Epidermal growth factor increases the level of the cyclin-dependent kinase (CDK) inhibitor p21/CIP1 (CDK-interacting protein 1) in A431 cells by increasing the half-lives of the p21/CIP1 transcript and the p21/CIP1 protein

Lene E. JOHANNESSEN, Sigrun L. KNARDAL and Inger Helene MADSHUS¹

Institute of Pathology, The National Hospital, The University of Oslo, 0027 Oslo, Norway

DNA synthesis was inhibited in A431 cells by epidermal growth factor (EGF) in a p21/CIP1-dependent manner [where CIP1 is cyclin-dependent kinase (CDK)-interacting protein 1]. When 1 or 10 nM EGF was added, the level of p21/CIP1 was increased to the same extent, and the protein level peaked after approx. 5 h of incubation. The increase in p21/CIP1 mRNA upon addition of EGF was rapid, and was enhanced in the presence of cycloheximide. The half-life of p21/CIP1 mRNA in EGF-treated A431 cells was increased approx. 2-fold; this is in contrast with the case in MCF-7 cells with normal p53, in which the half-life of p21/CIP1 mRNA was not increased upon addition of EGF. This increased stability accounts for most of the increase in mRNA levels observed in A431 cells during short incubation

periods. Additionally, upon prolonged incubation of A431 cells with EGF, the half-life of the protein was also increased compared with that in untreated cells and in cells treated with EGF for short time periods. Nuclear run-on assays demonstrated only marginal stimulation of transcription by 10 or 1 nM EGF, or by 10 ng/ml tumour necrosis factor α . Our results indicate that the most important mechanisms by which EGF increases p21/CIP1 protein levels in A431 cells are post-transcriptional and post-translational stabilization.

Key words: epidermal growth factor receptor, growth inhibition, mRNA stability, protein stability.

INTRODUCTION

A431 cells are growth-inhibited upon the addition of high concentrations of epidermal growth factor (EGF), in contrast with the situation in most other cells, where DNA synthesis is stimulated by EGF [1]. This inhibition of DNA synthesis has been demonstrated to be associated with a marked decrease in cyclin-dependent kinase 2 (CDK2) activity and persistent G1 arrest [2]. It has further been demonstrated that EGF induces mRNA and protein expression of the CDK inhibitor p21/CIP1 (CDK-interacting protein 1) in EGF-sensitive A431 cells [2,3], and that this p21/CIP1 is physically associated with both CDK2 and proliferating cell nuclear antigen [2]. An increase in the amount of p21/CIP1 relative to the amount of cyclin-bound CDKs has been demonstrated to result in the conversion of active CDK complexes into inactive ones [4–6].

The p21/CIP1 gene is a target of p53; however, the p53 protein appears to be non-functional in A431 cells due to a point mutation in codon 273 [7]. It has been suggested that p21/CIP1 can be induced by two distinct mechanisms: one pathway is p53-dependent and activated by DNA damage, while the other is p53-independent and can be activated by mitogens and cytokines [8,9]. p21/CIP1 was shown to be induced in a p53-independent manner by tumour necrosis factor α (TNF α) in human myeloid leukaemic cells; this induction occurred in the absence of new protein synthesis, being due mainly to the post-transcriptional stabilization of p21/CIP1 mRNA [9]. Furthermore, p21/CIP1 was shown to be induced by stabilization of mRNA in a p53-

independent manner upon exposure of cells to oxidative stress [10]. However, in colorectal carcinoma cells, p21/CIP1 was induced upon exposure to UV light mainly by stabilization of mRNA in a p53-dependent manner [11].

EGF and interferon- γ both inhibit the growth of A431 cells, and both induce tyrosine phosphorylation and activation of STAT (signal transducers and activators of transcription) proteins in A431 cells [12–15]. The promoter of the p21/CIP1 gene has three STAT-responsive elements [16,17], and the p21/CIP1 gene has been demonstrated to be a target gene of activated STAT1 and STAT3 [17]. It was furthermore suggested that p21/CIP1 is an immediate-early gene that is regulated directly by activated STAT1 [17], although this was not directly demonstrated.

Recently, high-intensity Raf signalling was shown to cause cell-cycle arrest mediated by p21/CIP1 in mouse fibroblasts [18] and in mouse embryo fibroblasts [19]. The induction of p21/CIP1 in mouse fibroblasts was reported to depend on protein synthesis, and therefore was not an immediate-early response [18]. Induction of p21/CIP1 by Raf in mouse embryo fibroblasts was demonstrated to occur in a p53-independent fashion [19]. Thus, originally described as a gene induced in response to p53 and upon cell senescence, p21/CIP1 has subsequently been shown to be subject to regulation by a variety of effectors in a number of different ways [2,17,20–26].

Given the complexity of the regulation of p21/CIP1 levels, we have carefully studied the mechanisms whereby the level of p21/CIP1 is increased upon addition of nanomolar concen-

Abbreviations used: CDK, cyclin-dependent kinase; CIP, CDK-interacting protein; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; STAT, signal transducers and activators of transcription; TNF α , tumour necrosis factor α .

¹ To whom correspondence should be addressed (e-mail i.h.madshus@labmed.uio.no).

trations of EGF to A431 cells. We found that the level of p21/CIP1 mRNA increased in the absence of protein synthesis, consistent with an immediate-early response. Furthermore, the stability of p21/CIP1 mRNA was increased approx. 2-fold in EGF-treated A431 cells. Upon long exposure times (5 h), the stability of the p21 protein was increased, whereas no increase in the half-life of the protein could be detected upon exposure of the cells to EGF for 2 h. The transcription rate of the p21/CIP1 gene was increased only slightly (approx. 1.2-fold) upon addition of 1 nM or 10 nM EGF.

EXPERIMENTAL

Materials

Human recombinant EGF was from Bachem Feinchemikalien AG (Budendorf, Switzerland). [³H]Thymidine (25 Ci/mmol), [α -³²P]UTP (800 Ci/mmol) and [α -³²P]dCTP (3000 Ci/mmol) were from Amersham International. All chemicals were from Sigma, unless otherwise stated.

Cell culture

A431 cells were from American Type Culture Collection (Rockville, MD, U.S.A.), and MCF-7 cells were provided by Dr. O. Fodstad (Radiumhospitalet, Oslo, Norway). Cells were grown in Costar 3275 flasks (Costar, Badhoevedorp, The Netherlands). The medium used was Dulbecco's modified Eagle's medium (DMEM) (3.7 g/l sodium bicarbonate) (BioWittaker, Walkersville, MD, U.S.A.) containing 10% (v/v) fetal bovine serum (FBS) (BioWittaker), 2 mM L-glutamine (BioWittaker), and 0.5 × penicillin/streptomycin/Fungizone mixture (17-745; BioWittaker). Cells were seeded at a density of 2.5×10^4 cells/cm² 2 days prior to the addition of EGF or TNF α .

Measurement of DNA synthesis

EGF was added to cells in 24-well microtitre plates. After 20 h, 1 μ Ci/ml [³H]thymidine was added, and the cells were incubated for an additional 4 h. The medium was removed, and 1 ml of 5% (w/v) trichloroacetic acid was added per well for 2 × 10 min at room temperature. The precipitated DNA was then solubilized with 0.3 ml of 0.1 M KOH, and 1.5 ml of Ultima Gold scintillation fluid (Packard Instrument B.V., Groningen, The Netherlands) was added before liquid scintillation counting.

Western blot analysis

EGF or TNF α was added to cells in 12-well microtitre plates. After incubation as indicated in the legends to the Figures, the cell lysates were subjected to Western blot analysis as described in [3]. Cells were lysed in lysis buffer on ice for 10 min (see below for buffer compositions), incubated at 95 °C for 10 min and centrifuged at 20000 g for 15 min, and then the supernatant fraction was subjected to SDS/PAGE [27] and the proteins were electrotransferred to nitrocellulose membranes (Micron Separations Inc., Westborough, MA, U.S.A.) [28]. The membranes were incubated with primary antibodies overnight at 4 °C before incubation with horseradish-peroxidase-conjugated secondary antibody for 2 h at room temperature. Immunobinding was detected by the ECL[®] method (Amersham), following the manufacturer's instructions. For quantification of band intensity, proteins were electrotransferred to PVDF membranes (Hybond-P; Amersham) after SDS/PAGE. The membrane was incubated with primary antibody as described above, followed by incubation with alkaline-phosphatase-conjugated secondary anti-

body for 2 h at room temperature. Immunobinding was detected by the enhanced chemifluorescence (ECF[®]) method (Amersham), and chemifluorescence was measured in a phosphorimager (STORM68; Molecular Dynamics).

Antibodies

Antibodies used were rabbit anti-p21 (diluted 1:3000) (sc-397; Santa Cruz Biotechnology Inc., CA, U.S.A.), rabbit anti-pSTAT3 (diluted 1:1000) (9131; New England Biolabs Inc., MA, U.S.A.), rabbit anti-pSTAT1 (diluted 1:2000) (9171; New England Biolabs Inc.), alkaline-phosphatase-conjugated anti-(rabbit IgG) (diluted 1:5000) (111-055-003; Jackson ImmunoResearch) and horseradish-peroxidase-conjugated anti-(rabbit IgG) (diluted 1:5000).

DNA probes

p21/CIP1 cDNA (2.1 kb) was excised with *Not*I from the pC-WAF1-S plasmid containing the p21/CIP1 cDNA in the pCEB vector (kindly donated by Dr. B. Vogelstein, Johns Hopkins University, Baltimore, MD, U.S.A.). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (1.1 kb) was excised with *Eco*R1 from the pGAPDH plasmid, and β -actin (1.7 kb) was excised with *Pst*I from the p β -actin plasmid. These plasmids and the pLDH plasmid, containing lactate dehydrogenase (LDH) cDNA, were obtained from LIIPAT, Institute of Pathology, The National Hospital, Oslo, Norway. DNA fragments were purified by gel electrophoresis in low-melting-point agarose (Gibco BRL, Paisley, Scotland, U.K.), excised from the gel and labelled with [α -³²P]dCTP by the random priming method, using a Ready-to-go kit (27-9240-01; Pharmacia LKB Biotechnology, Uppsala, Sweden) according to manufacturer's instructions.

Isolation of RNA and Northern blotting

EGF or TNF α was added to cells in 10 cm-diam. Petri dishes. After incubation as indicated in the legends to the Figures, the cells were washed in ice-cold PBS and then scraped loose in 5 ml of PBS and centrifuged for 5 min at 500 g at 4 °C. The pellets were stored at -80 °C. RNA was isolated with Tri-reagent (T-9424) according to the manufacturer's instructions. A 10–15 μ g portion of RNA was run on a 1.5% (w/v) agarose (Gibco BRL) gel in 1 × Mops running buffer, containing 6.67% (v/v) formaldehyde. The RNA was then transferred on to nylon membranes (Hybond-N; Amersham), and the membrane was exposed to UV light followed by baking at 80 °C for 2 h. Membranes were prehybridized for 2 h at 42 °C before hybridization with (1–2) × 10⁶ c.p.m./ml of the radioactively labelled cDNA probes in hybridization buffer at 42 °C for 16 h. The filters were washed to a stringency of 0.1 × SSC/0.1% (v/v) SDS at 60 °C before exposure to Kodak XAR 5 film for 12–72 h at -80 °C. The intensities of the bands were measured using a phosphorimager (445 SI; Molecular Dynamics).

Nuclear run-on assay

EGF or TNF α was added to cells in 10 cm-diam. Petri dishes. Two dishes were used for each condition. After incubation as indicated in the legends to the Figures, the cells were washed in ice-cold PBS and then scraped loose in 5 ml of PBS and centrifuged for 5 min at 500 g at 4 °C. The pellets were resuspended in ice-cold Nonidet P-40 lysis buffer and centrifuged at 500 g at 4 °C for 5 min. The pellet was resuspended in 100 μ l of ice-cold glycerol storage buffer before addition of 100 μ l of

transcription buffer and 2.5 μ l of [α - 32 P]UTP (800 Ci/mmol; 20 mCi/ml). After incubation for 30 min at 30 °C, the reaction was terminated with DNase I. The reaction mixture was digested with 100 μ g/ml proteinase K in 1 $\times$ SET for 30 min at 37 °C, followed by isolation of the newly transcribed RNA with Tri-reagent. The RNA was denatured with 0.2 M NaOH on ice and neutralized with 0.3 M Hepes. The RNA was run through a Sephadex G-50 spin column (27-5335-01; Pharmacia LKB Biotechnology) to remove unincorporated [32 P]UTP. Plasmid DNA was denatured with 0.1 M NaOH and neutralized with 5.5 $\times$ SSC. Portions of 5 μ g of the denatured plasmids were bound to nylon membranes (Hybond-N) using a dot-blot apparatus, and were immobilized by drying the membranes overnight at room temperature followed by baking at 80 °C for 2 h. A total of $(1-5) \times 10^6$ c.p.m. of newly transcribed mRNA was hybridized to the membranes in 1 ml of hybridization buffer. The membranes were washed to a stringency of $0.2 \times$ SSC at 60 °C before exposure to Kodak XAR-5 film for 1–2 days. The intensity of the spots was measured using a phosphorimager.

Buffers and solutions

Buffer compositions were as follows. PBS: 137 mM NaCl, 2.7 mM KCl, 1 mM Na_2HPO_4 and 2 mM NaH_2PO_4 , pH 7.4. SSC: 150 mM NaCl and 15 mM sodium citrate, pH 7.0. Mops running buffer: 40 mM Mops, pH 7.0, 10 mM sodium acetate and 1 mM EDTA. Lysis buffer: 10 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 5 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, 1% (v/v) β -mercaptoethanol, 1 mM PMSF, 1 mM NaVO_4 , 4% (v/v) glycerol and 0.005% (w/v) Bromophenol Blue. Nonidet P-40 lysis buffer: 10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl_2 and 0.5% (v/v) Nonidet P-40. Glycerol storage buffer: 50 mM Tris/HCl, pH 8.3, 40% (v/v) glycerol, 5 mM MgCl_2 and 0.1 mM EDTA. Transcription buffer: 10 mM Tris/HCl, pH 8.0, 5 mM MgCl_2 , 300 mM KCl, 5 mM dithiothreitol and 1 mM each of ATP, CTP and GTP. SET: 10 mM Tris/HCl, pH 7.5, 5 mM EDTA and 1% (v/v) SDS. Denhardt's solution: 0.02% (w/v) Ficoll-400, 0.02% (w/v) polyvinylpyrrolidone and 0.02% (w/v) BSA. Hybridization buffer (Northern blot analysis): 45% (v/v) formamide, 40 mM sodium phosphate buffer, pH 6.5, 5 $\times$ SSC, 0.1% (w/v) SDS, 5 $\times$ Denhardt's solution and 150 μ g/ml single-stranded salmon testis DNA. Hybridization buffer (run-on assay): 4 $\times$ SSC, 5 mM EDTA, 0.1% (v/v) SDS, 5 $\times$ Denhardt's solution, 50% (v/v) formamide and 100 μ g/ml single-stranded salmon testis DNA.

RESULTS

EGF-dependent inhibition of DNA synthesis and induction of p21/CIP1 in A431 cells

High concentrations of EGF have previously been demonstrated to inhibit growth in the carcinoma cell line A431 [2,29,30]. As demonstrated in Figure 1, growth (measured as DNA synthesis) was inhibited when 1 nM or 10 nM EGF was added to A431 cells, while 0.1 nM EGF did not significantly alter DNA synthesis. EGF-mediated growth suppression has previously been demonstrated to correlate with increased expression of the CDK inhibitor p21/CIP1 [2,3,17]. p21/CIP1 rapidly increased above control levels when 1 or 10 nM EGF was added, and the protein peak was observed approx. 5 h after addition of EGF (Figure 2A). EGF at 1 or 10 nM increased the level of p21/CIP1 to the same extent (see Figure 2A), while 0.1 nM EGF did not significantly increase the level of p21/CIP1 (Figure 2B).

Figure 1 Suppression of DNA synthesis by EGF in A431 cells

A431 cells were grown in DMEM containing 10% (v/v) FBS for 2 days, as described in the Experimental section. Then 0.01, 0.1, 1 or 10 nM EGF was added to the cells, which were further incubated for 24 h. [3 H]Thymidine (1 μ Ci/ml) was present during the final 4 h of incubation. The trichloroacetic acid-precipitable radioactivity was measured as described in the Experimental section.

Effect of cycloheximide (CHX) on the level of p21/CIP1 mRNA upon addition of EGF to A431 cells

In order to investigate whether p21/CIP1 mRNA induction was protein-synthesis-dependent, we incubated A431 cells with or without 20 μ g/ml CHX for 30 min before 1 nM EGF was added for various time periods. The cells were then lysed as described

Figure 2 Time- and concentration-dependent induction of p21/CIP1 by EGF in A431 cells

A431 cells were grown in DMEM containing 10% (v/v) FBS for 2 days, as described in the Experimental section. EGF at 10 or 1 nM (A) and at 1 nM or 0.1 nM (B) was added to the cells, which were subsequently incubated for 15 min, 30 min, 1 h, 2 h, 5 h or 10 h, as indicated. The cells were lysed and subjected to Western blotting with an antibody against p21/CIP1, as described in the Experimental section.

Figure 3 Induction of p21/CIP1 mRNA by EGF in A431 cells in the absence or presence of CHX

A431 cells were grown in DMEM containing 10% (v/v) FBS for 2 days, as described in the Experimental section. The cells were incubated in the absence or presence of 20 μg/ml CHX for 10 min before addition of 1 nM EGF. The cells were incubated without (lanes 1–7) or with (lanes 8–14) CHX in the presence of ligand for 15 min (lanes 2 and 9), 30 min (lanes 3 and 10), 1 h (lanes 4 and 11), 2 h (lanes 5 and 12), 5 h (lanes 6 and 13) or 10 h (lanes 7 and 14). Controls are shown in lanes 1 and 8. Total cellular RNA was purified, and 15 μg of total RNA was subjected to Northern blotting, as described in the Experimental section, using ³²P-labelled p21/CIP1 cDNA. The same blot was rehybridized with ³²P-labelled GAPDH cDNA as control for loading of RNA on to the gel.

Figure 4 EGF-induced increase in the half-life of p21/CIP1 mRNA in A431 cells

A431 cells were grown in DMEM containing 10% (v/v) FBS for 2 days, as described in the Experimental section. Then the cells were incubated with or without 1 nM EGF for 2 h before the addition of 6 μg/ml actinomycin D1. The cells were further incubated for the times indicated before total cellular RNA was purified. For EGF-stimulated cells and unstimulated cells (no addition), 10 μg and 15 μg of total RNA respectively was subjected to Northern blotting, as described in the Experimental section, using ³²P-labelled p21/CIP1 cDNA. The same blot was rehybridized with ³²P-labelled β-actin cDNA as a control for loading of RNA on to the gel (A). The intensity of the bands was measured using a phosphorimager. The intensity of the p21/CIP1 band was normalized to the intensity of the β-actin band and plotted as a percentage of the control (0 h) (B).

in the Experimental section, and the RNA was analysed by Northern blotting. The increase in mRNA was enhanced in the presence of CHX (Figure 3). The increase in mRNA was detectable after 30 min of incubation with EGF (Figure 3, lanes

Figure 5 Time-dependent stabilization of the p21/CIP1 protein upon incubation of A431 cells with EGF

A431 cells were grown in DMEM containing 10% (v/v) FBS for 2 days, as described in the Experimental section. Then the cells were incubated with 1 nM EGF for 2 or 5 h before the addition of 20 μg/ml CHX and incubation for the indicated time periods. (A) The cells were lysed and subjected to Western blotting with an antibody against p21/CIP1, as described in the Experimental section. (B) The samples were also subjected to SDS/PAGE and electrotransferred on to PVDF membranes; the intensity of the bands was quantified as described in the Experimental section and plotted as ln.(% of control) (0 h). The data represent the means of two independent experiments.

3 and 10), and the peak was observed after approx. 2 h (Figure 3, lane 5). As measured using phosphorimager analysis, the amount of mRNA was initially seen to double with time (Figure 3). Furthermore, in the presence of CHX, the increase in mRNA was sustained compared with that in the absence of CHX. The data could suggest that the gene encoding p21/CIP1 is transcribed as an immediate-early gene when A431 cells are incubated with growth-suppressing concentrations of EGF, as suggested previously [17].

Effect of EGF on stability of the p21/CIP1 mRNA and p21/CIP1 protein in A431 cells

Stabilization of p21/CIP1-encoding mRNA has been reported to occur in a p53-independent manner following the addition of TNFα [9] or interleukin-1 [31]. In order to investigate whether this was the case with EGF, we incubated A431 cells with or without 1 nM EGF for 2 h. Transcription was then arrested by addition of 6 μg/ml actinomycin D1. The cells were lysed, and the mRNA was analysed by Northern blotting as described in the Experimental section. The radioactivity was measured using a phosphorimager, and the measurements were corrected for loading differences by hybridizing the same filter with a probe recognizing β-actin. As shown in Figure 4, 1 nM EGF indeed increased the half-life of the p21/CIP1 mRNA. In three in-

Figure 6 Rate of transcription of the p21/CIP1 gene in EGF-treated A431 cells

A431 cells were grown in DMEM containing 10% (v/v) FBS for 2 days, as described in the Experimental section. Then the cells were stimulated with 10 or 1 nM EGF for 30 or 60 min before isolation of the nuclei. Nuclear run-on assays were performed as described in the Experimental section. Plasmids used as probes were pC-WAF1-S, which contains the p21/CIP1 cDNA, pLDH, which contains the LDH cDNA, pβ-actin, which contains the β-actin cDNA, and pCEB, which is a negative control for pC-WAF1-S (A). The intensities of the spots were measured using a phosphorimager. The intensity of p21/CIP1 was normalized to the intensity for LDH (the assumption being that the rate of transcription from the LDH gene is unaffected by EGF) and plotted as the fold increase compared with the control (0 h). The measurements represent means $\pm$ S.E.M. of three independent experiments (B).

dependent experiments, the half-life of the mRNA without EGF was 158 ± 8 min (mean $\pm$ S.E.M.), while after incubation with EGF the half-life was 298 ± 21 min. The half-life of the mRNA encoding p21/CIP1 was increased to a similar extent by 10 nM and 1 nM EGF (results not shown). A two-fold stabilization of

the mRNA could possibly account for the observed increase in mRNA levels (compare Figures 3 and 4).

Since stabilization of the p21/CIP1 protein has been described during preadipocyte differentiation [32], we also analysed the half-life of the protein after incubation of the cells for 2 or 5 h with or without 1 nM EGF and subsequent addition of CHX. No increase in the half-life of the p21/CIP1 protein could be detected after incubation of A431 cells with EGF for 2 h, while after incubation with EGF for 5 h an approx. 3-fold increase in protein stability was observed (half-life of approx. 6 h compared with 2 h) (Figure 5).

Rate of transcription of p21/CIP1 upon addition of different concentrations of EGF to A431 cells

Our results showed that p21/CIP1 was induced to the same extent by 10 nM and 1 nM EGF (Figure 2A), that increased levels of p21/CIP1 mRNA depended on stabilization of mRNA and that, with long incubation times, EGF increased the half-life of the p21/CIP1 protein. In order to study whether the encoding gene was transcribed, we carried out nuclear run-on assays to investigate the rate of transcription at both 10 and 1 nM EGF. Upon mitogenic stimulation, transcription of the β-actin gene is increased [33,34], and the pβ-actin plasmid was therefore used as positive control for the nuclear run-on assay. As a negative control we used the empty vector pCEB (see the Experimental section). In three independent experiments, the rate of transcription of p21/CIP1 was increased by a factor of 1.25 ± 0.03 (mean $\pm$ S.E.M.) at 30 min after addition of EGF. Transcription was increased to a similar extent at both concentrations of EGF (10 nM and 1 nM) at both 30 and 60 min (Figure 6).

EGF-induced activation of STAT1 and STAT3

EGF has been shown to induce activation and DNA binding of STAT1 and STAT3 in A431 cells [17]. However, such activation was demonstrated using 100 ng/ml EGF (~ 17 nM) [17,35]. Since transcription of p21/CIP1 is inefficiently induced at both 1 nM EGF and 10 nM EGF (see Figure 6), we studied activation of STAT1 and STAT3 at 10 nM and 1 nM EGF. Activation was studied using antibodies that specifically recognize the activated forms of the transcription factors. We found that both STAT1 and STAT3 were activated more strongly by 10 nM EGF than by 1 nM EGF (Figure 7). Our data clearly show that the extent of activation of STAT1 and STAT3 does not correlate with transcriptional activation of p21/CIP1.

Effect of TNFα in A431 cells on induction of p21/CIP1, transcription of the p21/CIP1 gene and stability of the p21/CIP1 mRNA

As TNFα has also been demonstrated to induce the expression of p21/CIP1 [9], we compared EGF- and TNFα-induced expression of p21/CIP1. EGF (1 nM) or TNFα (10 ng/ml) was added to A431 cells, and the cells were incubated for the times indicated in Figure 8(A). Both 1 nM EGF and 10 ng/ml TNFα induced expression of p21/CIP1 to approximately the same extent, but 1 nM EGF induced expression more rapidly than did 10 ng/ml TNFα.

TNFα at 10 ng/ml was observed to increase the level of p21/CIP1 mRNA (Figure 8B), as did 1 nM EGF (see Figure 4), and the induction was not dependent on new protein synthesis (results not shown). Nuclear run-on analysis showed that the rate of transcription increased approx. 1.2-fold upon incubation of A431 cells with 10 ng/ml TNFα for 30 min (Figure 8C). This is comparable with the transcriptional activation observed upon

Figure 7 EGF-induced phosphorylation of STAT1 and STAT3 in A431 cells

A431 cells were grown in DMEM containing 10% (v/v) FBS for 2 days, as described in the Experimental section. Then the cells were stimulated with 1 nM EGF (lanes 1–7) or 10 nM EGF (lanes 8–14) for 15 min (lanes 2 and 9), 30 min (lanes 3 and 10), 1 h (lanes 4 and 11), 2 h (lanes 5 and 12), 5 h (lanes 6 and 13) or 10 h (lanes 7 and 14). Lanes 1 and 8 contain control samples (no EGF). After stimulation with EGF, the cells were lysed and subjected to Western blotting with antibodies against pSTAT1 and pSTAT3, as described in the Experimental section.

Figure 8 Induction of p21/CIP1 by TNF α in A431 cells

A431 cells were grown in DMEM containing 10% (v/v) FBS for 2 days, as described in the Experimental section. **(A)** TNF α (10 ng/ml) or EGF (1 nM) was added, and the cells were incubated at 37 °C for the times indicated. The cells were lysed and subjected to Western blotting with an antibody to p21/CIP1, as described in the Experimental section. **(B)** TNF α (10 ng/ml) was added, and the cells were incubated for 0 min (lane 1), 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 5 h (lane 6) or 10 h (lane 7). Total cellular RNA was purified, and 10 μ g of total RNA was subjected to Northern blotting, as described in the Experimental section, using the 32 P-labelled p21/CIP1 cDNA. The same blot was rehybridized with 32 P-labelled GAPDH cDNA as control for loading of RNA on to the gel. **(C)** TNF α (10 ng/ml) was added, and the cells were incubated for the times indicated before isolation of the nuclei. Nuclear run-on assays were performed as described in the Experimental section. Plasmids used as probes were pC-WAF1-S, which contains the p21/CIP1 cDNA, pLDH, which contains the LDH cDNA, p β -actin, which contains the β -actin cDNA, and pCEB, which is a negative control for pC-WAF1-S. The intensity of the spots was measured as described in the legend to Figure 6. The data shown represent the means of two independent experiments. **(D)** A431 cells were incubated with or without 10 ng/ml TNF α for 2 h before addition of 6 μ g/ml actinomycin D1. The cells were further incubated for the times indicated before total cellular RNA was purified. A 10 μ g portion of RNA was subjected to Northern blotting, and the intensity of the 32 P-labelled p21/CIP1-cDNA-probed bands was measured and normalized to β -actin, as described in the legend to Figure 4. One representative experiment is shown.

incubation of A431 cells with EGF (Figure 6). TNF α has been demonstrated to increase the half-life of p21/CIP1 mRNA in p53-deficient human leukaemic cells [9]. We therefore compared the half-life of p21/CIP1 mRNA in A431 cells with or without addition of 10 ng/ml TNF α . No increased half-life of the p21/CIP1 mRNA was observed upon stimulation of A431 cells with 10 ng/ml TNF α for 2 h (Figure 8D). We further investigated how TNF α affected the half-life of the p21/CIP1 protein. In A431 cells, 10 ng/ml TNF α increased the half-life of the protein approx. 2-fold after incubation for 5 h, while there was no increase in p21/CIP1 half-life after a 2 h incubation (results not shown).

EGF-induced expression of p21/CIP1 in MCF-7 cells

As A431 cells have a mutated p53 protein, we studied the EGF-dependent induction of p21/CIP1 in MCF-7 cells, which are normal with respect to p53. In MCF-7 cells we observed a biphasic dose-response curve, with maximal DNA synthesis occurring at 0.1 nM EGF (results not shown), and p21/CIP1 protein was induced in MCF-7 cells incubated with 1 nM EGF for 5 and 10 h (results not shown). The p21/CIP1 mRNA was increased after 2 h, and the mRNA peak occurred upon incubation of MCF-7 cells for 5 h (Figure 9A). Both p21/CIP1 protein and mRNA were induced less efficiently in MCF-7 cells

compared with A431 cells (results not shown). When the stability of the mRNA was studied, we found that the half-life was not increased upon incubation of the cells with EGF. However, by comparing Figures 9(B) and 4, it is clear that, in the absence of EGF, the half-life of p21/CIP1 mRNA was significantly greater in MCF-7 cells (approx. 9.5 h) than in A431 cells (approx. 2.5 h).

DISCUSSION

The transcription factors STAT1 and STAT3 were reportedly activated upon addition of EGF to A431 cells, and were shown to bind to *cis*-inducible elements in the p21/CIP1 promoter [17]. We showed dose-dependent phosphorylation of both STAT1 and STAT3, with strong and rapid activation, upon addition of 10 nM EGF, while the activation upon addition of 1 nM EGF occurred later and less efficiently. However, our data clearly show that the activation of transcription at both ligand concentrations was almost negligible. This indicates that activation of STAT1/STAT3 is at best of minor importance for induction of p21/CIP1 upon incubation of A431 cells with EGF.

Our data show that EGF increased the half-life of the mRNA encoding p21/CIP1 approx. 2-fold when measured after a 2 h incubation. This accounts for most of the observed increase in mRNA upon incubation of A431 cells with EGF for short time periods. Additionally, the half-life of the p21 protein was

Figure 9 Induction of p21/CIP1 in MCF-7 cells

MCF-7 cells were grown in DMEM containing 10% (v/v) FBS for 2 days, as described in the Experimental section. **(A)** The cells were incubated with 1 nM EGF for 0 min (lane 1), 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 5 h (lane 6) or 10 h (lane 7). Total RNA was purified, and 15 µg was subjected to Northern blotting as described in the Experimental section, using the ³²P-labelled p21/CIP1 cDNA. The same blot was rehybridized with ³²P-labelled GAPDH cDNA as a control for loading of RNA on to the gel. **(B)** The cells were incubated with or without 1 nM EGF for 2 h before addition of 6 µg/ml actinomycin D1. The cells were further incubated for the times indicated before total cellular RNA was purified. For EGF-stimulated cells and unstimulated cells (no addition), 10 µg and 20 µg of total RNA respectively was subjected to Northern blotting, and the intensity of the ³²P-labelled p21/CIP1-cDNA-probed bands was measured as described in the legend to Figure 4.

increased upon longer incubation periods with EGF. After a 5 h incubation with EGF, the half-life of the protein was increased approx. 3-fold. This probably accounts for the finding that maximal p21 protein accumulation takes 5 h, even though the mRNA level is maximal at 2 h. Ubiquitination has been shown to be a reversible process, and several enzymes that deubiquitinate proteins have been identified (for a review, see [36]). It was demonstrated previously that, upon inhibition of proteasomal activity with lactacystin, the half-life of p21/CIP1 was increased [37].

TNFα increased the half-life of the p21/CIP1 mRNA in leukaemic KG-1 cells. Addition of TNFα was found to increase the rate of transcription by a factor of 1.4 and the half-life of the p21/CIP1 mRNA by a factor of 5 [9]. We found that, in A431 cells, 10 ng/ml TNFα increased the rate of transcription 1.2-fold. However, the half-life of the mRNA was approx. 2.5 h both with and without the addition of TNFα. It should be noted that five times more TNFα (1000 units/ml) and a four times longer incubation time was used with KG-1 cells, where the half-life of the p21/CIP1 mRNA was increased from 30 min to 2.5 h [9]. Both EGF and TNFα were found to stabilize the p21/CIP1 protein in A431 cells upon prolonged incubation times. The mRNA stabilization observed upon addition of 1 nM EGF, but not upon addition of 10 ng/ml TNFα, to A431 cells probably

explains the more rapid induction by EGF than by TNFα of the p21/CIP1 protein.

Regulation of mRNA stability is so far poorly understood. However, it has been demonstrated that *cis*-acting elements within the mRNA molecule can be recognized by regulatory proteins, and *cis*-elements have been demonstrated to modulate mRNA stability both positively and negatively (for reviews, see [38–40]). The 3′ untranslated regions of rapidly decaying mRNAs, such as those for granulocyte/macrophage-colony-stimulating factor, TNFα, interferon-γ, interleukin-2 and interleukin-3, usually contain an adenosine- or uridine-rich element, characterized by three or more copies of the pentanucleotide AUUUA [38–42]. The stability of these mRNA molecules can be regulated by extracellular stimuli [38,43]. The p21/CIP1 mRNA contains three AUUUA elements in the 3′-untranslated region [20]. The fact that EGF increased the stability of the mRNA encoding p21/CIP1 in a protein-synthesis-independent fashion could suggest that the mechanism responsible for the increased half-life is induced binding of a pre-existing protein to the mRNA or, alternatively phosphorylation or dephosphorylation of an mRNA-binding protein.

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