calculated by Scatchard analysis, was similar for both normal and c-*myc* primary hepatocytes. This suggests that the higher level of EGF receptor in the c-*myc* mouse does not affect the intrinsic binding property of EGF to the EGF receptor. However, dimerization of two EGF receptors and subsequent autophosphorylation is needed for complete activation of the tyrosine kinase domain [Heldin, 1995; Lemmon and Schlessinger, 1994]. The higher number of EGF receptors in the c-*myc* hepatocytes may facilitate the activation of the EGF receptor without significantly altering the affinity of the receptor for the ligand.

Subsequent to EGF binding and autophosphorylation of the receptor, a variety of cellular peptides are also phosphorylated by the EGF receptor kinase domain [Ullrich and Schlessinger, 1990]. This latter event activates several signaling pathways ultimately triggering an intracellular signaling cascade that propagates the mitogenic signal to the nucleus [Claesson-Welsh, 1994; Cohen et al., 1995; Naldini et al., 1991; Pawson and Schlessinger, 1993; White et al., 1988]. Comparison of phosphotyrosine containing proteins by Western blot analysis of primary hepatocytes from both the normal and c-*myc* mice show differences in the amount of phosphotyrosine incorporated into the EGF receptor and other proteins, both in the absence or in response to EGF, but no difference in the pattern of proteins that were phosphorylated. This suggests that the high number of EGF receptors in the c-*myc* transgenic mouse does not markedly affect the specificity of the receptor's tyrosine kinase for the proteins involved in the signal transduction pathway; however, it does alter quantitatively the amount of phosphotyrosine incorporated into the receptors and other phosphotyrosine-containing proteins.

The importance and consequence of this quantitative difference is still unknown but, except for the EGF receptor, the increase in phosphoty-

rosine content is not due to an increase in the proteins that are phosphorylated. Both normal and c-*myc* primary hepatocytes contain similar amounts of PLCγ and MAP kinases as analyzed by Western blot analysis of whole cell lysates (data not shown); however, the corresponding band detected by antiphosphotyrosine antibodies is more intense in samples from the c-*myc* mouse. Therefore, increased phosphorylation of these proteins in the c-*myc* primary hepatocytes triggered with EGF, demonstrates that a greater number of these proteins become phosphorylated. Since the state of phosphorylation of these proteins often correlates with an increase of kinase activity for these proteins, this suggests that the increased number of EGF receptors present on the hepatocytes of the c-*myc* mouse can initiate a larger and prolonged mitogenic signal than realized by hepatocytes from a normal mouse in response to the same concentration of ligand.

Precise regulation of the EGF receptor number is an important feature of its expression. With regard to EGF receptor protein levels and ligand binding, our studies have shown that prolonged exposure to EGF (72 h) only transiently reduced the number of receptors in the c-*myc* primary hepatocytes while as expected, it decreased the number of EGF receptors in hepatocytes from the normal mouse (Fig. 3). The number of EGF receptors decreased in both normal and c-*myc* hepatocytes at about the same rate after 24 h of EGF treatment, reflecting that the internalization of the receptor as the first level of downregulation of receptor number is similar. One possible mechanism for the return to the high number of EGF receptors in hepatocytes from the c-*myc* mouse could be due to an increase in receptor stabilization either by increased receptor recycling or decreased receptor degradation. Preliminary results from pulse-chase experiments in our laboratory indicate that the rate of EGF receptor synthesis is increased in the c-*myc* mouse; however, the rate of turnover is similar for hepatocytes from both normal and c-*myc* mice in the presence of EGF.

A possible mechanism for the increase in the number of EGF receptors found in the c-*myc* mouse is an increase in the rate of transcription of the EGF receptor gene. Using nuclear run-on assays of nuclei from normal and c-*myc* mice, we found that expression of c-*myc* increased the rate of transcription of the EGF receptor gene (Fig. 5). Since transcription of our control, S14, was also increased in the assay, normalization to S14 indicated that the rate of transcription of EGF receptor was 2.3-fold greater in the c-*myc* mouse. This increased level of transcription is consistent with the 2.5-fold increases of mRNA for the EGF receptor seen by Northern blot analysis of both c-*myc* mouse whole liver and primary hepatocytes in culture.

It has been demonstrated in Balb/3T3 cells that c-*myc* could restore EGF binding activity in cells that lost activity due to expression of activated H-*ras* [Okimoto et al., 1996]. Analysis of the human EGF promoter region did not identify any consensus sequences for c-*myc*/max binding sites [Johnson et al., 1988] but did identify several SP1 binding sites needed for maximal expression of the EGF promoter [Kageyama et al., 1988]. Gel-shift analysis of nuclear extracts from the c-*myc* mouse demonstrated higher amounts of SP1 binding in both whole liver and primary hepatocytes in culture (data not shown). While it has been demonstrated that other factors also bind in the promoter region of the human EGF receptor, an increase in SP1 activity in the c-*myc* mice suggests one possible mechanism for the steady-state increase of transcription and consequent increases of mRNA and protein for the EGF receptor.

Taken together these results suggest one mechanism leading to the transformation of cells overexpressing c-*myc*, namely, that c-*myc* causes the aberrant expression and regulation of the EGF receptor. In the liver of the c-*myc* mouse, hepatocytes are exposed to continuous signaling through the EGF receptor by elevated levels of endogenous TGF-α. Together the aberrant regulation of the EGF receptor and the elevated level of its ligand, TGF-α, lead to a constant and a higher constitutive level of signaling through the EGF receptor. The constant mitogenic signal of the EGF receptor causes an increase in hepatocyte proliferation and may increase the risk of the development of genetic alterations in normal cells or preferentially expand spontaneously initiated cells. These results further support the model of cooperation between c-*myc* and signaling through the EGF receptor as seen in the c-*myc*/TGF-α double transgenic mouse, in which c-*myc* allows for the aberrant expression and regulation of the EGF receptor, resulting in increased sensitivity to the mitogenic effect of the TGF-α transgene.

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