Minireview

Interactions between the Epidermal Growth Factor Receptor and Type I Protein Kinase A: Biological Significance and Therapeutic Implications

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Abstract

Peptide growth factors regulate normal cellular proliferation and differentiation through autocrine and paracrine pathways and are involved in cancer development and progression. Among the endogenous growth factors, the epidermal growth factor (EGF)-related proteins play an important role in the pathogenesis of human cancer. In fact, overexpression of EGF-related growth factors such as transforming growth factor α and amphiregulin and/or their specific receptor, the EGF receptor (EGFR), has been detected in several types of human cancers, including breast, lung, and colorectal cancers. Therefore, the blockade of EGFR activation by using anti-EGFR monoclonal antibodies (MAbs) has been proposed as a potential anticancer therapy.

The cAMP-dependent protein kinase (PKA) is an intracellular enzyme with serine-threonine kinase activity that plays a key role in cell growth and differentiation. Two PKA isoforms with identical catalytic (C) subunits but different cAMP-binding regulatory (R) subunits (defined as RI in PKAI and RII in PKAII) have been identified. Predominant expression of PKAII is found in normal nonproliferating tissues and in some types of tumors. PKAI expression and function is correlated recently with poor prognosis in breast cancer patients. Inhibition of PKAI expression and function by specific pharmacological agents such as the selective cAMP analogue 8-chloro-cAMP (8-Cl-cAMP) induces growth inhibition in various human cancer cell lines in vitro and in vivo.

We have provided experimental evidence of a functional cross-talk between ligand-induced EGFR activation and PKAI expression and function. In fact, PKAI is overexpressed and activated following transforming growth factor α-induced transformation in several rodent and human cell line models. Furthermore, PKAI is involved in the intracellular mitogenic signaling following ligand-induced EGFR activation. We have shown that an interaction between EGFR and PKAI occurs through direct binding of the RI subunit to the Grb2 adaptor protein. In this respect, PKAI seems to function downstream of the EGFR, and experimental evidence suggests that PKAI is acting upstream of the mitogen-activated protein kinase pathway.

We have also demonstrated that the functional interaction between the EGFR and the PKAI pathways could have potential therapeutic implications. In fact, the combined interference with both EGFR and PKAI with specific pharmacological agents, such as anti-EGFR blocking MAb and cAMP analogues, has a cooperative antiproliferative effect on human cancer cell lines in vitro and in vivo. The antitumor activity of this combination could be exploited in a clinical setting because both the 8-Cl-cAMP analogue and the anti-EGFR blocking MAB C225 have entered human clinical trial evaluation.

Finally, both MAB C225 and 8-Cl-cAMP are specific inhibitors of intracellular mitogenic signaling that have different mechanisms of action compared with conventional cytotoxic drugs. In this respect, a cooperative growth-inhibitory effect in combination with several chemotherapeutic agents in a large series of human cancer cell lines in vitro and in vivo has been demonstrated for anti-EGFR blocking MAb or for 8-Cl-cAMP. Therefore, the combination of MAB C225 and 8-Cl-cAMP following chemotherapy could be investigated in cancer patients.

Introduction

Growth factors regulate normal cellular proliferation and differentiation and are important in initiating and maintaining neoplastic transformation (1). Cancer cells generally exhibit a decreased requirement for exogenous growth factors as compared with normal cells (2). The relaxation in growth factor dependency is due in part to the ability of tumor cells to synthesize growth factors that can regulate their proliferation through autocrine and paracrine mechanisms by activating specific cell membrane receptors (2). Among the endogenously produced peptide growth factors, TGF-α, AR, and CRIPTO are
EGF-related proteins that play an important role in the pathogenesis of several human epithelial cancers (3). TGF-α, AR, and CRIPTO are expressed by the majority of human primary and metastatic breast and colorectal cancers (4–5). Suppression of synthesis of these growth factors by pharmacological tools such as a specific RNA or DNA antisense approach inhibits human colon and breast cancer cell growth (6–9). Both TGF-α and AR bind to and activate the EGFR. Enhanced expression of EGFR has been detected in the majority of glioblastomas, breast, lung, ovarian, colorectal, and renal carcinomas (3). Overexpression of EGFR has been associated with a poor prognosis in several human tumor types, including breast cancer (3). The EGFR is a Mr 170,000 transmembrane glycoprotein with an external binding domain and an intracellular tyrosine kinase domain. Following ligand binding, the EGFR is autophosphorylated on several tyrosine residues in the intracellular domain and dimerizes, creating a series of high-affinity binding sites for various adapter molecules that are involved in transmitting the mitogenic signaling to the ras/MAPK signal transduction pathway (10). In this respect, Grb2 is an adaptor molecule composed of one SH2 domain, which binds to phosphorylated tyrosines on tyrosine kinase receptors, and of two SH3 domains, which bind to proline-rich sequences of signaling proteins such as SOS (11). Grb2 allows the coupling of the activated EGFR to ras, phosphatidylinositol kinase, or phospholipase Cγ pathways (11–15). The specific cell membrane receptor for CRIPTO has not yet been identified, although the addition of recombinant CRIPTO protein to human mammary epithelial cells induces the intracellular signaling cascade that leads to MAPK activation (16).

The PKA is an intracellular enzyme with serine-threonine kinase activity that plays a key role in cell growth and differentiation. cAMP acts in mammalian cells by binding to either of two distinct isoforms of PKA, defined PKAI and PKAII. PKAI and PKAII share identical catalytic (C) subunits, but differ in the regulatory (R) subunits (termed RI in PKAII and RII in PKAII, respectively; Ref. 17). The PKA holoenzyme is a tetramer formed of two identical R subunits and two C subunits (17). Upon cAMP binding to the R subunits, the active C subunit is released (17). The synthesis of RI and RII and the relative abundance of PKAI and PKAII isoforms are differentially regulated during differentiation, cell growth, and neoplastic transformation (18). Predominant expression of PKAII is found in normal nonproliferating tissues and in growth-arrested cells, whereas enhanced levels of PKAI are detected steadily in tumor cells and transiently in normal cells exposed to mitogenic stimuli (19). In this respect, PKAI and its regulatory subunit RIIα are generally overexpressed in human cancer cell lines and primary tumors and are induced following transformation by certain oncogenes, such as ras (18–19). Overexpression of RIIα and PKAI has been correlated recently with poor prognosis in breast cancer patients (20). RIIα has also been identified in hepatoma × fibroblast cell hybrids as the tissue extenguisher of differentiation (TSE1), an inhibitor of the expression of several genes related to cell differentiation (21). In contrast, constitutive overexpression of PKAII, following infection with a recombinant retrovirus containing the human RIIβ gene, induces growth inhibition of human cancer cells, and reverts the transformed phenotype of ras-transformed mouse fibroblasts (22–23). Collectively, these data, along with the distinct subcellular location and the differential sensitivity to cAMP concentration for enzyme activation (17–18), suggest that PKAI and PKAII have different functions in the control of cell growth and differentiation.

In recent years, experimental evidence has been provided on a functional link between neoplastic transformation involving the TGF-α-EGFR autocrine pathway and PKAI expression and activity. In this review, we will discuss the biological relevance of the interactions between the activated EGFR and PKAI and we will present data suggesting that the blockade with specific biological agents of the PKAI serine-threonine kinase-dependent pathway and of the EGFR tyrosine kinase-dependent pathway is a potentially useful novel approach in cancer therapy.

### The EGFR-PKAI Connection

**PKAI Expression Is Linked to TGF-α- and ras-dependent Transformation.** Several studies have shown an increased expression of RIIα and PKAI following transformation by TGF-α or ras. On the other hand, an early inhibition of TGF-α and/or ras expression is observed after treatment with selective inhibitors of PKAI, such as 8-Cl-cAMP. These studies have suggested a functional involvement of PKAI in the mitogenic signals transmitted through the EGFR and/or p21ras pathways. For example, in NRK rat fibroblasts, TGF-α- and ras-dependent transformation causes an early induction of RIIα and PKAI expression and a parallel reduction of PKAII (24). The selective inhibition of PKAI by 8-Cl-cAMP is followed by inhibition of TGF-α and p21ras expression, by increased PKAII expression, and by cell growth arrest (24). In NOG-8 mouse mammary epithelial cells, stable overexpression of TGF-α, following transfection with a recombinant plasmid containing the human TGF-α cDNA, determines neoplastic transformation (25). This event is associated with a fall in RIIβ mRNA expression and a parallel rise of RIIα mRNA expression, without major changes in Ca expression (26). Therefore, PKAI becomes the predominant PKA isoform in TGF-α-transformed NOG-8 cells. Treatment of these cells with the specific PKAI inhibitor 8-Cl-cAMP down-regulates PKAI, induces PKAII, and inhibits TGF-α production, restoring the pattern of expression of non-transformed parental cells (26). Similarly to the NOG-8 mouse model, in MCF-10A normal human mammary epithelial cells, overexpression of TGF-α or of an activated ras gene causes neoplastic transformation (27). These events are associated with an increase of PKAI expression and a parallel reduction of PKAII. Down-regulation of PKAI by different pharmacological approaches, such as cAMP analogues or antisense oligonucleotides targeted against RIIα, inhibits TGF-α expression and decreases growth inhibition in MCF-10A cells transformed by either TGF-α or ras (28). Further experimental evidence of a functional cross-talk between TGF-α-EGFR-mediated cell transformation and PKAI expression and function has been provided recently in MDA-MB468 human breast carcinoma cells. In MDA-MB468 cells, a TGF-α-EGFR autocrine growth stimulatory pathway is involved in the control of cell growth and transformation (29). The constitutive inhibition of EGFR expression by transfection of MDA-MB468 cells with an antisense...
EGFR plasmid vector is accompanied by the selective down-regulation of PKAI expression (30).

**PKAI Is Induced by EGFR Activation in Normal Cells.** Different studies have disclosed a link between PKA activation and inhibition of EGFR- and/or ras-dependent activation of MAPK (31–33), but whether a specific PKA isoform is responsible for this effect has not yet been elucidated. On the other hand, it has been shown that an activated ras oncogene inhibits the function of PKA by interfering with the nuclear location of the Ca catalytic subunit, conceivably following release from PKAII (34). Recently, it has been demonstrated that treatment with the cAMP analogue 8-bromo-cAMP or with cAMP-specific phosphodiesterase inhibitors determines inhibition of smooth muscle cell proliferation in rat carotid arteries following injury (35). Because smooth muscle cell proliferation has been shown to be dependent on rasraf-1/MAPK signaling in this model (36), it seems likely that the growth-inhibitory effect is due to the interaction of PKA and ras-mediated signals. PKAI has been causally linked to positive regulation of mitogenic signals through the EGFR and ras pathways (24, 26, 28). We have shown that PKAI expression is induced in nontransformed MCF-10A human mammary epithelial cells following treatment with EGF or TGF-α, and it is functionally involved in S-phase entry (37). MCF-10A cells possess approximately 250,000 EGFR sites/cell and depend on the presence of EGFR or TGF-α in the culture medium for optimal cell growth, because their withdrawal determines growth arrest in the G1, G2 phases of the cell cycle. The addition of complete medium containing EGF to quiescent MCF-10A cells induces Rlα expression 6–9 h before cells enter S phase (37). Selective down-regulation of PKAI expression by pretreatment with an anti-Rlα antisense oligonucleotide blocks S-phase entry of MCF-10A cells following EGF addition, suggesting a role for PKAI in the EGFR-triggered G1-to-S transition (37). Furthermore, retroviral vector-mediated Rlα overexpression enables MCF-10A cells to grow in serum-free medium, bypassing EGF or TGF-α requirement and conferring a phenotype similar to MCF-10A cells transformed by either the TGF-α or the ras genes (27, 37). Taken together, these data suggest that PKAI mediates the mitogenic signaling by growth factors of the EGFR family in human mammary epithelial cells.

It is not yet clear whether PKAI activation is involved only in the downstream propagation of the EGFR-induced mitogenic signaling or whether PKAI is part of the signal transduction cascade induced by other growth factors. In this respect, an early involvement of PKAI following other mitogenic stimuli in different cell types has been reported. In normal human T lymphocytes, CD3 stimulation or phytohemagglutinin addition causes specific PKAI induction and activation within 5–10 min (38–39). In FRTL-5 rat thyroid cells, which depend on TSH for cell proliferation and thyroglobulin synthesis, a rapid induction of Rlα mRNA occurs within 30 min after TSH addition with an increase in PKAI that anticipates cell entry into S phase (40). Conversely, inhibition of PKAI synthesis by an anti-Rlα antisense oligonucleotide abrogates the TSH-induced mitogenic effect (40). Interestingly, a tumor-specific transforming sequence derived by the fusion of the ret tyrosine kinase receptor and the Rlα genes has been isolated in two human papillary thyroid carcinomas (41).

Direct Interaction of PKAI with the Activated EGFR through Grb2. It is possible that the different biological effects of the two PKA isoforms is due in part to their intracellular localization, which could allow interactions with potentially different adaptor molecules and/or substrates. The subcellular distribution of the PKA isoforms depends also on the interaction with a specific class of anchoring proteins (AKAPs), which may contribute to their functional role (42). PKAI has been found in association with the plasma membrane, the cytoskeleton, the secretory granules, and the nucleus (19, 43–45). PKAI is broadly distributed in the cytoplasm (46) and may also translocate to the cell membrane. In fact, in human T lymphocytes, PKAI is found in the inner face of the cell membrane, where it is associated with the T-cell receptor-CD3 complex after T-cell activation (39, 47). In EGF-stimulated MCF-10A cells, a cell membrane translocation of PKAI anticipates cell entry into S phase (37, 48).

Furthermore, we have shown recently that in MCF-10A both the Rlα and Ca subunits, but not the RIIβ subunit, coprecipitate with the ligand-activated EGFR and that they are present in the EGFR macromolecular signaling complex as an activatable PKAI holoenzyme (49). Whole-cell immunofluorescence studies have shown that Rlα staining is superimposable to that of EGFR, which, following ligand activation, translocates from the cell membrane to the cytoplasm by endocytosis (15).

PKAI provides a relevant contribution to the propagation of EGFR-activated mitogenic intracellular signaling. In fact, overexpression of PKAI in MCF-10A Rlα cells determines a constitutive activation of MAPK, mimicking the effect of EGF addition to quiescent MCF-10A cells (49). In contrast, inhibition of PKAI-mediated signaling by a Rlα antisense oligonucleotide or by 8-Cl-cAMP significantly reduces MAPK activation in EGF-stimulated MCF-10A cells (49).

Rlα contains a stretch of uncharged amino acids and a NH2-terminal proline-rich sequence (47), which may potentially bind to SH3 domains (11). We have found that PKAI interacts with the EGFR through Rlα binding to either NH2- or COOH-terminal SH3 domains of Grb2 (49). Rlα is associated with Grb2 independently from EGFR activation, suggesting that Rlα and Grb2 may form a complex before ligand activation of EGFR and recruitment of Grb2 to autophosphorylated tyrosine residue(s) (49). Because an activatable PKAI holoenzyme is present at the EGFR site following ligand-dependent activation, PKAI may interact with specific substrates involved in the EGFR-dependent signaling cascade. However, it is not yet defined whether PKAI is involved in a specific signaling pathway or participate to the integration of multiple growth factor-induced signals. The identification of PKAI-specific substrates will be an important step to elucidate the role of this PKA isoform in the transduction of mitogenic signals (Fig. 1).

Therapeutic Implications

**Inhibition of EGFR.** Because experimental and clinical studies have provided evidence for a TGF-α-mediated autocrine

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4 Unpublished results.
growth stimulatory pathway in a variety of human cancers, the blockade of the TGF-α-EGFR autocrine pathway by using anti-EGFR blocking MAbs, recombinant proteins containing TGF-α or EGF fused to toxins, or EGFR-specific tyrosine-kinase inhibitors has been proposed as a potential therapeutic modality (50–57). In this respect, several blocking anti-EGFR MAbs that inhibit the in vitro and in vivo growth of human cancer cell lines that express TGF-α and EGFR have been generated (58–61). Among these, MAb 528 and MAb 225 are two mouse MAbs that have been extensively characterized for their biological and preclinical properties and represent the first series of anti-EGFR blocking agents that have entered clinical evaluation in cancer patients (50–51, 58–59). MAb 528 and MAb 225 bind to the EGFR with affinity similar to EGF and TGF-α, compete with these ligands for receptor binding, and block EGF- or TGF-α-induced activation of EGFR tyrosine kinase (58–59). In addition, it has been shown that the combined treatment of mice bearing well-established human tumor xenografts with MAb 528 or with MAb 225 and with cytotoxic drugs, such as doxorubicin or cisplatin, significantly increases the antitumor activity of these drugs (62–63). To avoid human anti-mouse antibody production that can interfere with the therapeutic efficacy of repeated administrations of mouse MAbs in humans, a chimeric human-mouse MAb 225 (MAb C225), that contains the human IgG1 constant region, has been developed recently and purified for clinical use and is in early clinical trials in patients with advanced cancer (64–65).

**Inhibition of PKAI.** The potential usage of cAMP analogues for the therapy of cancer has been widely discussed in the past two decades. However, the lack of selectivity and the high doses required for the available cAMP analogues have been a major obstacle to the development of this approach into feasible clinical trials. A renewed interest has been fostered by the discovery of a new class of site-selective cAMP analogues, which are capable of distinguishing the activity of the PKA at micromolar concentrations (18). 8-Cl-cAMP, the most potent site-selective cAMP analogue, discriminates between the two cAMP binding sites on RI and RII and is able to down-regulate RII by facilitating the degradation of the protein, while up-regulating RII expression at the transcriptional level (18, 66). We have shown that down-regulation of RII by 8-Cl-cAMP is associated with growth inhibition (with IC₅₀'s ranging between 0.01 and 10 μM) and differentiation in a wide variety of human cancer cell lines in vitro and in vivo (66–68). These effects are accompanied by an increased RII:RI ratio and by inhibition in vitro and in vivo of different oncogenes and growth factor expression, including ras, myc, erbB2, TGF-α, basic fibroblast growth factor, and vascular endothelial growth factor (24, 26, 28, 66–69). The inhibition in the expression of these genes induced by 8-Cl-cAMP treatment is time and dose dependent and occurs at the mRNA level (26, 69). It has been shown recently that 8-Cl-cAMP is able to revert multidrug resistance in a variety of multidrug resistance cancer cell lines, restoring the sensitivity to cytotoxic drugs (70). Although it is still debated whether metabolites such as 8-Cl-adenosine may contribute to the 8-Cl-cAMP effect (19), 8-Cl-cAMP is presently under clinical investigation in Phase II trials, because we have shown in a Phase I clinical trial that 8-Cl-cAMP can be safely administered to cancer patients at doses that achieve plasma concentrations within the potential therapeutic range for growth inhibition (71).

A more direct approach to inhibit the synthesis and function of PKAI has been developed by the use of phosphorothio...
ate-modified antisense oligonucleotides targeted against the 5' coding sequence of the human RIa mRNA. Treatment with these antisense oligonucleotides suppressed RIa production and determined inhibition of in vitro proliferation in various human cancer cell lines and in vivo growth of LS-174T human colon cancer xenografts (72–74). Although phosphorothioate-modified antisense oligonucleotides have shown promising results as a first generation of oligonucleotides, a series of novel MBOs targeted against RIa has been generated to further improve their therapeutic potential. MBOs have appropriately placed segments of phosphorothioate oligonucleotides and segments of modified oligodeoxy- or oligoribonucleotides, such as methylyphosphonate linkages (75). The anti-RIa second generation antisense oligonucleotides have a significant antiproliferative effect in vitro (with IC_{50} ranging between 0.01 and 1 μM) and in vivo in a number of human cancer cell lines.4 Because MBOs have shown a significant reduction of side effects and a better pharmacokinetic profile in vivo as compared with phosphorothioate oligonucleotides (75), they are entering clinical evaluation in cancer patients.

**Combined Blockade of EGFR and PKAI.** The large body of experimental evidence suggesting a functional link between neoplastic transformation involving TGF-α-induced EGFR activation and PKAI has prompted studies to evaluate whether the double blockade of EGFR and PKAI may have an antiproliferative effect in human cancer cells and may improve the antitumor activity of either blockade alone. In a first series of experiments, we have evaluated the growth-inhibitory effects of the combined treatment with the anti-EGFR MAB 528 and 8-Cl-cAMP on two human colon cancer cell lines (GEO and CBS) and on a human breast cancer cell line (MDA-MB468; Ref. 76). The combination treatment with these two agents had a more severe TGF-α and express functional EGFRs (76). A 3- to 5-fold reduction in the 8-Cl-cAMP IC_{50} was observed when the tumor cells were exposed to low noninhibitory doses of MAb 528 in combination with 8-Cl-cAMP. Furthermore, treatment with higher concentrations of MAb 528 and 8-Cl-cAMP determined a similar degree of cooperative growth inhibition. We have next demonstrated that the combination of the humanized chimeric anti-EGFR MAb C225 and 8-Cl-cAMP is a highly effective anticancer treatment regimen in vivo using human GEO colon carcinoma xenografts as a model (77). The combined blockade of EGFR and of PKAI produced an antitumor effect that is not simply additive. Treatment with low doses of MAb C225 and 8-Cl-cAMP for 5 weeks resulted in a long-term suppression of GEO tumor growth, because tumors resume their growth only after ~8 weeks from cessation of the treatment (77). This effect was accompanied by a statistically significant benefit in animal survival in the group treated with both agents as compared with the groups treated with a single agent. The anticancer effect of the MAb C225 plus 8-Cl-cAMP combination was also accompanied by the suppression in tumor cell production of proteins that function as autocrine growth factors, such as TGF-α, AR, and CRIPTO, or as paracrine angiogenic growth factors, such as vascular endothelial growth factor and basic fibroblast growth factor (77). In addition, a strong inhibition in tumor-induced host neoangiogenesis was observed. The suppression of synthesis of endogenous growth factors has also potential therapeutic relevance. This effect could lead to tumor quiescence in terms of proliferation and neoangiogenic stimulation. Therefore, long-term treatment with anticancer agents that affect intracellular signaling, such as anti-EGFR MAbs and 8-Cl-cAMP, may obtain a control of cancer cell growth and spreading with no toxicity. In fact, MAb C225 and 8-Cl-cAMP treatments were well tolerated by the animals because no signs of toxicity were observed in any treatment group.

We are presently testing whether a similar cooperative antitumor effect could be also obtained by a combination of anti-RIa MBOs and anti-EGFR MAbs. Preliminary experiments in human breast and renal carcinoma cell lines have shown a supradditional growth-inhibitory effect with this approach.4

**Conclusions**

PKAI expression and activation is involved in the intracellular mitogenic signaling following EGFR activation. The interaction between EGFR and PKAI occurs through direct binding of the RIα subunit to the Grb2 adaptor. Therefore, PKAI seems to function downstream to the EGFR, and experimental evidence suggests that PKAI is acting upstream to the MAPK pathway.

The combined interference with EGFR and PKAI with specific pharmacological agents, such as anti-EGFR blocking MAbs and cAMP analogues, has a cooperative antiproliferative effect on human cancer cell lines in vitro and in vivo. The antitumor activity of this combination could be explored in a clinical setting because both 8-Cl-cAMP and MAb C225 have entered human clinical trial evaluation.

Finally, both anti-EGFR MAbs and 8-Cl-cAMP are inhibitors of intracellular mitogenic signaling with different mechanisms of action compared with cytotoxic agents. In this respect, a cooperative growth-inhibitory effect in combination with several conventional cytotoxic drugs in a large series of human cancer cell lines in vitro and in vivo has been demonstrated for anti-EGFR blocking MAbs or for 8-Cl-cAMP (62–63, 78). Therefore, the combination of MAb C225 and 8-Cl-cAMP following chemotherapy could be investigated in cancer patients.

**References**

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