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A novel effect of EGF on mRNA stability

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ABSTRACT

Expression of the epidermal growth factor (EGF) receptor gene is stimulated by EGF and the phorbol ester, 4β-phorbol 12-myristate 13-acetate (PMA). PMA elevates EGF receptor mRNA levels in human KB epidermoid carcinoma cells, but does not significantly affect the half-life of this mRNA when its decay is examined after the addition of actinomycin D. In contrast, EGF greatly prolongs the half-life of EGF receptor mRNA suggesting a possible mechanism for the stimulatory effect of EGF on EGF receptor mRNA levels. EGF also stabilizes β-tubulin and β-actin mRNAs but has very little effect on the degradation of total mRNA.

INTRODUCTION

Epidermal growth factor (EGF) induces a wide variety of cellular responses which include an increase in tyrosine kinase activity, stimulation of DNA synthesis and cell division (1) and stimulation of the transcription of a number of genes. These include c-fos (2,3), c-myc (2,3), prolactin (4) and actin (5). Previous results demonstrated that EGF stimulated the expression of the EGF receptor gene and suggested increased EGF receptor mRNA levels were most likely due to a posttranscriptional effect (6). Regulation of the expression of various oncogenes has been shown to occur at both the posttranscriptional and the transcriptional level (7-12). In this study we used a ribonuclease protection assay to measure the effect of EGF and PMA on the half-life of EGF receptor mRNA as well as actin and tubulin mRNAs. This was done by pretreating cells with PMA and EGF for 3-4 hours and then adding actinomycin D for a further 6 hours. The data shows that pretreatment with EGF stabilizes EGF receptor mRNA as well as β-tubulin and β-actin mRNAs but not total mRNA. This novel effect of EGF on mRNA stability may explain how EGF increases cell growth.

MATERIALS AND METHODS

Stability of RNA in the presence of actinomycin D

KB cells were plated at a density of $6x10^5$ cells per 100-mm dish and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. After 48 hrs, actinomycin D (Boehringer Mannheim) was added (final concentration, 5 µg/ml) and the cells were harvested at various times up to six hours. This was the longest time studied after actinomycin D treatment because cells began to detach from the dish after this time. Three or four hours prior to adding actinomycin D, 100 nM PMA (Sigma) or 10 nM EGF were added. Cells were homogenized in 5M guanidine isothiocyanate and RNA was prepared by centrifugation through a cesium chloride cushion (13). Total RNA was hybridized to riboprobes overnight and digested with RNase A and RNase T_1 (14). Samples of ribonuclease-treated RNA were fractionated by electrophoresis in a 6% polyacrylamide/8M urea gel and subjected to autoradiography. The intensity of the protected band was measured by a densitometer. For each RNA probe conditions were established to be sure that the intensity of the signal was directly proportional to the amount of RNA added.

RNA Analysis

A 479 base-pair (bp) XbaI-PstI DNA fragment from the 5' portion of EGF receptor cDNA, which includes the 1st exon and a part of the 2nd exon (16), was subcloned into a pGEM vector (Promega Biotec) and anti-sense RNA was transcribed from the SP6 promoter in the presence of [α-^{32}P]GTP (3000 ci/mmol, Amersham) *in vitro* (plasmid pGEMER7Δ5). Likewise, a 260 bp DNA fragment of a human ß-tubulin cDNA (pD-ß1, ref. 17), the gift of Dr. N. Cowan, which spans the 2nd, 3rd and part of the 4th exon, and a 420 bp BamHI-EcoRI DNA fragment of the 3' untranslated region of human ß-actin cDNA (pHFßA-1, ref. 18), a gift from Dr. L. Kedes, were subcloned into a pGEM vector and transcribed from the SP6 promoter. In each experiment, three concentrations of total RNA from the time zero sample (10, 5, or 0.2 µgs) were hybridized to the riboprobes of the EGF receptor, ß-tubulin or ß-actin. Usually, RNA from each time point was resuspended in the same volume as the time zero sample to facilitate comparison of the RNA levels after actinomycin D addition with the starting value. Hybridization and RNase digestion were performed according to supplier's recommendations. RNA blot hybridization was performed as previously described (6) using a nick-translated human ß-actin cDNA probe (18).

To examine the effect of EGF or PMA on the stability of the total mRNA

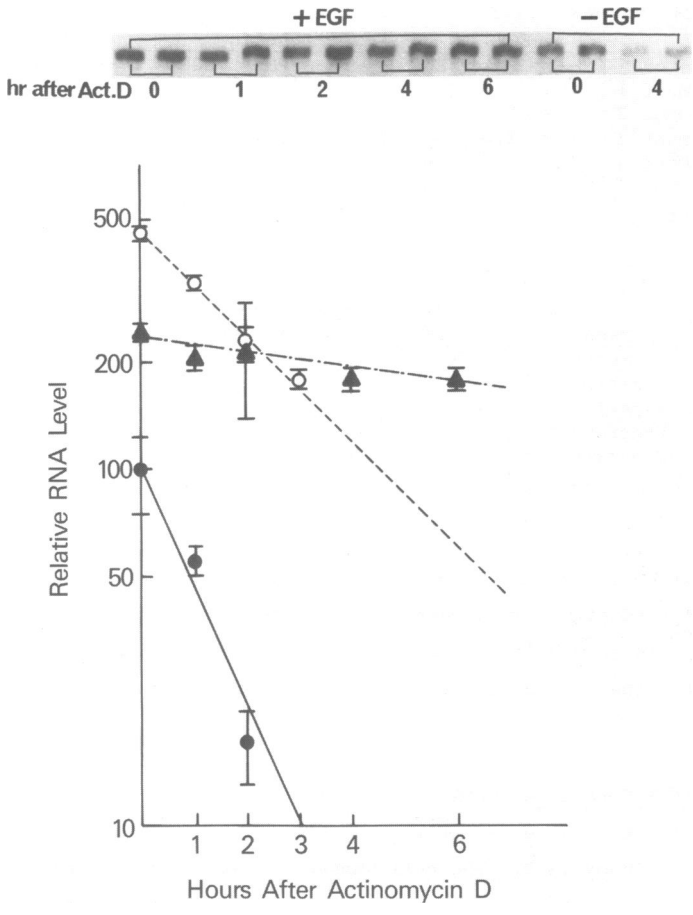

Figure 1. EGF receptor mRNA decay after actinomycin D addition in the presence or absence of EGF or PMA. KB cells were plated 48 hours before actinomycin D addition (5 µg/ml). Three or four hours prior to actinomycin D addition, PMA (100 nM) or EGF (10 nM) was added. Cells were harvested in 5 M guanidine isothiocynate at the indicated times after actinomycin D and RNA was prepared by centrifugation through a cesium chloride cushion. Total RNA was hybridized to riboprobe transcribed from EGF receptor cDNA, digested with RNase A and RNase T_1, fractionated by electrophoresis in a 6% polyacrylamide/ 8 M urea gel, and subjected to autoradiography. Relative RNA levels in the presence of EGF (▲-•-▲), presence of PMA (o---o), or with nothing added (●—●) were plotted after desitometric scanning of protected bands. Upper panel shows autoradiograph of protected RNA fragments after RNase digestion following total RNA-riboprobe hybridization.

population, decay of total poly(A)+ RNA was measured by an S1 nuclease assay according to Tracy and Kohne (15) with minor modifications. In this experiment, 2 µg of total RNA from the time-zero sample were hybridized to

TABLE 1A: Half-lives of EGFR, β-tubulin and β-actin mRNA (hr).

mRNA	EGFR			β-tubulin			β-actin		
Stimulator	--	PMA	EGF	--	PMA	EGF	--	PMA	EGF
Experiment 1	1.3								
Experiment 2	0.9	1.2		1.2	1.0		3.1	2.9	
Experiment 3	1.9	2.0			2.1		1.4	0.8	
Experiment 4	1.9		>6	1.8		>6	1.2		>6
Experiment 5	2.2		>6				5.6		>6
Average	1.6	1.6	>6	1.5	1.5	>6	2.8	1.9	>6

TABLE 1B: Effect of PMA and EGF on EGFR, β-tubulin, and β-actin mRNA levels (fold increase)

mRNA	EGFR		β-tubulin		β-actin	
Stimulator	PMA	EGF	PMA	EGF	PMA	EGF
Experiment 2	4.2		1.7		2.6	
Experiment 3	4.6		1.9		1.2	
Experiment 4		2.0		1.0		1.3
Experiment 5		2.5				1.1
Average	4.4	2.3	1.8	1.0	1.9	1.2

approximately 0.8 pmole of a P-32 end-labeled synthetic oligo(dT) 65-mer. Under these conditions, mRNA measurements were linear. After S1 nuclease digestion, acid-insoluble radioactivity was counted. All results were corrected for the self-protectd oligo(dT) 65-mer background radioactivity.

RESULTS

Half-life of EGF receptor mRNA

KB cells were harvested at varying times up to 6 hrs. after the addition of 5 μg/ml actinomycin D. The cell number did not change significantly during the six hour time period but the amount of RNA recovered gradually decreased indicating that the half-life of total RNA was 8 to 11 hours. Pretreatment of the cells with EGF or PMA had no effect on the amount of total RNA recovered after actinomycin D. To measure the amount of EGF receptor mRNA present at various times after actinomycin D addition, a ribonuclease protection assay was used. As shown in Figure 1 and Table 1, the half-life of EGF receptor mRNA was short, ranging from 0.9-2.2 hr.

To assess the effect of EGF or PMA on EGF receptor RNA levels and half-life, cells were treated with these substances for 3 or 4 hours prior to the addition of actinomycin D. In the experiment shown in Figure 1, EGF increased EGF receptor mRNA levels about 2.5-fold and increased the stability of this mRNA so that its half-life was greater than 6 hrs. A similar result was obtained in two other experiments (Table I). The effect of PMA was

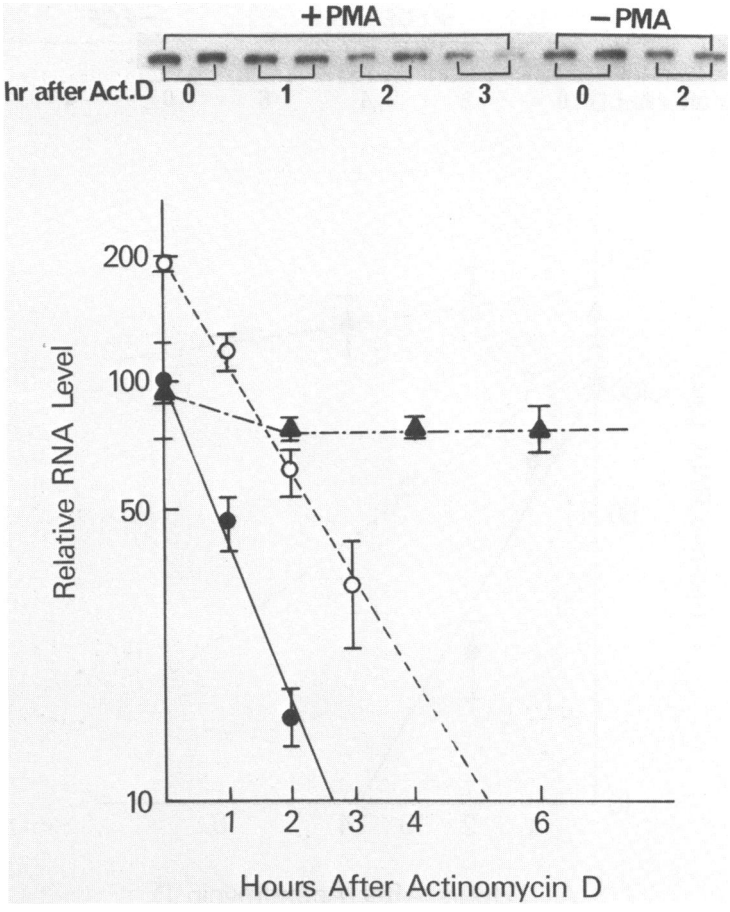

Figure 2. β-Tubulin mRNA decay after actinomycin D in the presence or absence of EGF or PMA. Explanation is in the legend of Figure 1. A riboprobe from β-tubulin cDNA was used in this RNase protection experiment. Symbols are: presence of EGF (▲-•-▲), presence of PMA (o---o), or nothing added (•——•).

different. It increased EGF receptor mRNA levels about 5-fold, but had only a minor effect on EGF receptor mRNA half-life (Figure 1 and Table I).

Half-lives of β-tubulin and β-actin mRNAs

To investigate whether this effect of EGF on RNA stability was EGF receptor mRNA specific, or more universal, we examined two other cellular mRNAs, those for β-tubulin and β-actin. First we measured the effect of PMA and EGF on mRNA levels for these proteins. PMA increased β-tubulin and

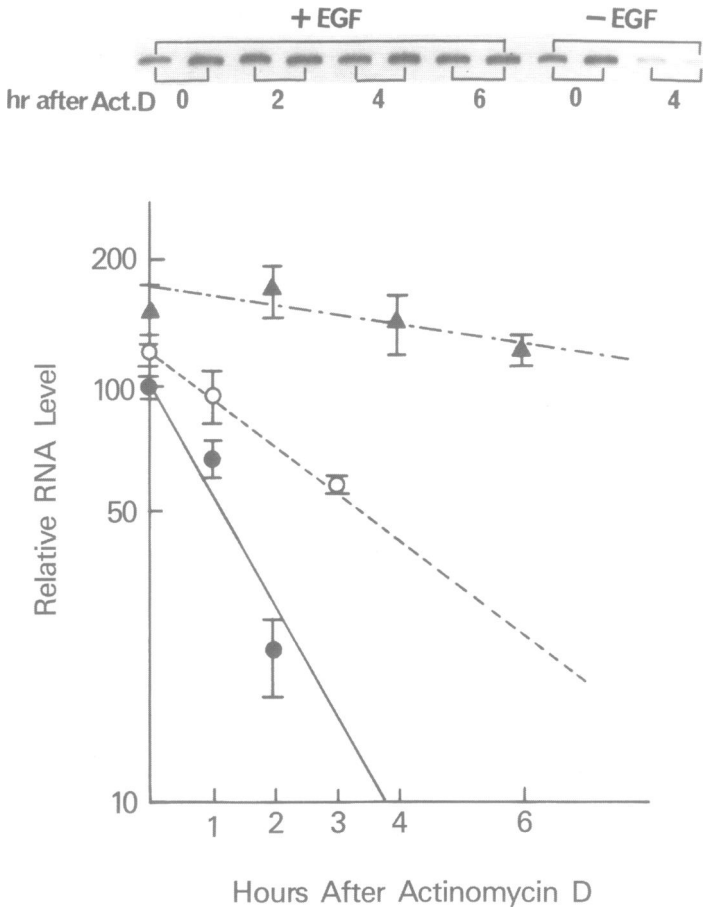

Figure 3. β-Actin mRNA decay after actinomycin D in the presence or absence of EGF or PMA. Explanation is in the legend of Figure 1. A riboprobe from β-actin cDNA was used in this RNase protection experiment. Symbols are: presence of EGF (▲-•-▲), presence of PMA (o---o), or nothing added (●—●).

β-actin mRNA levels about 2-fold whereas EGF had no effect on the steady-state levels of these mRNAs.

In untreated KB cells, both β-tubulin mRNA (Figure 2) and β-actin mRNA (Figure 3) had half-lives that were in the range of the EGF receptor. The half-life of β-tubulin mRNA averaged 1.5 hours and that of β-actin mRNA 2.8 hours (Table I). EGF pretreatment increased the half lives of both mRNAs to greater than 6 hours, whereas pretreatment of the cells with PMA had little to no effect on the half lives of these mRNAs (Figure 2 and Figure 3). Thus,

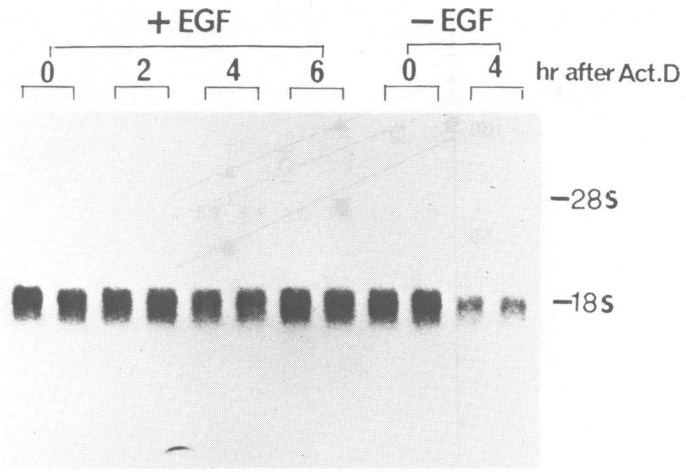

Figure 4. Northern blot analysis of β-actin mRNA decay after actinomycin D in the presence or absence of EGF. 2 µg of total RNA were fractionated in a 1% agarose gel in MOPS buffer, transferred to nitrocellulose filter and probed with nick-translated β-actin cDNA.

the effect of EGF on mRNA stability was evident with three different mRNAs.

These RNA half-lives were all measured by the very sensitive RNase protection assay. This assay was chosen because EGF receptor mRNA is not abundant in KB cells. However, to ensure that no RNase protection artifact was responsible for the observed stability results, we used Northern RNA blot analysis to measure the half-life of the relatively prevalant β-actin mRNA. β-actin mRNAs from actinomycin D-treated KB cells were electrophoretically fractionated on agarose, transferred to nitrocellulose, and visualized by hybridization to the β-actin cDNA probe. Figure 4 confirms by Northern blot analysis that EGF extends the half-life of the β-actin mRNA to greater than 6 hours. This finding confirms the RNase protection results and supports our conclusion that EGF increases the half-life of some mRNAs.

To test whether EGF increases the average half-life of the mRNA population, the poly(A) content of total RNA from treated and untreated cells was measured by S1 nuclease digestion of hybrids of poly(A)+ RNA and a synthetic ^{32}P-labeled oligo(dT) 65-mer. Figure 5 shows that untreated, EGF-treated, and PMA-treated cells contained poly(A)+ RNA with approximately the same half-life (4.4 to 5.2 hrs). This result indicates that EGF extends the half-life of a specific subset of mRNA, and not the entire mRNA population.

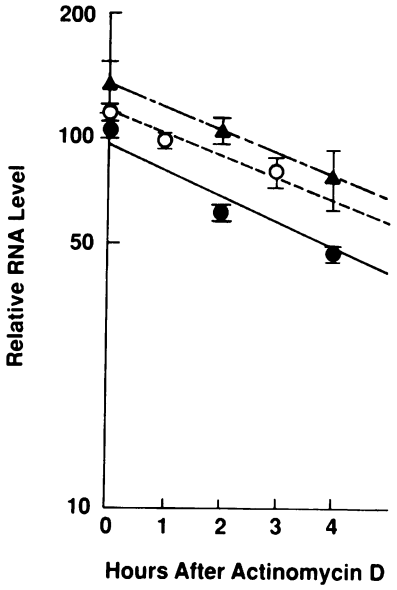

Figure 5. Decay of total poly(A)+ RNA after actinomycin D in the presence or absence of EGF or PMA. S1 nuclease-protected probe was precipitated in TCA and assayed for radioactivity. Data points are expressed relative to the radioactivity of the control (-EGF, -PMA) at time zero (100). Symbols are: presence of EGF (▲-•-▲), presence of PMA (o---o), or nothing added (●—●).

DISCUSSION

We have investigated the effect of EGF on mRNA stability and found that pretreatment of human KB cells with EGF greatly increased the stability of the mRNA for the EGF receptor, as well as those for β-actin and β-tubulin. Little effect on the degradation of total RNA was observed. Furthermore, treatment of cells with PMA had very little effect on the stability of these mRNAs.

We and others have previously found that EGF increased the levels of EGF mRNA (6,19,20) but did not appear to increase transcription of the EGF receptor gene (6). The current results suggest that EGF may be acting to increase EGF receptor mRNA levels by stabilizing EGF receptor mRNA. However, EGF also stabilizes the mRNAs for β-tubulin and β-actin without significantly increasing the steady-state levels of these mRNAs. The explanation for this is not known but it is possible that EGF simultaneously inhibits transcription of these genes but not the EGF receptor gene (5).

The mechanisms by which EGF affects mRNA stability has not yet been

investigated. Recently, Paek and Axel (21) found that glucocorticoid stabilized growth hormone mRNA by increasing polyadenylation of this mRNA; however, a change in polyadenylation is not always concomitant with a change in mRNA stability (22). Furthermore, 3'-specific sequences that affect mRNA stability have been identified (12). It is therefore possible that EGF exerts its effect by altering the 3' end of the EGF receptor RNA. However, the alteration could not be large, for EGF receptor mRNA sizes are not visably changed due to EGF treatment of KB cells, as judged by Northern RNA blot analysis (6). Furthermore, the ratio of the two major EGF receptor RNA species (10 kb and 5.6 kb) remains the same after EGF addition (6), indicating that one species of RNA is not stabilized by EGF treatment relative to the other.

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