Up-regulated EGF receptors undergo to rapid internalization and ubiquitin-dependent degradation in human cancer cells exposed to 8-Cl-cAMP

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Abstract 8-Cl-cAMP, a cAMP analogue that antagonizes type I cAMP-dependent protein kinase, is a novel anti-tumor agent presently under investigation in clinical trials. Herein we report the effects of this agent on epidermal growth factor receptor expression and degradation in human KB cancer cells. Exposure to 10 μM 8-Cl-cAMP for 48 h induced a 65% increase in epidermal growth factor receptor surface expression while the receptor synthesis was 22-fold enhanced. Analysis of epidermal growth factor-dependent receptor internalization in 8-Cl-cAMP-treated cells showed a higher endocytosis rate as well as an accelerated degradation which occurred together with an increased receptor ubiquitination. The enhanced degradation of epidermal growth factor receptor correlated with the lack of epidermal growth factor-induced proliferation and mitogen-activated protein kinase stimulation. The disregulation of epidermal growth factor receptor internalization and ubiquitin-dependent degradation could underlay a new mechanism of the anti-tumor activity of 8-Cl-cAMP suggesting its combination with agents that disrupt epidermal growth factor receptor signalling.

Key words: cAMP analogue; Protein kinase A; Epidermal growth factor receptor; Endocytosis; Ubiquitin; Mitogen-activated protein kinase

1. Introduction

Epidermal growth factor (EGF) is a polypeptide which has a strong mitogenic activity both in vitro and in vivo [1] and exerts its effects through a cell surface receptor. The specific binding of EGF to the extracellular domain of the 170 kDa EGF receptor (EGF-R) triggers dimerization of the receptor and activation of its intrinsic tyrosine kinase activity [2]. The latter event leads to the receptor autophosphorylation followed by the recruitment and activation of multiple signalling proteins which initiate a complex series of intracellular events regulating the cell responsiveness to the growth stimuli [2,3]. A key point in the EGF-induced mitogenic cascade is the stimulation of mitogen-activated protein kinase (MAPK) that transmits the signal into the nucleus leading to gene expression regulation and, finally, to the induction of cell proliferation [4,5]. Together with the activation of the signal transduction cascade, receptor/ligand complexes are rapidly internalized via the endocytic pathway and are routed to lysosomes where the receptors are degraded [6]. The latter event causes receptor down-regulation and attenuates the signal transduction cascade [6,7].

Several studies have suggested that modulation of the expression of peptide growth factor receptors (PGF-R) is a common event in tumor cells that have been exposed to low concentrations of cytotoxic drugs or to biological agents [8–12]. We have previously reported that cytostatic concentrations of α2-recombinant interferon (IFNα) induced up-regulation of EGF-R expression in human epidermoid cancer KB cells [12] and that this effect potentiated the mitogenic activity of EGF on these cells [13]. 8-Cl-cAMP, a synthetic cAMP analogue which can be considered an antagonist of type I cAMP-dependent protein kinase (PKA-I), is a novel anti-tumor agent that has undergone extensive preclinical evaluation and is presently under investigation in clinical trials on cancer patients [14–17]. 8-Cl-cAMP changes the ratio between the two regulatory subunits, RI and RII, of PKA [18]. In particular, it has been shown that this agent reduces the expression and activity of RI/PKA-I which is overexpressed in tumor cells and is enhanced in normal cells upon exposure to mitogenic stimuli [18,19]. On the other hand, 8-Cl-cAMP increases RII/PKA-II which has been correlated with cell growth inhibition and differentiation [18,19]. It has also been demonstrated that 8-Cl-cAMP interferes with growth factor autocrine pathways and the growth factor-induced cell transformation [20]. Furthermore, 8-Cl-cAMP synergizes with a blocking anti-EGF-R monoclonal antibody (MAb) on the growth inhibition of tumor cells [21]. The concomitant and selective disruption of different growth regulating pathways, by the use of pharmacological or biological agents, is presently an important tool for the development of new anti-tumor therapeutic strategies [22–24].

In this study we have investigated the EGF/EGF-R signal pathway in KB cells which are growth inhibited upon 8-Cl-cAMP treatment and are dependent on EGF for their proliferation [25]. Specifically, we have studied the effects of 8-Cl-cAMP on EGF-R cell surface expression and synthesis. We have found a discrepancy in magnitude between the EGF-R up-regulated expression on the cell membrane and the increase in receptor synthesis induced by 8-Cl-cAMP. Therefore, we have evaluated the effects of the cAMP analogue

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Abbreviations: EGF, epidermal growth factor; EGF-R, EGF receptor; MAPK, mitogen-activated protein kinase; PKA, cAMP-dependent protein kinase; MAb, monoclonal antibody; IFNα, α2-recombinant interferon; PGF-R, peptide growth factor receptor

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on EGF-R internalization and on ubiquitin-dependent degradation which has been described as a possible pathway for EGF-R down-regulation [7]. Furthermore, we have investigated the effect of 8-Cl-cAMP on the EGF-R transduction pathway studying the EGF-induced MAPK activation and cell proliferation in KB cells.

2. Materials and methods

2.1. Cell culture and cell proliferation assays

The human epidermoid carcinoma KB cell line (ATCC, Rockville, MD, USA) was grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), penicillin (50 U/ml), streptomycin (500 μg/ml) and 4 mM glutamine, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. KB cells throughout all the experiments have been maintained in serum-containing medium and no EGF deprivation has been performed to enhance the EGF effects on cell growth or protein phosphorylation. For cell growth assays, 10_5 cells were seeded in triplicate in 6-multiwell plates. 8-Cl-cAMP (sodium salt, provided by K.P. Flora, National Cancer Institute, Bethesda, MD, USA) or EGF (Sigma, St. Louis, MO, USA) were added at indicated concentrations after 3 h of seeding and every 48 h thereafter when medium change was performed. At the indicated time points, cell growth assessment was performed by haemocytometric cell count and a trypan blue viability assay, following gentle trypsinization.

2.2. [^{125}I]EGF-binding

The cells were seeded in 24-multiwell plates at $2\times10_4$ cells/well and treated as described above. 8-Cl-adenosine (BioLog Life Science Institute, Bremen, Germany) was used here and throughout all further experiments at a concentration of 1 μM which is the IC_{50} for KB cells after 48 h of treatment. The treated and untreated cells were incubated overnight in serum-free medium (i.e. DMEM supplemented with non-essential amino acids and vitamines). The cells were washed twice with ice-cold phosphate buffered saline (PBS) in the presence of 1 mg/ml of bovine serum albumin (BSA) and further incubated for 4 h at 4°C with 200 μl/well binding buffer (DMEM, HEPES 25 mM, BSA 1 mg/ml) containing 30 000 cpm/well of [^{125}I]EGF (164 μCi/mg, Amersham, Buckinghamshire, UK) and increasing concentrations of unlabelled EGF, as previously described [8]. Cells were washed four times with PBS/BSA and lysed in 0.5 μl/well of 20 mM HEPES, 1% Triton X-100, 10% glycerol. The cell-associated radioactivity was counted in a Beckman γ-counter. The value of the EGF-R-binding affinity and the receptor number were determined by Scatchard analysis as previously described [8].

2.3. EGF-R live cell radioimmunoassay (RIA)

KB cells were seeded in 96-multiwell plates at 5×10^3 cells/well and exposed for 48 h to 8-Cl-cAMP or 8-Cl-adenosine. After overnight incubation in serum-free medium, 100 μl 5% BSA (w/v) in DMEM was added to each well. After 60 min of incubation at 37°C, the cells were washed with DMEM/BSA and 50 μl of appropriately diluted anti-EGF-R MAb 108.1 (kindly donated by Prof. J. Schlessinger, Department of Pharmacology, NYU Medical Center, NY, USA) was added to each well for 3 h at 4°C. The cells were washed twice with 5% PBS/BSA (w/v) and ^{125}I-labelled sheep anti-mouse IgG (75 000 cpm in 50 μl, Amersham) was added to each well. Following a 60 min incubation (at 37°C), the cells were washed three times with PBS/BSA 5% (w/v), adsorbed with a cotton swab and the radioactivity was counted in a Beckmann γ-counter, as previously described [13].

2.4. EGF-R endocytosis

The cells were seeded in 24-multiwell plates at $2\times10_4$ cells/well and treated as described above. After overnight incubation in serum-free medium, cells were washed twice with ice-cold PBS/BSA and exposed for 1 h at 4°C to binding buffer containing 4 ng/ml [^{125}I]EGF (∼120 000 dpm/well). Then, untreated or 8-Cl-cAMP-treated cells were washed three times with ice-cold PBS/BSA and exposed to binding buffer, pre-warmed at 37°C, containing 0.1 μM non-labelled EGF for the indicated times at 37°C and washed twice with PBS/BSA at 4°C. The cells were incubated twice with acid buffer (0.2 M acetic acid, pH 2.5 and 0.2 M NaCl) for 3 min at 4°C and lysed. Acid sensitive and acid resistant cell-associated radioactivity was determined by a γ-counter. The % EGF-R endocytosis was calculated for each time of exposure to EGF as:

$$\frac{\text{acid resistant } [^{125}\text{I}]\text{EGF radioactivity}}{\text{acid resistant + acid sensitive } [^{125}\text{I}]\text{EGF radioactivity}}\times100$$

2.5. Metabolic labelling of EGF-R and solubilization of intact cells

KB cells were starved for 30 min at 37°C in methionine-free medium and then incubated for 3 h in methionine-free medium in the presence of 200 μCi/ml L-[^{35}S]methionine (1300 Ci/mM, Amersham). For cell extract preparation, cells were washed twice with ice-cold PBS/BSA, scraped and centrifuged in lysis buffer (20 mM, Tris(hydroxymethyl) methylamine-HCl (Tris), pH 7.4, 100 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 mM $MgCl_2$, 0.1 mM pepstatin, 0.1 mM antipain, 0.1 mM chymostatin, 0.2 mM leupeptin, 0.4 mg/ml aprotinin and 0.5 mg/ml soybean trypsin inhibitor) as previously described [13]. The samples were equalized on radioisotope incorporation by β-counting the radioactivity contained in trichloroacetic acid (TCA)-precipitated aliquots.

2.6. Immunoprecipitation, electrophoresis and Western blot analysis

Supernatants from L-[^{35}S]methionine-labelled cells were subjected to immunoprecipitation with 25 μg anti-EGF-R 108.1 MAb for 90 min at 4°C with 50 μl of protein A-Sepharose conjugated. The immunoprecipitated were separated by 8% SDS-PAGE and then fixed and dried before autoradiography [13].

For Western blot analysis, equal amounts of protein extracts, determined by the Lowry method using serum albumin as standard, were immunoprecipitated and separated as described above. The blots, obtained from electrotransfer to nitrocellulose filter, were probed for 12 h with anti-EGF-R 108.1 or anti-phosphotyrosine 4G10 (Upstate Biotechnology, Lake Placid, NJ, USA) or anti-ubiquitin (DAKO, Glostrup, DK, USA) antibodies. The immunocomplexes were detected with an enhanced chemiluminescence (ECL) system from Amersham.

2.7. MAPK assay

The cell pellets, after two washes with ice-cold PBS, were suspended in buffer H (50 mM β-glycerophosphate, 1.5 mM ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA), 0.1 mM Na_3V0_4, 1 mM dithiotreitol (DTT), 30 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin), homogenized and centrifuged at $10000\times g$ for 10 min. The resulting supernatants were processed for a MAPK assay using myelin basic protein (MBP, Sigma) and [γ^{32}P]ATP (Amersham) as substrates. 10 μl of cell extract was added to an assay mixture (15 μl) containing 50 mM β-glycerophosphate, 1.5 mM EGTA, 0.1 mM Na_3V0_4, 1 mM DTT, 15 mM $MgCl_2$, 0.66 μM PKA inhibitor (Sigma), MBP at 0.5 mg/ml and 22.5 μM [γ^{32}-P]ATP (5–20 cpm/fmol). After 30 min incubation at 30°C, the reaction mixtures were spotted onto phosphocellulose filters (Whatman P81) and washed three times in 175 mM phosphoric acid. Filters were air-dried and then counted by liquid scintillation using Omnifluor/toluene (DuPont, New England Nuclear, Boston, MA, USA).

3. Results

3.1. Effects of 8-Cl-cAMP on EGF-R expression and synthesis

8-Cl-cAMP inhibits the KB cell growth without any cytotoxic effect [16] while EGF induces proliferation both in vitro and in vivo [25]. We found a dose- and time-dependent increase of [^{125}I]EGF binding sites on KB cells after 8-Cl-cAMP treatment (Fig. 1A). In fact, increased EGF-binding was detectable after 24 h of treatment with 5–10 μM 8-Cl-cAMP, reached a peak after 48 h and decreased beyond this time point as compared with untreated cells at the same time points (Fig. 1A).

It was previously shown that 8-Cl-cAMP can be hydrolyzed to 8-Cl-adenosine by the serum-phosphodiesterases and 5′-nucleotidase and has been hypothesized that this molecule can be indeed responsible for the effects of 8-Cl-cAMP

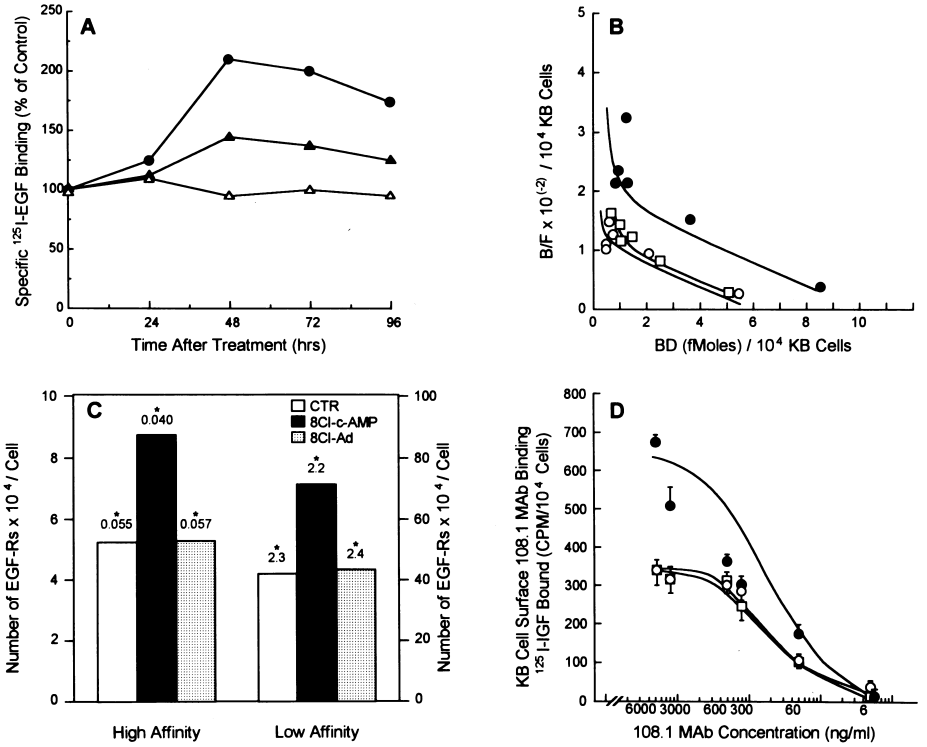

Fig. 1. Effects of 8-Cl-cAMP on EGF-R expression on the KB cell surface. (A) Effects of different concentrations of 8-Cl-cAMP on [^{125}I]EGF-binding. After medium withdrawal, fresh medium without 8-Cl-cAMP or with 1 μM (Δ), 5 μM (▲) and 10 μM (●) 8-Cl-cAMP was added. [^{125}I]EGF-specific binding was expressed as a percentage of that measured in control cultures for each time point. [^{125}I]EGF-specific binding was determined in the presence of 100 nM unlabelled EGF. The data show means of three separate experiments of triplicate determinations. S.E. never exceeded 10%. (B) Scatchard analysis of EGF-binding data on untreated controls and 8-Cl-cAMP-treated KB cells. [^{125}I]EGF-binding to untreated (○) or 48 h 10 μM 8-Cl-cAMP-treated (●) or 1 μM 8Cl-adenosine-treated (□) KB cells. [^{125}I]EGF-binding has been performed in the presence of different concentrations of [^{125}I]EGF. Points, averages from triplicate determinations. (C) Number and K_d of the low and high affinity EGF binding sites evaluated as described above. (D) Anti-EGF-R MAb binding curve on KB cells. Binding of anti-EGF-R MAb 108.1 to untreated (○) or 10 μM 8-Cl-cAMP- (●) or 1 μM 8-Cl-adenosine-treated (□) KB cells as assessed by live cell RIA, performed through the use of [^{125}I]sheep anti-mouse IgG and expressed as cpm/10^4 cells. The data show means of three separate experiments of triplicate determinations. Bars, S.D.

[26,27]. However, further investigations have demonstrated that the effects of 8-Cl-cAMP are not mediated by its metabolite [19,28]. EGF-R up-regulation induced by 8-Cl-cAMP was not due to the metabolite because, in our experimental conditions, 8-Cl-adenosine did not modify the EGF-binding (Fig. 1B and C). Scatchard plot analysis of EGF-binding data revealed two classes of EGF receptors (Fig. 1B and C) with K_d values of 0.04–0.055 nM (high affinity) and 2.2–2.8 nM (low affinity), respectively (Fig. 1C). An about 65% increase of both low and high affinity EGF-R number was detectable after 48 h exposure of KB cells to 8-Cl-cAMP without any change in the receptor affinity (Fig. 1B and C). In order to determine whether the increase of EGF-binding on the cell surface was really due to an increase of EGF-R proteins and not to an unmasking of unoccupied EGF binding sites, we have performed a live cell RIA experiment using the anti-EGF-R MAb 108.1 that is directed against an epitope outside the EGF binding site. Again, this assay shows that 8-Cl-cAMP increased the number of EGF-Rs on KB cells while no effects were demonstrated in 8-Cl-adenosine-treated cells (Fig. 1D).

In order to evaluate whether the synthesis of EGF-R was modified by 8-Cl-cAMP, we have performed an immunoprecipitation of the EGF-R after metabolic labelling with L-[^{35}S]methionine. We have found that 18 h and 36 h of treatment with 8-Cl-cAMP induced a 18 and a 22-fold increase of EGF-R synthesis, respectively (Fig. 2A and B). Therefore, the up-regulation induced by 8-Cl-cAMP at the tumor cell surface was likely due to the increased synthesis of EGF-R. However, the two effects resulted much different in magnitude because the 22-fold enhancement of receptor synthesis resulted only in a 65% increase in receptor expression at the tumor cell surface.

3.2. *Effects of EGF and 8-Cl-cAMP on EGF-R endocytosis and ubiquitin-dependent degradation*

In order to investigate the discrepancy between the extents of the magnitude of EGF-R surface up-regulation and the increased synthesis, we have investigated if the internalization rate of EGF-R was also modified by 8-Cl-cAMP in KB cells. We have found that 8-Cl-cAMP induced an increase in the rate of EGF-R endocytosis as compared with untreated control cells (Fig. 3A). EGF-R internalization was again not affected by KB cell treatment with 8-Cl-adenosine (Fig. 3A). Since EGF-R is not recycled on the cell membrane and degradation follows endocytosis [2,7], we have investigated by Western blotting analysis if 8-Cl-cAMP could modulate also the whole cell content of EGF-R in KB cells following exposure to EGF. We found that 8-Cl-cAMP alone induced about a 2-fold increase of the EGF-R expression in KB cells (Fig. 3B). The exposure to EGF alone for 5–30 min caused a slight decrease in the EGF-R electrophoretic mobility shift (Fig. 3B)

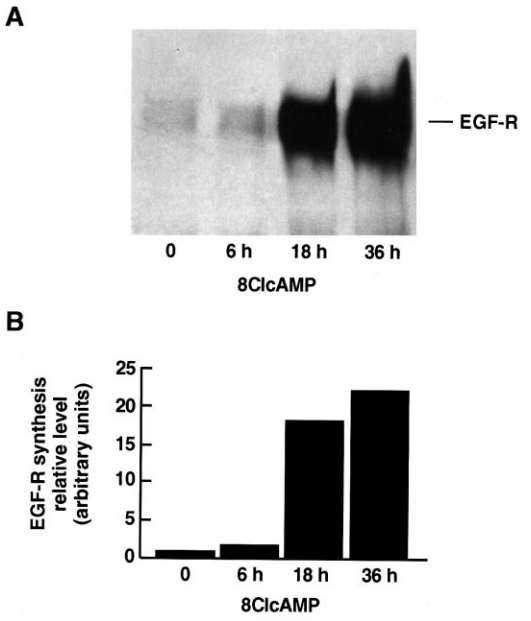

Fig. 2. Effects of EGF and 8-Cl-cAMP on the EGF-R synthesis in KB cells. Immunoprecipitation of EGF-R with anti-EGF-R MAb 108.1 after metabolic labelling of KB cells with L-[^{35}S]methionine. (A) The samples were equalized on radioisotope incorporation by β-counting the radioactivity contained in TCA-precipitated aliquots. After PAGE of immunoprecipitates and autoradiography of dried gels, EGF-R appears as a 170 kDa band. Cells have been exposed for 6 h, 18 h and 36 h to 10 μM 8-Cl-cAMP as shown in the figure. The experiment shown is one of three experiments that gave similar results. (B) Laser scanning of the bands corresponding to EGF-R in the immunoprecipitation experiment expressed as realtive level and arbitrary units derived from a common software (Gel-Pro Analyzer, Media Cybernetics, Silver Spring, MD, USA).

that could be due to EGF-R covalent modifications such as phosphorylation or ubiquitination, as previously demonstrated [7]. The further exposure of KB cells to EGF reduced the receptor expression. As shown in Fig. 3B, after 6 h of treatment with EGF, a 2-fold decrease of EGF-R cell content was recorded. In 8-Cl-cAMP-treated cells, a 6-fold reduction of EGF-R expression was already detectable after 3 h of exposure to EGF (Fig. 3B). Moreover, 8-Cl-cAMP had no effect on the EGF-induced tyrosine phosphorylation of the receptor (Fig. 3C).

EGF-induced down-regulation of the EGF-R is followed by receptor degradation that can be partially ubiquitin-dependent [7]. We have therefore studied if the rapid fall of the receptor levels in 8-Cl-cAMP-treated cells was associated to an increased ubiquitination of EGF-Rs. We have indeed found that the formation of ubiquitin-EGF-R complexes following exposure to EGF was significantly increased in 8-Cl-cAMP-treated cells as compared with an untreated control. As shown in Fig. 3D, a rapid increase in EGF-R ubiquitination was evident after exposure of 8-Cl-cAMP-treated cells to EGF for 5 min and correlated with EGF-R tyrosine phosphorylation (Fig. 3C), while in untreated cells a slight increase was observed after 15 min of treatment with EGF. At later times, a progressive loss of ubiquitin-EGF-R complexes correlated with EGF-R degradation (Fig. 3B). Therefore, 8-Cl-cAMP increased the EGF-induced internalization and ubiquitin-dependent degradation of EGF-R.

3.3. *Effects of 8-Cl-cAMP on the EGF-induced cell proliferation and MAPK activity*

In order to assess if the changes in EGF-R expression and degradation induced by 8-Cl-cAMP could modify the biological response of KB cells to EGF, we have evaluated the interaction between 8-Cl-cAMP and EGF on the cell proliferation. 48 h treatment with 10 μM 8-Cl-cAMP induced 50% growth inhibition without cytotoxicity (Fig. 4A), as demonstrated by a trypan blue assay (data not shown), or major morphological changes as demonstrated by FACS and electron microscopic analysis (A. Budillon, unpublished results). On the other hand, 1 nM EGF, which was the concentration approximately equivalent to the K_d value of the low affinity receptors (Fig. 1C), brought about the maximal stimulation of KB cell growth after 4 days of treatment (Fig. 4A). The proliferative response to EGF was, however, completely abolished in the presence of 10 μM 8-Cl-cAMP and this effect was time-dependent (Fig. 4A and B). The induction of MAPK activity is a crucial event in the signalling cascade which transmits the EGF-mediated mitogenic effect into the nucleus through phosphorylation of transcription factors and regulation of gene expression [4]. We have therefore investigated whether 8-Cl-cAMP could interfere with the EGF in-

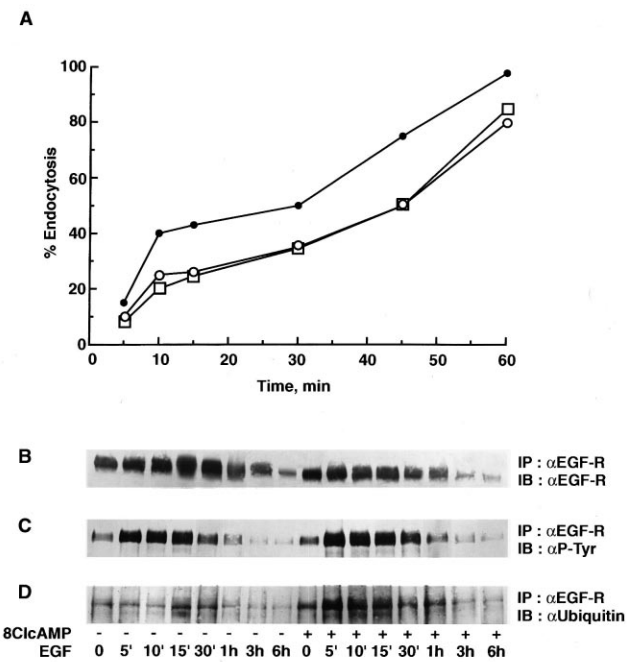

Fig. 3. Effects of EGF and 8-Cl-cAMP on EGF-R endocytosis, protein levels, ubiquitination and phosphorylation in KB cells. (A) We have evaluated the effects of 8-Cl-cAMP on the endocytosis of EGF-R in KB cells. $2\times10_5$ cells/well were seeded in 24-multiwell plates. Cells were grown for 48 h in the absence (●) or presence of 10 μM 8-Cl-cAMP (●) or 1 μM 8-Cl-adenosine (□). [^{125}I]EGF endocytosis was measured as described in Section 2. The data show means of three separate experiments of triplicate determinations. S.E. were always less than 10%. (B) Expression, (C) tyrosine phosphorylation and (D) ubiquitination of EGF-R have been assessed by Western blot analysis as described in Section 2. Cells have been cultured for the indicated times with 10 μM 8-Cl-cAMP and/or 10 nM EGF. Cell extracts, equalized by protein determination, were immunoprecipitated with EGF-R polyclonal antiserum and then subjected to hybridization with anti-EGF-R 108.1, anti-phosphotyrosine 4G10 and anti-ubiquitin MAbs, respectively. The ECL (Amersham) detection system was used.

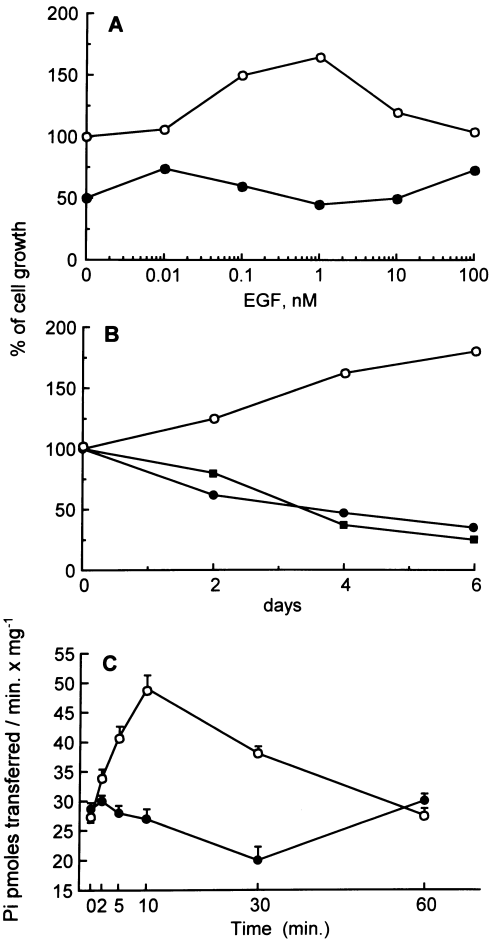

Fig. 4. Effects of 8-Cl-cAMP on the proliferative response of KB cells to EGF and on MAPK activity. (A) Cells have been exposed for 48 h to different concentrations of EGF in the presence (●) or absence (○) of 10 μM 8-Cl-cAMP. Cell growth was expressed as a percentage of untreated controls. The data show means of three separate experiments of triplicate determinations. The S.E. never exceeded 10%. (B) Cells have been cultured for the indicated times in the presence of the following agents: (○) 10 nM EGF, (●) 10 μM 8-Cl-cAMP, (■) 10 nM EGF+10 μM 8-Cl-cAMP. The cell growth was expressed as a percentage of untreated controls. The data show means of three separate experiments of triplicate determinations. The S.E. never exceeded 10%. (C) A MAPK assay performed after different times of exposure to 10 nM EGF on untreated (○) or 10 μM 8-Cl-cAMP-treated (●) KB cells, as described in Section 2. The data were expressed as inorganic phosphates (P_i) pmoles transferred/min/mg of protein lysate. The data show means of three separate experiments of triplicate determinations. Bars, S.D.

duction of MAPK activity. Treatment of KB cells with EGF for 10 min induced an almost 100% increase in the MAPK activity (Fig. 4B). This effect was however completely lost in KB cells which have been exposed for 48 h to 10 μM 8-Cl-cAMP (Fig. 4B). Therefore, the 8-Cl-cAMP-induced disregulation of EGF-R internalization and degradation is associated to a lack of EGF-induced mitogenic response and to a block in the EGF-R signalling cascade.

4. Discussion

Cellular receptors for peptide growth factors and other down-stream signal transducers are important regulators of normal and tumor cell proliferation [1]. EGF-R has been found to be overexpressed on neoplastic cells of epithelial origin and has been implicated in the process of cell growth and transformation [1].

8-Cl-cAMP, a synthetic cAMP analogue which can be considered an antagonist of PKA-I, inhibits the cell proliferation without exerting cytotoxic effects [14–16,28]. Moreover, 8-Cl-cAMP is under clinical evaluation in phase I trials as anticancer agent [17]. Several reports showed that 8-Cl-cAMP may interfere with growth factor autocrine pathways and growth factor-induced transformation [20,29,30]. On the basis of these considerations, we have investigated the effects of 8-Cl-cAMP on the EGF-R expression in KB cells, which are dependent on EGF for their proliferation [25]. We have found that growth inhibition induced by 8-Cl-cAMP was paralleled by an about 65% increase of EGF-R expression on the cell membrane. We have previously shown that also IFNα or cytotoxic drugs used at cytostatic concentrations, such as cytosine arabinoside and 5-aza-2′ deoxy cytidine, increase the expression of EGF-R on epithelial cancer cells [8,9,12,13]. Therefore, such an effect could be ascribed to a homeostatic response of cancer cells to the growth inhibitory stimuli induced by the drugs. However, in the present study, a discrepancy in the magnitude of 8-Cl-cAMP effects on the EGF-R synthesis and expression was described. These effects occurred together with an increase of EGF-induced EGF-R down-regulation and degradation. Moreover, the EGF-mediated ubiquitination of EGF-R was dramatically increased in 8-Cl-cAMP-treated KB cells. The internalization and the subsequent degradation process is an important mechanism of modulation of EGF-R expression and function. It is possible that the ubiquitin pathway may account, at least in part, for the degradation of cell surface membrane proteins. Also ubiquitination may serve as a regulator of cell proliferation since the human oncogene tre-2 bares a de-ubiquitinating property [31]. Recently, it has been demonstrated that EGF-R is ubiquitinated after EGF-mediated internalization [7]. However, it has also been reported that a mutant EGF-R that fails to be ubiquitinated is only partially defective in the EGF-stimulated down-regulation [7]. Therefore, ubiquitination is not an obligatory requirement for EGF-R degradation and the redundancy of pathways for EGF-R down-regulation may reflect the importance of receptor degradation for the attenuation of signal transduction. In our experimental model, the preservation of the catalytic activity of EGF-R in 8-Cl-cAMP-treated cells correlated with the increased ubiquitination of the receptor. The requirement of an active kinase for the ubiquitination process has been described for the protein kinase C family and for the receptor tyrosine kinase c-kit [32]. In the case of EGF-R, the kinase activity is required for EGF-stimulated ubiquitination although the role of the receptor tyrosine phosphorylation has not been completely established [6,7]. Interestingly, a direct interaction between cAMP-dependent protein kinase PKA-I isoenzyme and EGF-R has been recently described [33]. Since 8-Cl-cAMP can be considered an antagonist of PKA-I, it may be involved in the disruption of this association leading to modulation of the EGF-R expression and trafficking. Moreover, in our experimental model, an increased receptor degradation was paralleled by the loss of mitogenic response and of MAPK activity, induced by EGF in KB cells. Therefore, the increase of EGF-R ubiquitination could be involved in a general mechanism of negative regulation of receptor-mediated activation of a growth promoting

pathway. Furthermore, it is important to consider that the EGF-R up-regulation induced by other agents, such as IFNα, was associated with a sensitization of tumor cells to the growth promoting activity of EGF [13].

The understanding of the molecular mechanisms regulating the expression and function of PGF-Rs in tumor cells exposed to cytostatic agents could be useful for the design of innovative approaches to tumor cell growth inhibition, combining anti-proliferative agents and bioreagents that target such receptors [21–24]. Moreover, the pharmacological increase of the ubiquitination process, as induced by 8-Cl-cAMP, could be a further approach for the control of PGF-R-dependent tumor cell growth.

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