Cyclic AMP Distinguishes Between Two Functions of Acidic FGF in a Rat Bladder Carcinoma Cell Line

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Abstract. The rat bladder carcinoma cell line NBT-II exhibits two completely different responses to acidic FGF (aFGF): at high cell density, aFGF is a potent mitogen whereas at low cell density, aFGF acts as a scattering agent that can convert the epithelial NBT-II cells into fibroblastic-like, motile cells. The basis of the dual action of aFGF has been approached by using substances interfering with the transducing pathways known to be activated by growth factors. Genistein and tyrphostin, two inhibitors of tyrosine kinases, inhibit both cell scattering and mitogenesis induced by aFGF. Conversely, sodium orthovanadate, a potent inhibitor of tyrosine phosphatases can reproduce the two effects of aFGF, indicating that protein tyrosine phosphorylations are determinant in the two pathways. In contrast, transforming growth factor (TGF)-β1 is a strong inhibitor of DNA synthesis induced by aFGF but has no effect on cell scattering, providing evidence that the two pathways are divergent. In an attempt to determine the specificity of the pathways of aFGF we found that the level of cAMP, which can be externally elevated, is of pivotal importance in distinguishing between the two transducing pathways leading to either DNA replication or cell dispersion. Forskolin, 8-bromo cAMP, dibutyryl-cAMP, and cholera toxin are all capable of potentiating the mitogenic effect of aFGF while strongly inhibiting its scattering action. Moreover, addition of any of these substances to NBT-II cells converted into fibroblasts immediately induces their reversion towards an epithelial phenotype. These findings support a role for cAMP as a modulator of the effects of aFGF. Moreover, basal cAMP synthesis, which is not affected by aFGF, is higher in sparse than in dense cultures indicating that the level of cAMP depends on the status of the cell. Altogether, these results suggest that establishment and maintenance of the epithelial state require a precise regulation of cAMP level.

CELL migrations have been described as a major event in tissue formation and remodeling that take place during embryonic and adult life. They are also components of certain pathological situations such as invasion and metastasis of tumor cells (27). Even though the cellular mechanisms responsible for the acquisition of cell motility are not yet fully understood, it is clear that they are multiple, depending in part on the state of differentiation of the cells induced to migrate. One of the most puzzling examples of cellular transformations leading to cell motility is provided by the epithelial–mesenchymal transition (EMT),[1] which has been characterized in many morphogenetic processes (13, 23, 37). In such cases, the acquisition of cell motility correlates with dramatic changes in the program of cell differentiation: the cohesive epithelial cells lose their epithelial features and are converted into individual, motile fibroblastic cells. Because of the general importance of EMT, several groups have established in vitro model systems, using well-defined cell cultures to approach the individual steps of such complex processes (17). The search for inducer molecules of these processes has led to the discovery that growth factors, primarily known as regulators of cell proliferation, can also stimulate in vitro cell motility (2, 17, 34, 45, 47) and control in vivo embryonic EMT (8, 35). Among them, scatter factor (SF), which is identical to hepatocyte growth factor (HGF) and therefore elicits scattering as well as mitogenic functions, is a morphogenetic factor for aggregates of MDCK cells (29). These results suggest that factors that have been described in vitro as scattering agents promoting EMT-like changes of epithelial cells could act in vivo as morphogens involved in the plasticity of epithelial tissues.

Because many growth factors, such as SF/HGF, are multifunctional it is important to understand the mechanisms that trigger the specificity of the cellular response to a given growth factor. Although this question is far from being elucidated (9) there is a growing body of evidence indicating that signal transduction generated by a given growth factor can follow multiple pathways (40). The choice of the pathways that are activated may be dictated in part by the cell itself.

We have used the rat bladder carcinoma-derived cell line

1. *Abbreviations used in this paper*: aFGF, acidic FGF; DP, desmoplakin; EMT, epithelium–mesenchyme transition; IBMX, 3-isobutyl-1-methylxanthine; TGF, transforming growth factor.

NBT-II (38) as a convenient in vitro model for studying the multifunctionality of acidic FGF (aFGF). On addition of aFGF, subconfluent cultures of NBT-II cells dissociate and give rise to individual, elongated, actively migrating cells (41), whereas confluent cultures enter mitosis in response to the same factor (42). Similarly, EGF had already been reported to induce confluent cultures of rat intestinal epithelial cells to proliferate and subconfluent cultures to migrate (6). Here, we have analyzed the two biological responses of NBT-II cells stimulated by aFGF after treatment with agents known to synergize or antagonize second messenger molecules. Although the transducing pathways activated by the interaction of peptide hormones, referred to as growth factors, with their cognate receptors remain poorly defined, there are three major enzymatic activities potentially important in the signaling cascades. First, tyrosine-specific protein kinases have been shown to play a role in transducing signals of several growth factors which directly bind to receptors harboring a tyrosine kinase activity (40). Second, activation of phospholipase (PLC) has also been proposed as a primary event in signal transduction in response to several growth factors (4). The third pathway involves the production of cyclic AMP (cAMP) by stimulated adenylate cyclase (14). Our results demonstrate that tyrosine-specific kinase activity is central in the two biological responses to aFGF. Furthermore, a sustained elevation of cAMP level antagonizes the dispersing response to aFGF while potentiating the mitogenic action of aFGF. These results strongly support the existence of second messenger molecules restricted to each biological response to aFGF.

Materials and Methods

Reagents

Mouse mAb against vimentin was purchased from Amersham International (Buckinghamshire, UK). Mouse mAb against desmosomal proteins desmoplakins (DPs) I and II was the generous gift of Professor W. W. Franke (German Cancer Research Center, Heidelberg, FRG). Texas red-coupled goat anti-mouse IgG (Immunotech, Marseille, France) was used as secondary antibody. Human recombinant aFGF (22) was kindly provided by Dr. M. Jaye (Rhône-Poulenc, King of Prussia, PA). FCS was from Biological Industries (Kibbutz Beth Haemek, Israel). Forskolin, pertussis toxin, and sodium orthovanadate were purchased from Sigma Chemical Co. (St. Louis, MO), cholera toxin from Calbiochem Corporation (La Jolla, CA), and genistein from ICN Biochemicals (Cleveland, OH). Transforming growth factor (TGF)-β1 was obtained from British Biotechnology Limited (Oxford, UK). Tyrphostin AG18 was the generous gift of Dr. A. Levitzki (The Alexander Silberman Institute of Life Sciences, Jerusalem, Israel).

Cell Culture

The NBT-II cell line, originally established by Tohoshima and colleagues (38), was obtained from Prof. M. Mareel (University Hospital, Ghent, Belgium). The original tumor from which NBT-II cell line was derived is a squamous bladder carcinoma induced by carcinogenesis. Under standard conditions of culture, the cells assume an organization and morphology typical of epithelial cells. The cells were routinely grown (37°C in 5% CO_2) in DME containing 10% heat-inactivated FCS (standard medium), as described previously (7). Sparse/subconfluent/low-density cultures are defined by 30–50% cell density, compared with 100% cell density for confluent/high-density cultures.

Cell motility assay was done using videocinematography as described previously (41).

In Vitro Wound Model

NBT-II cell monolayers were obtained after 48 h of culture in standard medium. They were gently scratched with a Gilson pipette yellow tip, and extensively rinsed with DME to remove all cellular debris. The procedure left a cell-free area of substratum ("wound"). The cultures were then allowed to grow overnight in the presence or absence of various factors and reagents. The next morning, cultures were rinsed with PBS, stained with Coomassie blue, and photographed on T-MAX 100 film (Eastman Kodak Co., Rochester, NY) with an Olympus camera mounted on an inverted microscope (Leitz, Wetzlar, FRG).

Indirect Immunofluorescence Microscopy

NBT-II cells were seeded on glass coverslips. 2 d after plating, standard medium was replaced by DME. aFGF (10 ng/ml) plus heparin (10 μg/ml), or various reagents were added for 8 h. Subsequently, cells were fixed and processed for immunocytochemistry as described previously (7).

Mitogenic Assay

Cells were seeded in 24-well Nunc plates and grown to confluency in standard medium after which the medium was changed to DME for 24 h. The cells were then incubated with growth factors or reagents for 24 h. [methyl-^{3}H] thymidine (specific activity, 42 Ci/mmol, Amersham) was added at 1 μCi/ml for the last 4 h of the experiment. At the end of the labeling period,

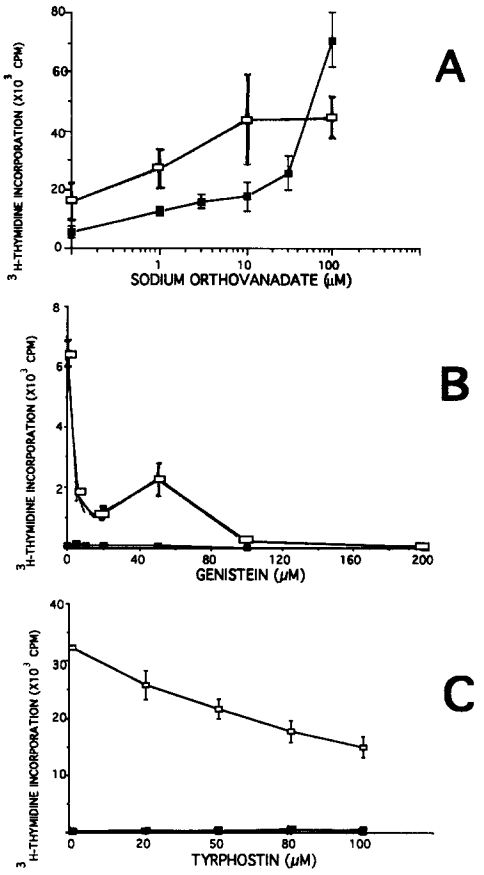

Figure 1. Reinitiation of DNA synthesis in confluent NBT-II cells is dependent on tyrosine phosphorylation. NBT-II cells arrested by serum starvation for 24 h were stimulated for 20 h before a 4 h incubation with [^{3}H]thymidine. The number of counts per minute incorporated was determined as indicated in Materials and Methods. Each point represents the mean value of three identical wells ± standard error. In *A*, cells were stimulated by increasing concentrations of sodium orthovanadate alone (■) or in conjunction with 0.1 ng/ml aFGF (□). In *B*, cells were incubated with genistein alone (■) or genistein in conjunction with 1 ng/ml aFGF (□). In *C*, cells were incubated with tyrphostin AG18 alone (■), or tyrphostin AG18 and 1 ng/ml aFGF (□).

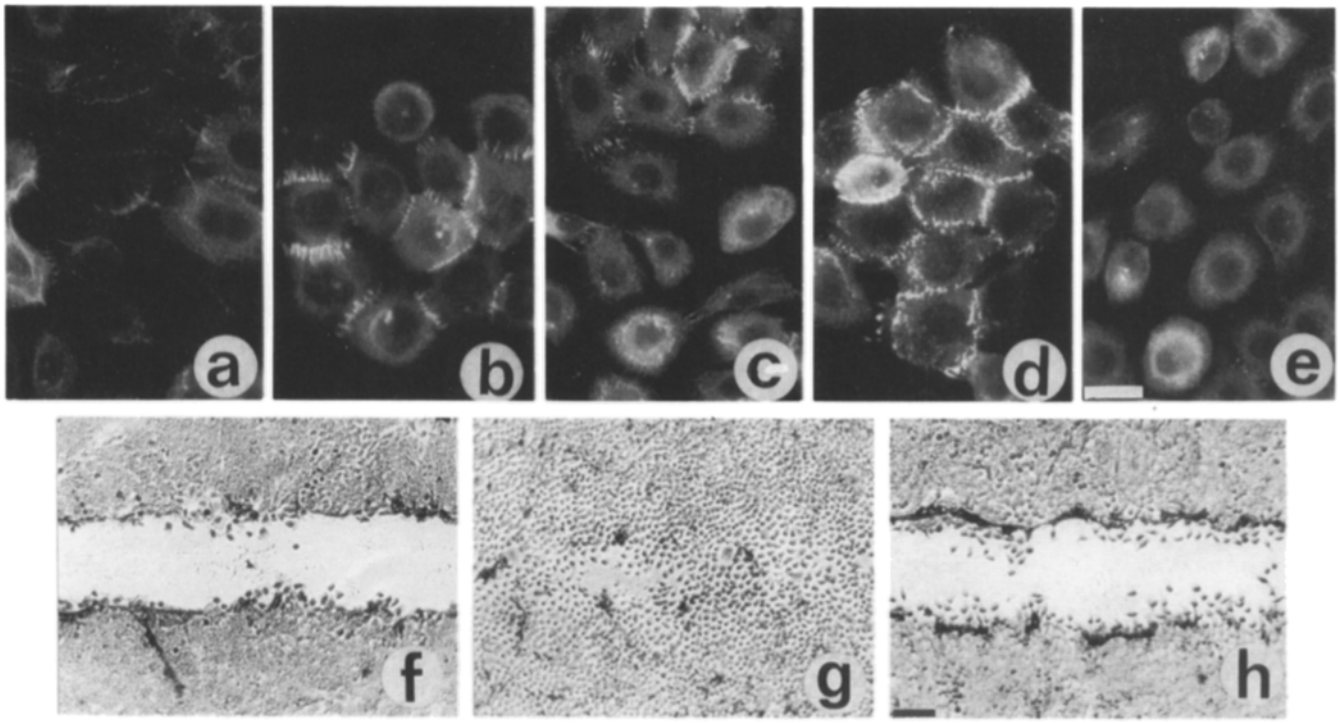

Figure 2. Induction of NBT-II cell dispersion is dependent on tyrosine phosphorylation events. (*a–e*) DP immunolabeling of NBT-II cells incubated for 8 h with 10 ng/ml aFGF (*a*), 100 μM tyrphostin AG18 plus 10 ng/ml aFGF (*b*), 50 μM genistein + 10 ng/ml aFGF (*c*), 100 μM genistein + 10 ng/ml aFGF (*d*), or 100 μM sodium orthovanadate (*e*). After the incubation period with the various reagents, cell cultures were fixed and processed for immunofluorescence microscopy. Note the partial loss of DP peripheral immunostaining in *c*, and the persistence of DP immunoreactivity in cell contacts in *b* and *d*. (*f–h*) Penetration of isolated NBT-II cells into wounded areas of cell monolayers under standard conditions (*f*), or with 20 ng/ml aFGF (*g*), or in the presence of 100 μM sodium orthovanadate (*h*). Bars: (*a–e*) 20 μm; (*f–h*) 200 μm.

each culture plate was washed twice with ice-cold PBS followed by the addition of 1 ml ice-cold 10% TCA and incubated for 30 min at 4°C. After four washes with water, cells were solubilized with 0.1 N sodium hydroxide, and radioactivity was quantitated by liquid scintillation counting. Experiments were conducted in triplicate cultures.

Binding Studies

Human recombinant aFGF was labeled by a modification of the chloramine T method (21) as described (33). Specific activities of the iodinated ligand varied from 960 to 2,150 dpm/fmol of aFGF.

Equilibrium binding assays on NBT-II cells were carried out as previously described (41). When appropriate (see Results), cells were preincubated for 4 h in DME containing the reagents to be tested.

Cyclic AMP Radioimmunoassay

G_0-arrested NBT-II cells were washed with DME and equilibrated for 30 min in this medium before the additions indicated in the legend to Table II. At the end of the incubation the medium was removed, the cultures were rinsed twice with ice-cold PBS, and cells were lysed for 20 min at 4°C with 0.5 ml ethanol/5 mM EDTA (2:1). Cell extracts were evaporated and their cAMP content was determined using a radioimmunoassay kit obtained from ERIA Diagnostics Pasteur (Marnes-la-Coquette, France), after reconstitution of the extracts with the sodium acetate buffer, pH 6.2, used in this assay.

Results

Tyrosine Kinases Are of Central Importance in the Biological Responses Elicited by aFGF

As previously described (7, 41), sparse cultures of NBT-II cells assume an epithelial organization under standard conditions of culture. On exposure to aFGF, the epithelial clusters readily dissociate, giving rise to individual, fibroblastic-like cells that actively migrate. The overall changes in NBT-II cell morphology, appearing after addition of aFGF, are highly reminiscent of an EMT (i.e., an eventually reversible process of conversion between the epithelial and mesenchymal states). In our system we have used the term EMT to indicate that NBT-II cells lose some epithelial characteristics and acquire certain properties of fibroblastic cells. We also demonstrated that aFGF which is a potent mitogen for confluent cultures of NBT-II cells, is unable to promote cell division under subconfluent conditions of culture. Other factors that were found to induce dispersion of NBT-II cells (EGF, TGFα, IGF-I) (16 and A. M. Vallés, unpublished results) reproduce the dual function of aFGF under the two different culture conditions. Interestingly, all these scatter factors bind to tyrosine kinase receptors.

Because of the unique property of growth factors activating receptor tyrosine kinase to induce either dispersion or proliferation of NBT-II cells, we carried out a series of experiments in which tyrosine kinase activity was specifically inhibited or enhanced. Genistein and tyrphostin AG18 were used to specifically inhibit protein-tyrosine phosphorylation (1, 48) and sodium orthovanadate to inhibit tyrosine phosphatases (19). As shown in Fig. 1, genistein and tyrphostin AG18 dramatically reduced [^{3}H]thymidine incorporation in cells stimulated with aFGF. Conversely, sodium orthovanadate was a potent mitogen for NBT-II cells. Interestingly, it was active on confluent cultures only and had no mitogenic

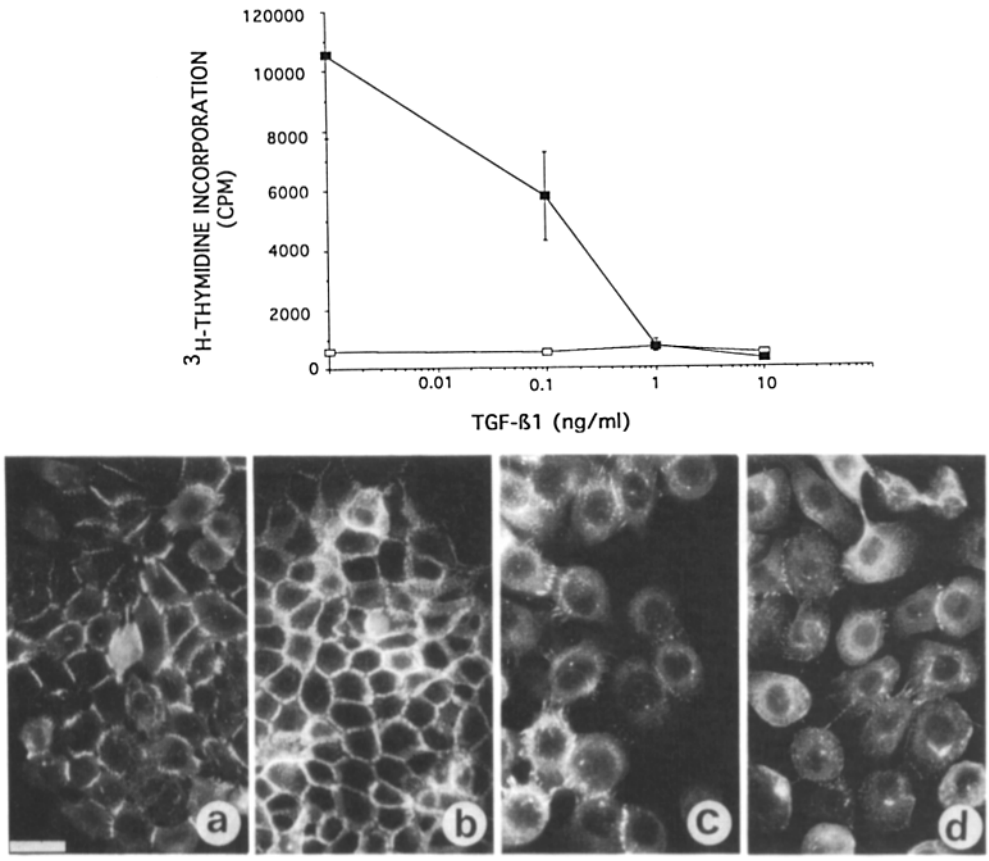

Figure 3. TGF-β1 abolishes the mitogenic action of aFGF without affecting its dispersing effect. (*Upper panel*) The reinitiation of DNA synthesis was estimated by [^{3}H]thymidine incorporation in confluent cultures of cells deprived of serum for 24 h before addition of TGF-β1 alone (□) or in conjunction with 1 ng/ml aFGF (■). The number of counts per minute incorporated was determined as indicated in Materials and Methods. Each point represents the mean value of three identical wells ± standard error. (*Lower panels*) Sparse cultures of NBT-II cells were incubated for 8 h in standard medium without (*a*) or with TGF-β1 at 10 ng/ml (*b*). Alternatively, cells were incubated for the same period of time with aFGF at 10 ng/ml alone (*c*) or in conjunction with 10 ng/ml TGF-β1 (*d*) before fixation and processing for immunofluorescence labeling with anti-DP antibody. Note that TGF-β1 had no effect on the accumulation of DP immunoreactivity in lateral contacts of control cells (*b*) and did not perturb its internalization in cells stimulated with aFGF (*d*). Bar, 20 μm.

effect on subconfluent cultures (data not shown). In addition, it markedly increased [^{3}H]thymidine incorporation in cells stimulated with aFGF (Fig. 1). These results demonstrated that sodium orthovanadate could mimic the mitogenic action of aFGF thus suggesting that tyrosine kinase activation was a central event in aFGF-induced mitogenesis. The effects of the compounds were also tested on the induction of cell dispersion (Fig. 2). Cell dispersion was examined in two ways: cell dissociation and acquisition of motility. Cell dissociation was estimated by the modulation of intercellular junctions, and specifically by the alteration of desmosomes. We had previously shown that the immunoreactive pattern observed with the mAb to desmoplakin (anti-DP), which recognizes a subset of proteins of the desmosomal plaque (15) disappeared from the boundaries of cells dissociated by aFGF (7, 41). DP immunoreactivity was redistributed in intracellular dots, suggesting that desmosomes were split and internalized during the aFGF-induced EMT (e.g., Fig. 2 *d*). Cell motility was estimated by the ability of cells located at the edge of an artificially produced wound to colonize the wounded area. As already stated (7, 42), wound repair was due solely to the ability of NBT-II cells to migrate actively into the wound and did not depend on cell proliferation.

As shown in Fig. 2, in the presence of 100 μM tyrphostin AG18 aFGF was no longer able to induce the disappearance of DP immunoreactivity from cell boundaries. Genistein also fully inhibited cell dissociation when used at 100 μM, which was the concentration giving 100% inhibition of DNA synthesis (see Fig. 1). The same doses of tyrphostin and genistein blocked cell migration into the wounded areas of cultures stimulated by aFGF (data not shown). As tyrphostin AG18 and genistein did not reduce the level of DNA synthesis in cells stimulated with FCS (not shown), their inhibiting action of aFGF-induced mitogenesis and cell scattering was not due to nonspecific, toxic effects. Sodium orthovanadate alone was able to reproduce the effects of aFGF: it induced the dissociation of cell clusters as visualized by the internalization of DP immunoreactivity. Interestingly, the response to sodium orthovanadate was quite rapid, beginning 2 h after its addition into the culture medium as compared with the response to aFGF which begins 5 h after addition of aFGF. Although less effective than aFGF, sodium orthovanadate induced acquisition of cell motility as visualized by the penetration of isolated cells within wounded areas of cell monolayers (Fig. 2, compare *g* and *h*). Thus, because compounds interfering with tyrosine phosphorylation cannot distinguish between the two pathways involved in transducing the aFGF message, our results indicate that tyrosine phosphorylation constitutes a central event in both cell scattering and mitogenesis induced by aFGF.

TGF-β1 Distinguishes between the Two Responses to aFGF

Because it is well established that TGF-β1 is a potent growth inhibitor in many cell types, it was interesting to test whether it was able to antagonize the effects of aFGF on NBT-II cells. As illustrated in Fig. 3, TGF-β1 abolished the mitogenic response elicited by aFGF with 50% inhibition at a low concentration (0.1 ng/ml). In contrast, the dissociating effect of aFGF, when used at a concentration similar to that of the mitogenic assay, was not altered by addition of TGF-β1 tested within a wide range of concentrations. In Fig. 3 the effect of addition of 10 ng/ml of TGF-β1 is shown. TGF-β1 is thus a factor that discriminates between the two pathways elicited by aFGF stimulation.

An Elevation in cAMP Blocks the EMT of NBT-II Cells and Potentiates the Mitogenic Response to aFGF

Another agent which discriminates between the two pathways of aFGF is intracellular cAMP. The levels of intracellular cAMP can be externally manipulated by using various toxins and chemicals: 8-bromo cAMP and dibutyryl cAMP are diffusible analogs of natural cAMP, forskolin reversibly stimulates adenylate cyclase by binding directly to it, cholera toxin stimulates a GTP-binding protein that selectively activates adenylate cyclase, and pertussis toxin inactivates an inhibitory G protein (39). However, some of these compounds might have complex effects; for example, pertussis toxin also inactivates a G_p involved in the activation of PLC (31).

Mitogenesis induced by aFGF was dramatically increased in the presence of cAMP-elevating agents (Fig. 4). Cholera toxin alone at 10 μg/ml had a mitogenic effect (Fig. 4 *C*). All compounds potentiated the action of aFGF at a concentration of aFGF that was either sufficient to induce DNA synthesis (Fig. 4, *C* and *D*) or not (Fig. 4, *A* and *B*). The rather modest effect of pertussis toxin can be attributed to the fact that the toxin interferes with two different transducing pathways.

Upon exposure of arrested cells to a combination of aFGF and one of the cAMP-elevating agents, DNA synthesis was reinitiated 7 h after addition of the factors (data not shown). Comparison with the lag period of 12–14 h after which aFGF was able to induce an increase in [^{3}H]thymidine incorporation suggests that cAMP-modulating agents may bypass some steps of the pathway leading to cell proliferation.

None of the cAMP-elevating compounds tested were able to induce, alone or in combination with aFGF, the reinitiation of DNA synthesis in cells arrested at subconfluency (data not shown). This result demonstrated that an increase in cAMP, which facilitated aFGF-induced mitogenesis at confluency, was not sufficient to overcome the inhibitory action of aFGF at subconfluency.

The effect of cAMP-elevating compounds was also tested on induction of EMT initiated by aFGF. Cell dissociation was assessed by internalization of DP immunoreactivity in cells incubated with a combination of 20 ng/ml aFGF, and each compound used at a concentration that was the most effective in the mitogenic assay. As shown in Fig. 5, cAMP analogs, forskolin, and cholera toxin dramatically affected the internalization of DP immunoreactivity induced by aFGF. Here again, pertussis toxin had a reduced inhibitory

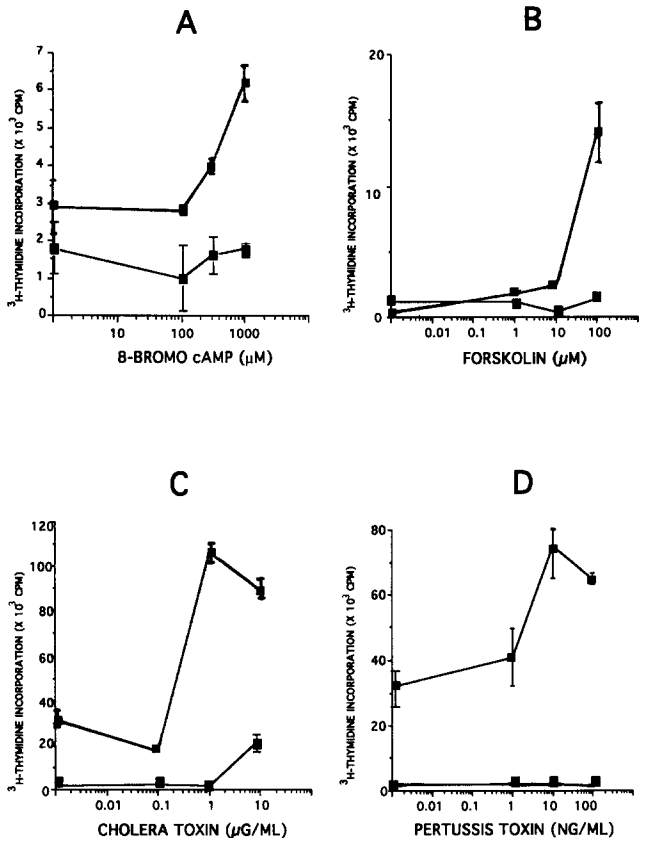

Figure 4. Incorporation of [^{3}H]thymidine in replicating DNA of confluent NBT-II cells arrested by serum starvation for 24 h before stimulation with different agents for 20 h and incubation with [^{3}H]thymidine for 4 h. (▨) Stimulation with cAMP-elevating agents at the indicated concentration. (■) Stimulation with cAMP-elevating reagents in conjunction with 0.1 ng/ml aFGF (*A* and *B*) or 1 ng/ml aFGF (*C* and *D*). The number of counts per minute incorporated was determined as indicated in Materials and Methods. Each point represents the mean value of three identical wells ± standard error.

effect. The inhibition of cell dissociation cannot be accounted for by a toxic effect of the compounds tested because they did exert a positive effect on DNA synthesis.

The same products were assayed on cell motility tested in wound healing experiments (Fig. 6). Depending on the compound, migration of cells within the wound was partially or totally abolished. Once again the less pronounced effect was obtained with pertussis toxin. Altogether, these data indicate that an augmentation in intracellular cAMP content markedly synergized the mitogenic function of aFGF on NBT-II cells while drastically inhibiting its dispersing effect.

We subsequently examined whether the effects of cAMP-elevating agents on aFGF-induced cell responses were due to modifications in aFGF binding on NBT-II cells. At saturating concentrations of the growth factor, the amount of cell-surface bound ^{125}I-aFGF was not affected by preincubation of cells with the different compounds tested (Table I). Therefore, the biological effects of cAMP-elevating agents were not due to changes in the binding of aFGF to its receptors, and arose most likely from modifications in the signaling pathways.

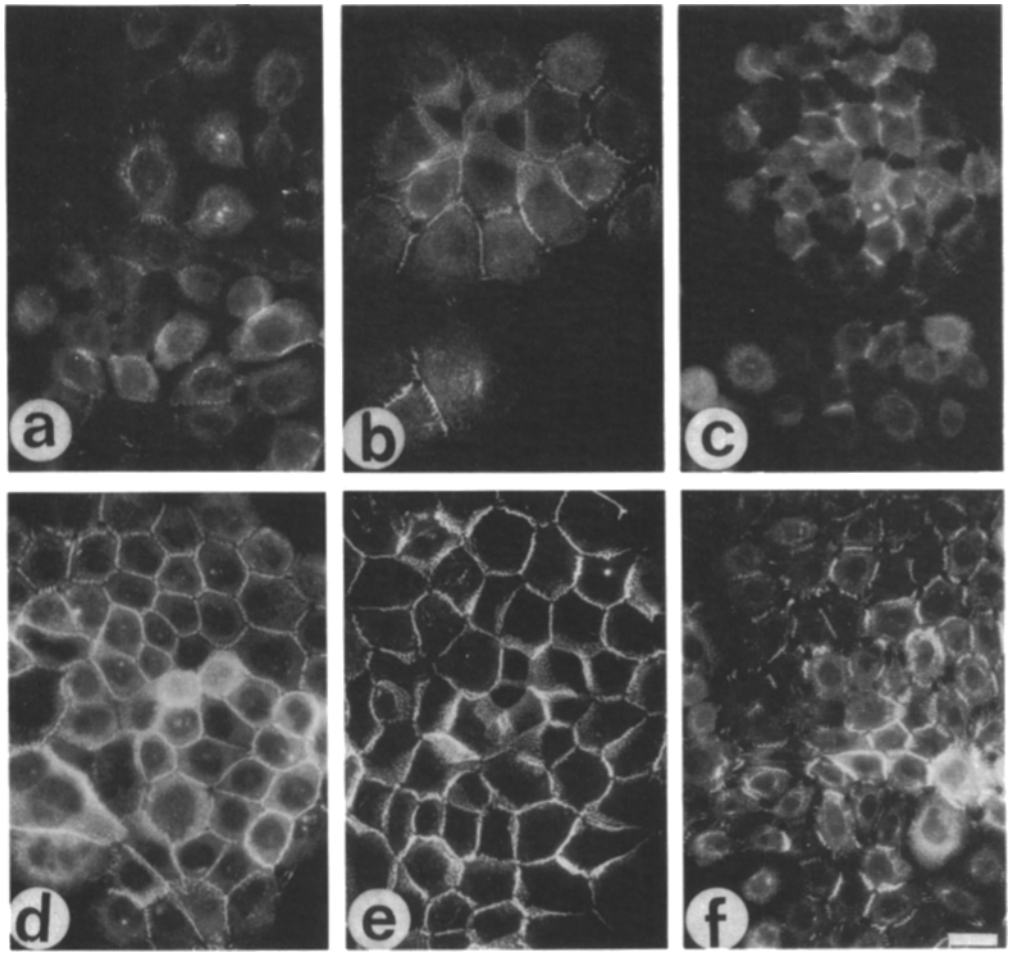

Figure 5. DP immunostaining of NBT-II cells incubated for 8 h with 10 ng/ml aFGF and various cAMP-elevating agents before fixation and processing for immunofluorescence microscopy. Cells were incubated with 10 ng/ml aFGF alone (*a*), or plus 10 μg/ml cholera toxin (*b*), plus 100 ng/ml pertussis toxin (*c*), plus 100 μM forskolin (*d*), plus 1 mM 8-bromo cAMP (*e*), plus 1 mM dibutyryl-cAMP (*f*). Bar, 20 μm.

Cyclic AMP-elevating Agents Can Reverse the Fibroblastic Phenotype Induced by aFGF

Since artificial elevation of the intracellular cAMP level completely abolished the transition towards a fibroblastic phenotype, it was important to investigate the effects of cAMP-elevating agents on cells that were already established in their fibroblastic-like phenotype by long term culture with aFGF. In the presence of any of the agents tested, the reformation of intercellular desmosomes was induced very rapidly, with DP immunoreactivity reexpressed at cell boundaries only 2 h after addition of the compounds (Fig. 7). In keeping with the effects of pertussis toxin on the initiation of EMT, this toxin was not very efficient in the reformation of desmosomal contacts between cells. Concomitantly with the reformation of desmosomes, cells stopped migrating: for example, videomicrocinematography recordings showed that the average velocity of cells cultured for 2 wk in the presence of aFGF dropped from 30 to 10 μm/h after a 2 h exposure to 10 μg/ml cholera toxin. Moreover, the vimentin intermediate filaments that were induced by long-term culture with aFGF (see reference 7), underwent dramatic modifications. As an example, Fig. 7 shows the immunoreactive pattern obtained by incubating cells with cholera toxin: the network of filaments running through the cytoplasm was replaced by a dense immunoreactivity accumulated in a region near the nucleus; some cells were even devoid of vimentin immunostaining (Fig. 7, compare *g* and *h*).

aFGF Does Not Significantly Modify Basal or Activated Adenylate Cyclase Activity

Due to the discriminating effects of cAMP-elevating agents on the dual function of aFGF, it was of importance to determine whether intracellular cAMP content was affected by aFGF. In the absence of adenylate cyclase activator, no significant increase of the basal cAMP production could be detected upon addition of aFGF to sparse or confluent G_0-arrested cells (Table II). Stimulation of adenylate cyclase activity by forskolin (10 μM) or cholera toxin (1 μg/ml) resulted in the expected augmentation of intracellular cAMP content. The presence of aFGF (30 ng/ml) did not significantly modify forskolin or cholera toxin-stimulated cAMP production, even in the presence of the phosphoesterase inhibitor IBMX, which was included at 1 mM to allow accumulation of intracellular cAMP. These results suggest that aFGF-induced stimulation of its cognate receptors does not generate a modification in the adenylate cyclase system. Therefore, the synergy between cAMP-elevating agents and aFGF in the reinitiation of DNA synthesis is likely to arise from events involved downstream in the cAMP transducing pathway.

Interestingly, subconfluent cultures had an elevated content of intracellular cAMP as compared with confluent cultures (Table II). The amount of cAMP in sparse cells is nevertheless too small to prevent cells from being dispersed by aFGF at a concentration of 10 ng/ml. However, in other

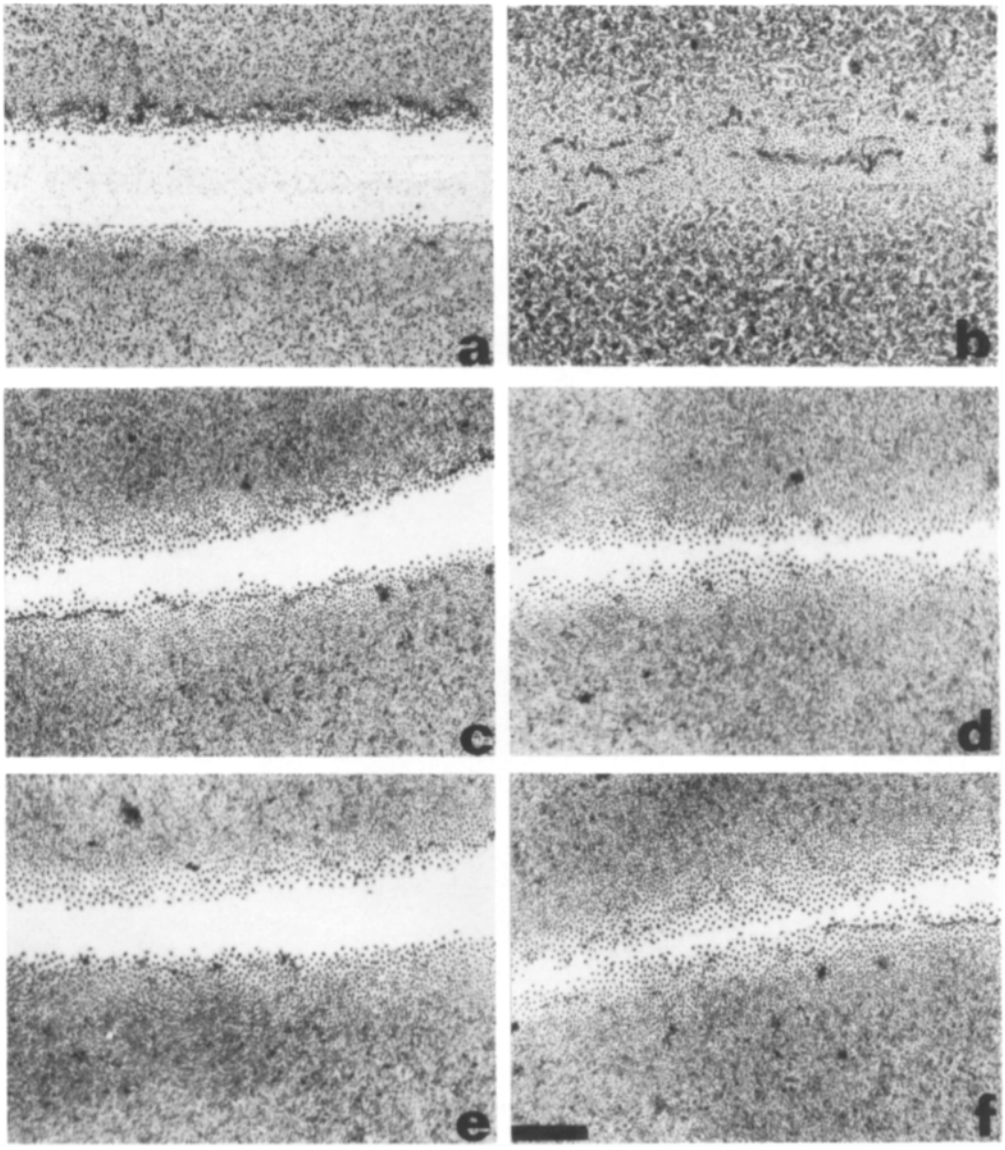

Figure 6. Closure of in vitro wounds made in monolayers of NBT-II cells. Cell cultures were fixed and examined 18 h after producing an artificial wound in cultures incubated with standard medium (*a*). Alternatively, wounded cell cultures were stimulated with 10 ng/ml aFGF alone (*b*), or plus 10 μg/ml cholera toxin (*c*), plus 100 ng/ml pertussis toxin (*d*), plus 1 mM 8-bromo cAMP (*e*), plus 1 mM dibutyryl-cAMP (*f*). Bar, 200 μm.

situations in which local concentrations of aFGF would be in a more physiological range than those used here, the amounts of intracellular cAMP could be sufficient to inhibit the scattering effect of this growth factor.

Discussion

aFGF promotes two different biological responses in the NBT-II rat bladder carcinoma cell line. At confluency, it induces reentry to cell division while at subconfluency, it triggers cell scattering (42). Resistance of subconfluent cultures to the mitogenic effect of aFGF does not arise from the intrinsic inability of sparse G_0-arrested cells to reinitiate DNA synthesis. Indeed, bradykinin, a mitogenic peptide that stimulates the hydrolysis of phosphoinositides (5) promotes the proliferation of sparse cultures of NBT-II cells and has no dispersing effect (data not shown). This result raises the attractive possibility that the induction of EMT would be coupled to inhibition of proliferation and furthermore would be under the control of factors binding tyrosine kinase receptors.

The two functions of aFGF are mutually exclusive since the growth factor cannot simultaneously elicit dispersion and DNA synthesis. The opposing activities of aFGF depending on the status of the cell cannot be accounted for by differences in prevalence or affinity of aFGF receptors on the surface of confluent versus subconfluent cells (42). Moreover, NBT-II cells are devoid of receptors recognizing basic FGF (bFGF) (41) and do not scatter in response to this growth factor. Transfection of NBT-II cells with a plasmid

Table I. cAMP-elevating Agents Do Not Influence aFGF Binding on NBT-II Cells

Treatment	Binding of radiolabeled aFGF
	cpm ± *SE*
–	12696 ± 56
pertussis toxin	11795 ± 289
cholera toxin	13552 ± 1279
forskolin	14291 ± 211

Binding of 10 ng/ml ^{125}I-aFGF on the surface of NBT-II cells preincubated for 4 h with 100 ng/ml pertussis toxin, 10 μg/ml cholera toxin, or 100 μM forskolin. The number of cpm bound is determined under conditions described in Materials and Methods and represents the mean value of three identical wells ± standard error.

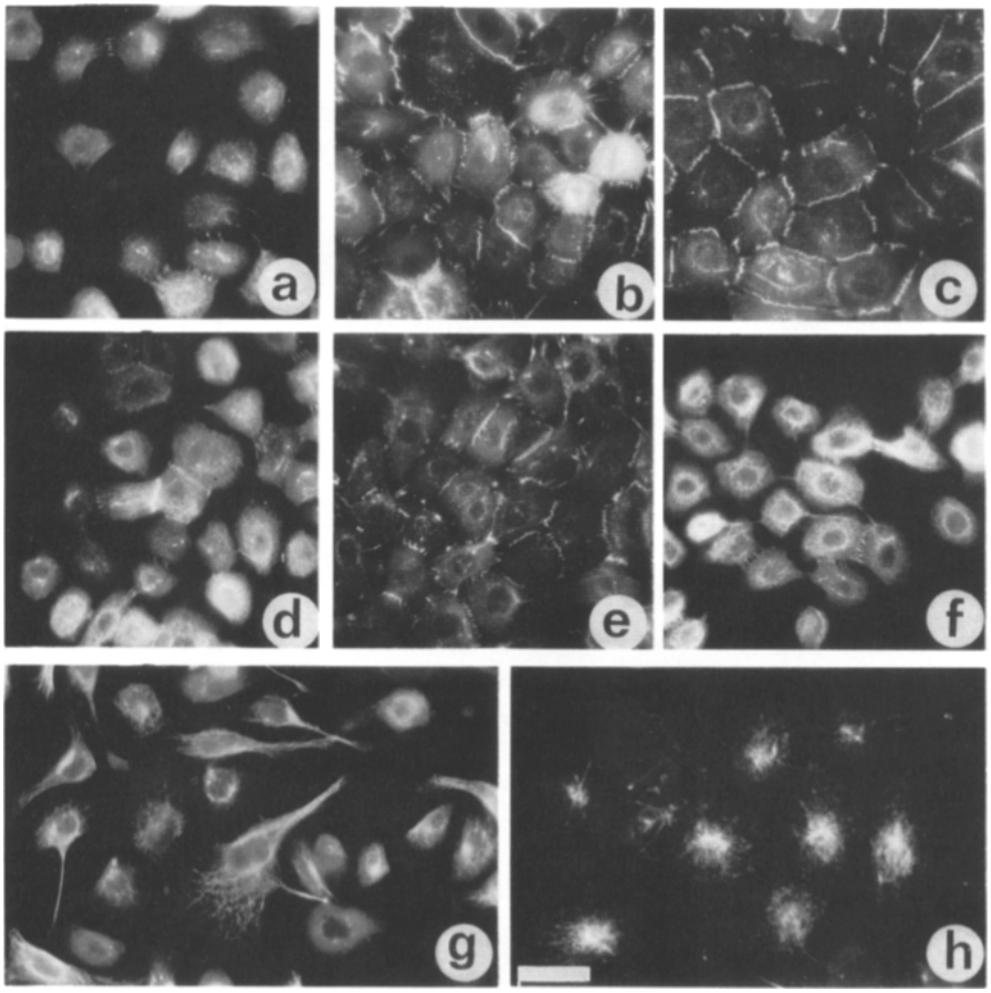

Figure 7. The transition towards a dispersed state is reversed by cAMP-elevating agents. NBT-II cells were maintained in the presence of 10 ng/ml aFGF for 2 wk before addition of various reagents for 6 h. Cells were fixed and processed for immunofluorescence microscopy with a mAb against DPs (*a–f*) or vimentin (*g* and *h*). (*a* and *g*) Control cells permanently stimulated by aFGF; (*b* and *h*) addition of 10 μg/ml cholera toxin; (*c*) addition of 100 μM forskolin; (*d*) addition of 100 ng/ml pertussis toxin; (*e*) addition of 1 mM 8-bromo cAMP; and (*f*) addition of 100 μM genistein.

encoding the well-defined receptor for acidic and basic FGF *flg* induces cells to disperse in response to bFGF stimulation (Savagner, P., A. M. Vallés, J. Jouanneau, Y. Yamada, and J. P. Thiery, unpublished data). These data indicate that the scattering activity of growth factors toward the NBT-II cell line can be triggered by receptors endowed with a mitogenic activity and is therefore not harbored by a special type of receptor. It is thus important to determine whether the alternative action of aFGF is dependent on alternative signaling pathways.

Our results clearly demonstrate the role of tyrosine phosphorylation in both responses. Tyrosine phosphorylations have already been shown to be implicated in the mitogenic response of 3T3 cells to aFGF (11). This is not an unexpected result, since FGFs bind to receptors endowed with a tyrosine kinase activity (20, 40). Moreover, our results indicate that sustained tyrosine phosphorylation activity is able to reproduce by itself the scattering effect of aFGF. These data are consistent with the observation that sodium orthovanadate dissociates MDCK cells and reproduces the alterations of

Table II. Effect of aFGF on the Basal and Stimulated Adenylate Cyclase Activity in Sparse and Confluent Cultures of NBT-II Cells

Condition	Confluent culture	Subconfluent culture
	pmol cAMP/10⁵ cells	*pmol/10⁵ cells*
+ IBMX	0.03 ± 0.005	0.18 ± 0.04
+ IBMX + aFGF	0.05 ± 0.004	0.18 ± 0.05
+ IBMX + forskolin	1.5 ± 0.4	1.8 ± 0.25
+ IBMX +aFGF + forskolin	1.8 ± 0.3	1.6 ± 0.3
+ IBMX + cholera toxin	1.6 ± 0.4	1.1 ± 0.3
+ IBMX + aFGF + cholera toxin	2.0 ± 0.5	1.0 ± 0.2

Two million cells (confluent cultures) or 2 × 10^5 cells (subconfluent cultures) were preincubated for 30 min in DME before receiving the various agents. Cholera toxin (1 μg/ml) was present during the 30-min incubation. aFGF (30 ng/ml) was added for 15 min before addition of IBMX (1 mM). Forskolin (10 μM) was added 15 min after IBMX. The accumulation of cAMP was measured 10 min after addition of IBMX (when forskolin was omitted) or 10 min after addition of forskolin. Determination of cAMP content was done by radioimmunoassay as described in Materials and Methods. Values are the means ± standard error of four different determinations.

adherent junctions that were previously described in src-transformed chick cells (43, 44). Since one of the primary events in aFGF-induced scattering consists of the removal of desmosomal components from the cell periphery (7), our results suggest that intercellular junctions may be direct targets for growth factor action. Involvement of a specific signaling pathway in the disruption of intercellular contacts induced by low extracellular calcium had already been reported (10). On the other hand, cell adhesion molecules that participate in establishing cell–cell interactions mediate their function through pertussis-sensitive G protein (12), suggesting that modifications in the balance of messenger molecules could in turn be directly reflected by changes in intercellular adhesiveness.

From our results, it appears clear that tyrosine kinase-modulating agents cannot distinguish between the two responses elicited by aFGF. In an attempt to demonstrate that the transducing pathways of aFGF leading to the two biological responses of NBT-II cells are different, we found that TGF-β1 has the unique property of blocking aFGF-induced DNA replication without affecting aFGF-mediated EMT. Because they are not equally sensitive to TGF-β1, the two biological responses of NBT-II cells are therefore not triggered by the same set of transducing molecules. Mysterious for a long time, the nature of the signaling mechanisms of TGFβ is now being progressively elucidated (28, 30). Most interestingly, the growth inhibitory signals of TGF-β1 are likely to take place late during the cell cycle by preventing the phosphorylation of pRB (24). This result suggests that the two pathways of aFGF in NBT-II cells diverge at a branching point taking place before late G1 phase.

Activation or inhibition of PKC did not have any effect on the two responses elicited by aFGF in NBT-II cells (data not shown). Consequently, we sought to determine whether the effect of cAMP-elevating agents on the two responses to aFGF could provide additional evidence for the existence of a diverging point in the transduction pathways of aFGF. Our results point out to the discriminative effect of sustained elevation of cAMP: cAMP-elevating agents indeed synergize with aFGF to trigger DNA synthesis and strongly repress aFGF-induced EMT. Moreover, cAMP elevation is also capable of reversing the fibroblastic-like phenotype, indicating that the level of cAMP not only regulates EMT but is also probably involved in the maintenance of the epithelial phenotype.

Implication of cAMP in FGF-induced mitogenesis has been a subject of controversy (25, 36). More generally, it appears that cAMP could be involved in epithelial but not in fibroblastic mitogenesis (14). However, there are exceptions to this rule. For example, cAMP-elevating agents still potentiate aFGF-induced DNA synthesis in fibroblastic-like NBT-II cells that have been maintained in the presence of aFGF for one month (data not shown); cAMP enhancers also synergize with insulin to induce DNA synthesis in 3T3 fibroblasts (32).

Cyclic AMP not only regulates cell division but also cell differentiation. In many cells, there is an obvious link between growth and inhibition of differentiation or conversely, between growth arrest and differentiation processes. Our system provides evidence for such a linkage, since cAMP elevation stimulated DNA synthesis and inhibited EMT, which can be regarded as a process of differentiation. In addition it should be stressed that the status of the cell itself participates in the choice between proliferation and differentiation (42). In agreement with our results, O'Neill and coworkers reported that elevation in intracellular cAMP stimulates the proliferation of quiescent fibroblasts and inhibits their motility (32).

Measurements of intracellular cAMP content in subconfluent and confluent cultures indicated that no change in the cAMP level was detected in FGF-stimulated cells, even in the presence of cAMP-elevating agents. The discrepancy between our results and others' (26) might arise from the fact that in our system cAMP stimulates cell proliferation while in the other report it was a strong inhibitor of FGF-induced mitogenesis. The absence of effect of aFGF on cAMP production suggests that the pathways of aFGF and cAMP are largely separate. Moreover, our results raise the question as to whether cAMP elicits its discriminative effects on aFGF functions by activating cyclic AMP-dependent protein kinases (PKA) or by acting on gene expression. Cyclic AMP could indeed display both stimulatory and inhibitory effects by acting only on DNA transcription: whereas transcriptional activation of cyclic AMP-responsive element (CRE)-containing genes is a common effect of cAMP, cAMP inhibits fibronectin gene expression in a granulosa cell line by acting indirectly on a DNA sequence other than CRE (3). Therefore, it would be interesting to study induction of gene expression in aFGF-stimulated NBT-II cells in the presence or absence of cAMP-elevating agents.

There is increasing evidence that many signals emanating from a single growth factor receptor follow multiple cellular pathways. This could explain the pleiotropic effects of growth factors that elicit their biological responses by inducing a wide range of cellular modifications. The molecular bases underlying the generation of complex networks of branching pathways are far from being elucidated. However, there is evidence that divergence of the signals might occur at the receptor level (40, 47). In the case of the dual response elicited by aFGF in NBT-II cells, our experiments suggest the existence of a precocious branching point that can be artificially modulated by elevation of intracellular cAMP. It is therefore conceivable that signal transfer particles created between the stimulated receptor and intracellular effector molecules might specify the type of biological response. This hypothesis is under current investigation.

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