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Possible Involvement of Normal p21 H-*ras* in the Insulin/Insulinlike Growth Factor 1 Signal Transduction Pathway

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Expression of a mutant H-*ras* gene confers a transformed phenotype to rat-1 fibroblasts which is basically independent of exogenous growth factors (GFs). Rat-1 cells induced to express high levels of the normal H-*ras* gene were also found to display a transformed phenotype. In contrast to cells expressing mutant H-*ras*, these cells were dependent on GFs. We used this difference in GF dependence to analyze a possible involvement of exogenous GFs in H-*ras* function. Compared with untransformed rat-1 cells, cells overexpressing normal H-*ras* displayed an elevated response toward insulinlike growth factor 1 (IGF-1), insulin, and bombesin and an increased sensitivity toward phosphatidic acids. It was found that 8-bromo-cyclic AMP inhibited the responses to all GFs in rat-1 cells but had no effect on mutant-H-*ras*-transformed cells. In cells overexpressing normal H-*ras*, 8-bromo-cyclic AMP inhibited the responses to all GFs except those to insulin and IGF-1. This implies that overexpression of normal H-*ras* in the presence of insulin/IGF-1 is functionally similar to the expression of mutant H-*ras*, since mutant H-*ras* can circumvent this block by itself. These and other results strongly suggest a functional linkage between insulin/IGF-1 and normal p21 H-*ras*.

All members of the *ras* gene family, H-*ras*, K-*ras*, and N-*ras*, share several important biochemical features (43, 47, 51, 55, 62) with a family of GTP-binding proteins most commonly referred to as G proteins (7, 52). Because it is becoming clear that GTP-binding proteins are involved in most of the growth factor (GF) receptor-induced responses (8), it is conceivable that normal p21 *ras* may be a regulatory G protein that mediates the effect(s) of external GFs (52). However, the nature of this putative GF(s) as well as the (cellular) level at which the normal *ras* protein participates in the signal transduction pathways of this GF(s) are, as yet, unknown. Moreover, the role of external GFs in the process of transformation by the *ras* oncogene is still poorly understood. In general, *ras*-transformed cells appear to lack responsiveness toward exogenous GFs (2, 49, 53). In part, this might be explained by autocrine stimulation (4). *ras*-transformed cells are known to produce at least one GF: transforming growth factor α (TGFα) (18, 30). However, several observations indicate that TGFα production is not a prerequisite for transformation by *ras* (24, 48). Enhanced responsiveness has been reported for bradykinin-induced phosphatidylinositol 4,5-biphosphate (PIP2) breakdown (31), yet this is most likely due to elevated receptor numbers (9a).

Most of the experiments described above have been done with cells transformed by mutated *ras* genes, which encode proteins with a defective intrinsic GTPase activity (51). This may result in a protein that is constitutively activated. As a result of this, the mutant p21 *ras* transforms cells by activating the second messenger system(s) without the need for GF stimulation. However, not only mutant *ras* but also overexpression of normal p21 *ras* can transform cells (24, 25, 35, 60). It is not known whether such cells, like mutant *ras*-transformed cells, lack responsiveness toward exogenous GFs. In analogy to the epidermal growth factor (EGF)-dependent transformation of cells overexpressing the EGF receptor (9), it seems reasonable to assume that transformation resulting from elevated levels of postreceptor components, such as normal p21 *ras*, also requires stimulation by the relevant GF(s). This concept of ligand-induced transformation of cells overexpressing signal transduction intermediates is illustrated by several reports studying cell lines expressing either amplified GFs or GF receptors (9, 11, 16, 38, 39, 48, 59).

On the basis of this concept, we investigated whether one or more specific GFs are required to induce the transformed phenotype in cells expressing high levels of normal p21 H-*ras*. Identification of such GF(s) might elucidate a signal transduction pathway in which normal p21 H-*ras* is involved. To this end, we compared the GF responsiveness of three different cell lines: untransformed rat-1 cells (R1), rat-1 cells expressing transforming levels of normal H-*ras* (HE^+), and rat-1 cells transformed by mutant H-*ras* (EJ). In addition, we investigated the effect of 8-bromo-cyclic AMP (8-bromo-cAMP) on GF responsiveness. Addition of this synthetic compound to culture medium results in elevated intracellular levels of cAMP and has been shown to phenotypically revert mutant-H-*ras*-transformed cells (6, 32, 33). The results presented in this report provide evidence that insulin in nonphysiological concentrations and insulinlike growth factor 1 (IGF-1) in physiological concentrations are involved in the induction of some aspects of the transformed phenotype of cells expressing high levels of normal p21 H-*ras*.

MATERIALS AND METHODS

Materials. Sera and tissue culture media were purchased from GIBCO Laboratories (Grand Island, N.Y.). All tissue culture plastics were obtained from Greiner (Nürtingen, Federal Republic of Germany) except 24-well plates, which were from Costar (Cambridge, Mass.). Dexamethasone, agarose for soft-agar assays, 8-bromo-cAMP, and GFs were obtained from Sigma Chemical Co. (St. Louis, Mo.), except IGF-1 (Kabi-Gen), EGF (Collaborative Research, Inc., Waltham, Mass.), and platelet-derived GF, which was

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kindly provided by C.-H. Heldin. Wheat germ lectin-Sepharose 6MB was purchased from Pharmacia Fine Chemicals (Piscataway, N.J.).

Cell culture. Cells were routinely cultured in minimal essential medium containing 8% fetal calf serum (FCS). Subconfluent cultures were arrested on minimal essential medium plus 0.5% FCS and 10 μg of transferrin per ml. For [^{3}H]thymidine assays, cells were seeded in 24-well Costar plates. For RNA analysis, cells were seeded into 9-cm dishes. For isolation of glycoproteins, cells were grown on 15-cm dishes and medium was changed 24 h before isolation. Clonal *ras*-transformed cell lines were obtained by standard transfection procedures (57). Cell line HE contains plasmid pLTR-H-ras (N) (15), and cell lines EJ1 and EJ2 contain plasmid pEJ 6.6 (46). Unless stated otherwise, EJ$^+$ refers to the EJ1 cell line.

[^{3}H]thymidine assay. Cells were seeded into 16-mm wells in the culture medium used routinely and grown to subconfluency. Subsequently, the medium was changed to medium containing 0.5% FCS and 10 μg of transferrin per ml. After 24 h, the medium was refreshed, and after 48 h, the appropriate GF, in the absence or presence of 8-bromo-cAMP, was added in fresh serum-free medium. [^{3}H]thymidine incorporation (0.5 μCi per well) was measured between 16 and 20 h after mitogenic stimulation in triplicate, and cells were then treated as described previously (58).

Growth curves. Cells were seeded at the indicated densities in 5-cm plates. After the cells attached to the culture dish, the medium was changed to medium containing the desired additives and cells were counted in duplicate (= day 0). After 2 days, the cell number was determined in triplicate and the remaining plates were refed. This procedure was repeated at days 4 and 7.

Soft-agar assay. Agar bases (2 ml in 3.5-cm plates) consisted of minimal essential medium supplemented with 0.6% agar. Cells were seeded into agar containing growth medium (0.3% agar and 2 or 10% FCS) at densities of 5,000 cells per 3.5-cm plate. After 1 week, the percentage of cells forming colonies (clusters of more than 12 cells) was determined. In different experiments performed at different times, colony formation varied around 10%.

Insulin and IGF-1 binding. Binding of ^{125}I-labeled insulin or ^{125}I-labeled IGF-1 was essentially done as described by Maassen et al. (21). Only tracer amounts of labeled IGF-1 or insulin were used (3×10^{-11} M). The specific binding is represented by the amount of radioactivity bound which can be suppressed by the addition of 1 μM unlabeled insulin or 100 nM IGF-1, respectively.

Preparation of glycoprotein fractions and protein phosphorylation assays. Glycoprotein fractions were isolated by chromatography over wheat-germ lectin-Sepharose 6MB. Glycoprotein fractions were stored in WgA buffer (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid] [pH 7.4], 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1% Triton X-100, 1 kU of Trasylol per ml, 1 μg of leupeptin per ml, 0.1 mM phenylmethylsulfonyl fluoride, 0.3 M *N*-acetylglucosamine). Protein content was determined by the Bradford method (Bio-Rad Laboratories, Richmond, Calif.). Concentration was made 1 mg/ml by adding WgA buffer and 1 mg of bovine serum albumin. Insulin or IGF-1 was added to a 10-μl fraction of glycoproteins, and incubation was for 1 h at 22°C. Phosphorylation was initiated by the addition of $MnCl_2$ (final concentration, 6 mM) and [γ-^{32}P]ATP (final concentration, 25 μM; 200 Ci/mmol), the final incubation volume being 15 μl. Incubation was for 6 min at 22°C. The reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid. The protein pellet was washed and dissolved in 50 μl of $NaHCO_3$–0.1% sodium dodecyl sulfate and used for sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis or immunoprecipitation.

Immunoprecipitation. Cell labeling with [^{35}S]methionine and immunoprecipitations were done as described by Maassen et al. (21). The monoclonal antibody used was Y13-259 (13), which recognizes all forms of p21 *ras*, and a rabbit polyclonal antiserum directed against the human insulin receptor which shows no substantial cross-reactivity with the IGF-1 receptor (22) was used.

RNA analysis. Cells were seeded onto 9-cm plates and grown to subconfluent cultures. Control RNA was isolated from these log-phase subconfluent cultures by LiCl-urea lysis. Subsequently, the medium of the remaining plates was changed for medium containing 0.5% FCS and 10 μg of transferrin per ml. After 24 h, the indicated mitogens were added. At various time points, RNA was isolated. Control RNA was isolated at the same time points from plates not treated with either agent. RNA isolation and Northern (RNA) blotting analysis were performed as described previously (42).

Probes. The fragments used for nick translation were HEF, a nearly full length cDNA clone of human elongation factor 1 (5); *myc*, two fragments, covering exon 2 (*Pst*I fragment of 420 base pairs) and exon 3 (*Bgl*II-*Pst*I fragment of 1,400 base pairs), respectively, isolated from clone pGmyc 7.5, kindly provided by G. Klein (Karolinska Institutet, Stockholm, Sweden); and *fos*, a partial human cDNA clone, kindly provided by W. Kruijer (Hubrechts Laboratory, Utrecht, The Netherlands).

RESULTS

Characteristics of cells. To examine the GF responsiveness of H-*ras*-transformed cells, we constructed a panel of H-*ras*-transformed rat-1 cell lines. The properties of the three cell types used are shown in Table 1 and Fig. 1.

The cell line designated HE, harboring a normal H-*ras* gene under the control of the mouse mammary tumor virus promoter, was selected for optimal induction kinetics of p21 H-*ras* after the addition of the synthetic glucocorticoid dexamethasone to the culture medium. Cells grown in the absence of dexamethasone (HE$^-$) expressed a low level of p21 H-*ras*, whereas cells grown in the presence of dexamethasone (HE$^+$) contained large amounts of p21 H-*ras* (at least a 100- to 150-fold increase in expression, Fig. 1). This level of normal H-*ras* protein confers to these cells transformation characteristics nearly identical to those of cell lines transformed by the expression of mutant H-*ras* (EJ). HE$^+$ cells were morphologically transformed and no longer contact inhibited (Fig. 1). Initially, they were not very refractile, but after growing to higher densities, the cells began to pile up and form multilayered foci, instead of filling up the open spaces. This resulted in the typical starlike appearance of these cell cultures. When grown to confluency, HE$^+$ cells reached higher saturation densities (equivalent to those of some EJ$^+$ cells) than R1$^+$ cells, and they were able to grow in soft agar (Table 1).

Besides these general transformed growth properties, HE$^+$ cells also displayed other features characteristic of *ras* transformation. They appeared to produce TGFα, since the response to EGF (see Fig. 3) was impaired; in addition, HE$^+$ cells expressed elevated levels of transin mRNA (data not shown) (23). The elevated expression of this gene in *ras*-transformed cells is mediated most likely by a TGFα auto-

TABLE 1. Biological characteristics of cell lines

Cell line	Transfected DNA	Saturation density[a]	Anchorage-independent growth[b] (% colony formation)			
			2% FCS		10% FCS	
			−cAMP	+cAMP	−cAMP	+cAMP
Rat-1 ($R1^-$)		45				
Rat-1 + dexamethasone ($R1^+$)		48				
HE (HE^-)	pLTR-Hras (N)	45				
HE + dexamethasone (HE^+)	pLTR-Hras (N)	113				
EJ1 + dexamethasone (EJ^+)	pEJ 6.6	200[c]				
EJ2 + dexamethasone	pEJ 6.6	128				
$R1^+$			0	0	10[d]	0
HE^+			30	1	50	1
EJ^+			30	4	90	5[e]
EJ^+			ND[f]	ND	90	5[g]

[a] Expressed as the number of cells × 10^5 per 5-cm dish.
[b] For experimental conditions, see Materials and Methods.
[c] At this density, an equilibrium exists between attached and nonattached cells (EJ1 and EJ2 express different levels of H-ras protein; see also Fig. 1).
[d] Growth of $R1^+$ cells in soft agar at high serum concentrations has been described by Kaplan and Ozanne (19).
[e] Normal plating density of cells (5 × 10^3 cells per 3.5-cm dish).
[f] ND, Not done.
[g] High plating density of cells (2 × 10^4 cells per 3.5-cm dish).

crine loop (24). There is some disagreement whether overexpression of normal H-*ras* can transform normal fibroblasts (37). However, this may be explained by the background of the parental rat-1 cell line (19).

The addition of dexamethasone is a prerequisite for obtaining high levels of p21 *ras* expression in HE cells and, hence, a transformed phenotype. Therefore, to serve as proper controls, both the rat-1 cells and the EJ cells were also grown in the continuous presence of dexamethasone. This is indicated by the superscript $^+$ ($R1^+$, EJ^+). The addition of dexamethasone did not have any major effect on the basic characteristics of these cells.

HE^+ cells depend on GFs for growth. Since our intention was to analyze the possibility of a linkage between H-*ras* and GFs, we first compared the GF dependence of the HE^+ and EJ^+ cells. When assayed for their ability to grow in GF-defined media, HE^+ cells were found to depend on the presence of low amounts of FCS (0.2 to 0.5%), whereas EJ^+ cells were able to grow in absolutely serum-free medium (Fig. 2). This latter property has already been described for v-K-*ras*-transformed rat-1 cells (18). Although EJ^+ cells are capable of growing in serum-free medium, this does not necessarily mean that these cells will not benefit from components present in FCS. Indeed, the addition of 10% FCS still increases the number of cell doublings. Thus, in contrast to EJ^+ cells, HE^+ cells depend on GFs to sustain their growth and consequently their transformed phenotype.

Induction of DNA synthesis by GFs. Since HE^+ cells are transformed cells and depend on GFs for their growth, we decided to analyze this dependence in more detail. Since normal p21 H-*ras* appears to be functionally linked to certain GF receptor-mediated signals, one would expect cells expressing high levels of normal p21 H-*ras* to display at least a different response toward these GFs. This could be either an increase in sensitivity for a particular GF or an elevated response.

As a parameter for GF responsiveness, we used the ability of GFs to induce [^{3}H]thymidine incorporation. The addition of dexamethasone had a small but reproducible effect on the induction of [^{3}H]thymidine incorporation by some of the GFs. However, these effects were also seen when different serum batches, probably differing in steroid concentrations, were used.

The patterns of relative [^{3}H]thymidine incorporation values for $R1^+$, HE^+, and EJ^+ cells are shown in Fig. 3. $R1^+$ cells responded strongly to EGF and, to a lesser but significant extent, also to the other GFs used. As might be expected for a mutant-*ras*-transformed cell, EJ^+ cells incorporated high amounts of [^{3}H]thymidine even in the absence of GFs. The addition of any of the GFs tested did not give rise to further substantial incorporation. Consequently, expression of mutant p21 H-*ras* rendered these cells largely independent of GFs for their DNA synthesis.

HE^+ cells responded strongly to GFs. However, when compared with $R1^+$ cells, HE^+ cells responded differently toward a subset of the GFs used. The responses to insulin, IGF-1, and bombesin were elevated, in the sense that they (nearly) equaled the response to FCS. In this case, the elevated responsiveness might be explained by a synergism between TGFα, a GF produced by *ras*-transformed cells, and the added GF. When, for instance, EGF, replacing TGFα, and insulin are added simultaneously to serum-deprived $R1^+$ cells, a strong synergism is seen in a [^{3}H]thymidine assay (data not shown). However, this experimental interpretation does not completely reflect the situation for *ras*-transformed cells, since these cells continuously produce TGFα. Therefore, to study this possible synergism more properly, $R1^+$ cells were grown to subconfluent cultures and the medium was replaced with serum-free medium containing 20 ng of EGF per ml. After 24 h, these cells were stimulated with insulin and treated as described in Materials and Methods. Control levels (= 0 in Fig. 4D) were then comparable with those of the HE^+ cells (compare with 0 in Fig. 3), and a clear desensitization toward EGF was observed. These results indicated that in a [^{3}H]thymidine assay, certain features of *ras* transformation can be mimicked by this treatment. Again, a strong synergism was seen when insulin was added (compare with normal insulin response of $R1^+$ cells shown in Fig. 3). A similar synergism is also found for IGF-1 but not for bombesin (data not shown).

For several GFs, we determined the concentrations giving half-maximal stimulation to investigate whether HE^+ cells display an increase in sensitivity to certain GFs. HE^+ cells were sensitized in their response to phosphatidic acids (Fig. 5). Compared with $R1^+$ cells, HE^+ cells were approximately 150 times more sensitive to phosphatidic acids from egg

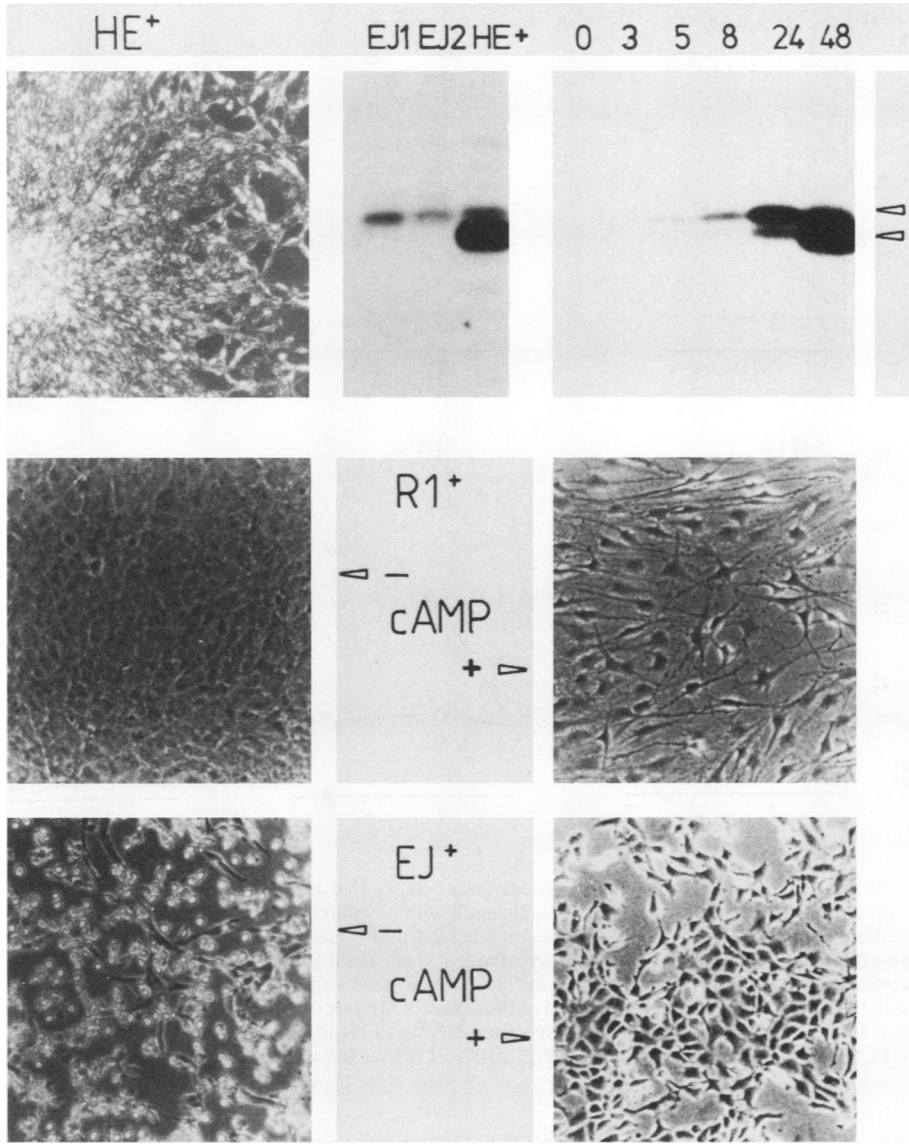

FIG. 1. Cellular morphology and p21-H-*ras* expression levels. Upper panel, left: Photomicrograph of HE^+ cells at a density that is comparable to that of $R1^+$ cells at (sub)confluent density. Upper panel, middle: Comparison of the steady-state levels of *ras* protein in the cell lines used. [^{35}S]methionine-labeled cell lysates were analyzed for the presence of p21 c-H-*ras* and p21 H-$ras^{12\rightarrow Val}$ with monoclonal antibody Y13-259 (13). The EJ^+ cells contain the mutated H-*ras* protein, which migrates somewhat slower on sodium dodecyl sulfate-polyacrylamide gels than the normal H-*ras* protein. In HE^+ cells, the upper and lower protein bands, indicated by the arrows, represent the nonprocessed precursor protein and the mature p21 from the normal H-*ras* gene, respectively. Upper panel, right: Induction of normal p21 H-*ras* expression in HE cell line. Shown is the time course (hours) of induction of normal p21 H-*ras* after the addition of 1 μM dexamethasone to the culture medium. One hour before lysis, the cells were pulse-labeled with 100 μCi of [^{35}S]methionine per ml. Equal amounts of radioactivity were assayed for the presence of p21 H-*ras* with monoclonal antibody Y13-259. Middle and lower panels: Morphology of $R1^+$ and EJ^+ cells. Cells were grown in the continuous presence of dexamethasone (1 μM). Cultures were split 1:4 and grown to confluent density ($R1^+$) or, for EJ^+ cells, to a density that equals the amount of $R1^+$ cells at confluent density. Subsequently, half of the cells were treated with 0.5 mM 8-bromo-cAMP, whereas the other half remained untreated. Photomicrographs were taken after 48 h. The absence or presence of 8-bromo-cAMP is indicated by − and +, respectively.

lecithin and approximately 1,000 times more sensitive toward L-α-dipalmitoyl phosphatidic acid. In contrast to the elevated responsiveness toward insulin, IGF-1, and bombesin, this increased sensitivity is not the result of a synergism between TGFα and these GFs (data not shown).

In summary, by GF-induced [^{3}H]thymidine incorporation, we showed that the ability of several GFs to induce DNA synthesis is affected by the overexpression of normal p21 H-*ras*. These results, however, did not reveal a specific involvement of normal p21 H-*ras* in any particular signal transduction pathway.

Effect of 8-bromo-cAMP on GF-induced DNA synthesis. To further elaborate on the differences between untransformed and H-*ras*-transformed cells in a [^{3}H]thymidine incorporation assay, we searched for a compound that could be used as a specific inhibitor of GF-induced responses. Ideally, such a compound should have a clear effect on the responses of untransformed $R1^+$ cells and no effect on the responses of

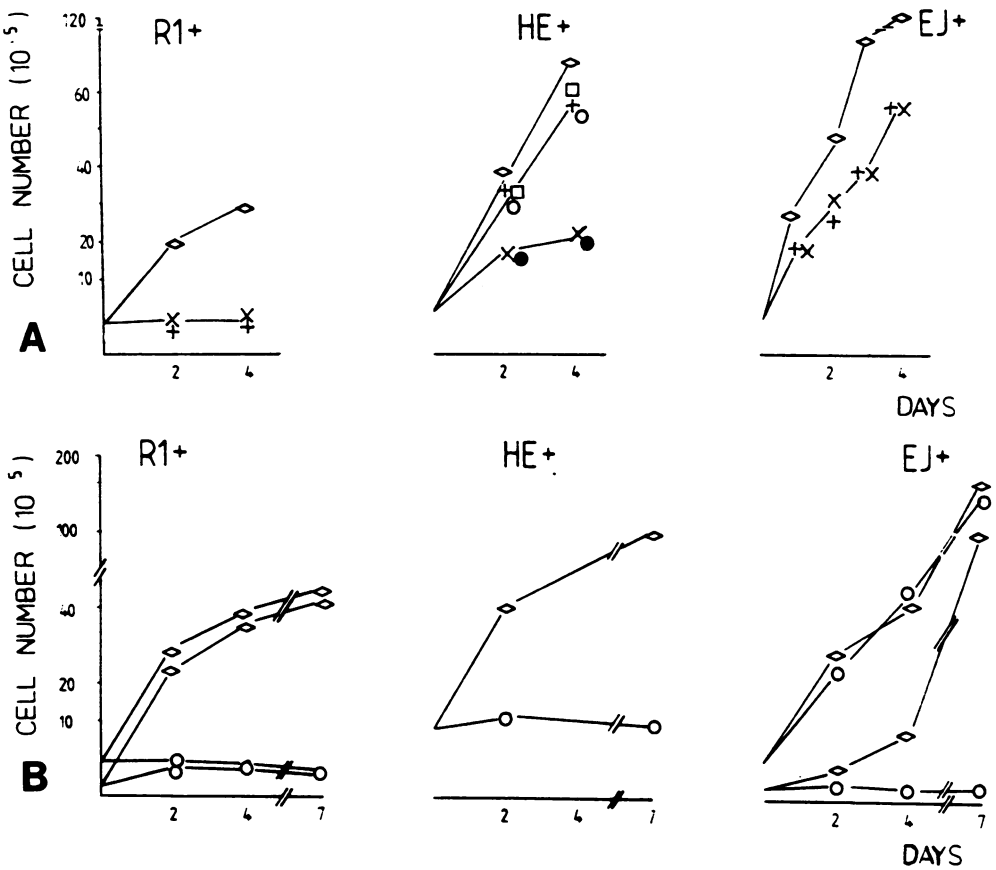

FIG. 2. Influence of 8-bromo-cAMP and insulin/IGF-1 on anchorage-dependent growth. Cells were seeded at the indicated densities in 5-cm dishes. After cell attachment to the plates, medium was changed with absolutely serum-free medium supplemented with the indicated additions (A) or with medium supplemented with 10% FCS containing 0.5 mM 8-bromo-cAMP or lacking 8-bromo-cAMP (B). For analysis of the effect of 8-bromo-cAMP, both $R1^+$ and EJ^+ cells were seeded at different initial plating densities (see text for explanation). Cell counting was done in triplicate. Standard deviations did not exceed 10% of mean values. Cell death, as measured by trypan blue exclusion, was less than 5% during the experiments. The concentrations of the different GFs were identical to the concentrations used in the experiment described in the legend to Fig. 3. Symbols: (A) ×, absolutely serum-free; +, plus insulin; ○, plus insulin and EGF; □, plus 0.5% FCS; ●, plus bombesin; ◇, 10% FCS; (B) ◇, 10% FCS minus cAMP; ○, 10% FCS plus 0.5 mM cAMP.

H-*ras*-transformed cells or vice versa. 8-Bromo-cAMP fulfilled these conditions. To measure the effect of 8-bromo-cAMP on the induction of [^{3}H]thymidine incorporation by GFs, we pulse-labeled serum-starved cells between 16 and 20 h after the simultaneous addition of the GF and 0.5 mM 8-bromo-cAMP. 8-Bromo-cAMP exerted an inhibitory effect on GF-induced [^{3}H]thymidine incorporation in $R1^+$ cells but not on that in EJ^+ cells (Fig. 3). Thus, 8-bromo-cAMP revealed a clear difference in GF responsiveness between untransformed and *ras*-transformed cells. HE^+ cells showed an effect intermediate between those of $R1^+$ and EJ^+ cells. 8-Bromo-cAMP did not repress serum-induced [^{3}H]thymidine incorporation but in general inhibited [^{3}H]thymidine incorporations induced by individual GFs; however, those induced by insulin and IGF-1 remained largely unaffected. These results imply that in HE^+ cells, in contrast to $R1^+$ cells, insulin-IGF-1 action is not inhibited by 8-bromo-cAMP. Most importantly, this effect is not due to a synergism between TGFα and insulin-IGF-1, since in $R1^+$ cells 8-bromo-cAMP also inhibited the stimulation of [^{3}H]thymidine incorporation by a combination of insulin and EGF (Fig. 4D).

These results suggest that in our working hypothesis of ligand-induced transformation, insulin-IGF-1 is the prime candidate as the ligand responsible for the transformed phenotype of normal p21 H-*ras*-overexpressing cells.

Effects of 8-bromo-cAMP on biological characteristics of cells. Apart from its effect on the induction of DNA synthesis, we also looked at other effects of 8-bromo-cAMP on the cell lines used. Addition of 0.5 mM 8-bromo-cAMP to the culture medium resulted within 48 h in changes in cell morphology. An example is given in Fig. 1. $R1^+$ cells began to stretch, and their cytoplasm formed long thin fibers. Both HE^+ and EJ^+ cells also showed changes in cellular morphology. Attachment to the culture dish was enhanced, resulting in a relatively flat appearance instead of the normal rounded shape. The peculiar membrane knobbing, most pronounced when EJ^+ cells are grown to higher densities, disappeared. This phenomenon has also been observed for CHO cells after treatment with cAMP analogs (34). These effects have been previously designated as "cAMP-mediated reversion" (32, 33).

Besides inducing morphological reversion, 8-bromo-cAMP also inhibited cell growth of some of the cell lines used ($R1^+$, HE^+) (Fig. 2). For EJ^+ cells, this effect depended on the initial plating density. At plating densities of approximately 4×10^5 cells per 50-mm dish, 8-bromo-cAMP did not substantially inhibit the growth or saturation density

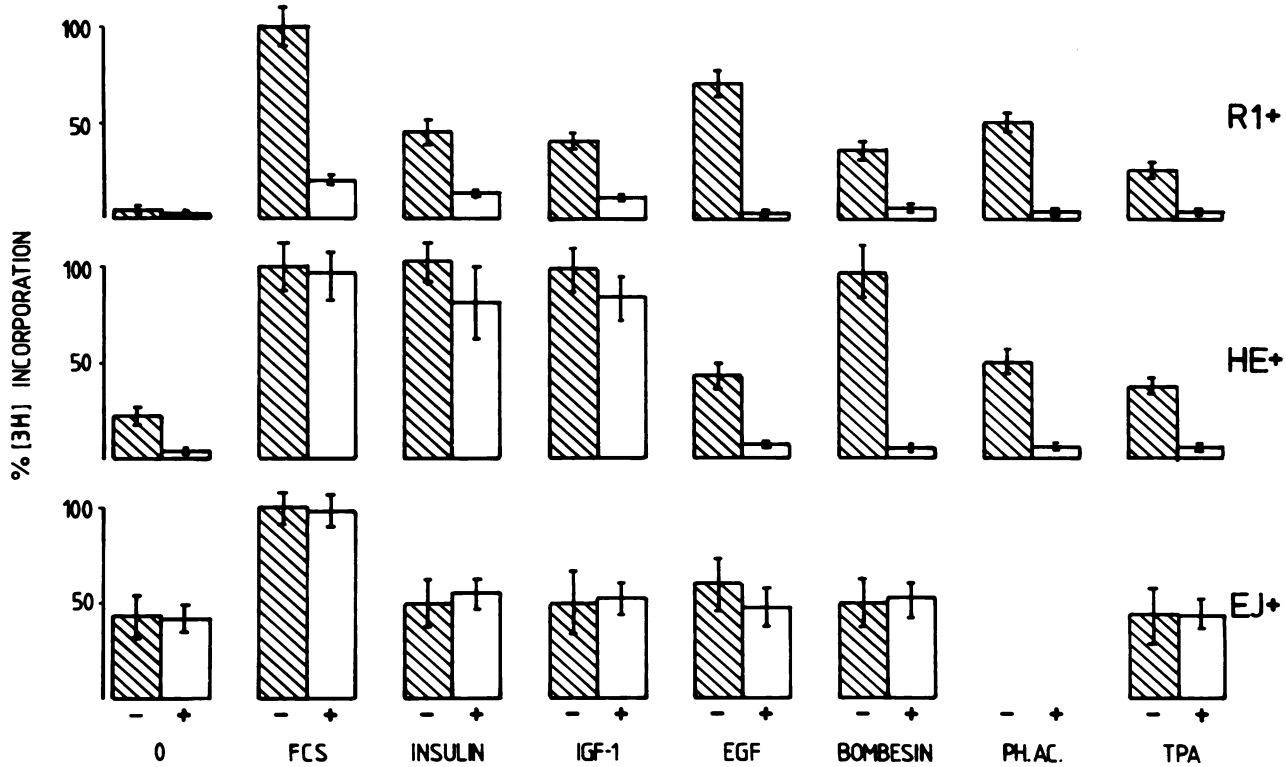

FIG. 3. GF-induced [^{3}H]thymidine incorporation in R1$^+$, HE$^+$, and EJ$^+$ cells. Cells were treated as described in Materials and Methods. The [^{3}H]thymidine incorporation induced by the different GFs is expressed as the percentage of the [^{3}H]thymidine incorporation of cells stimulated with 5% FCS in the absence of 8-bromo-cAMP, which arbitrarily was set at 100% (actual, 2×10^5 to 3×10^5 cpm per well, interexperimental variation owing to cell number). Absence or presence of 0.5 mM 8-bromo-cAMP is indicated by − (open bars) or + (hatched bars), respectively. Under all conditions, cells were pulse-labeled from 16 to 20 h after the addition of the indicated agent. GFs were used at the following final concentrations: insulin, 5 μg/ml; IGF-1, 10 ng/ml; bombesin, 10 ng/ml; EGF, 10 ng/ml; 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 100 ng/ml; phosphatidic acid (PH. AC.) from egg lecithin, 50 μg/ml. Other GFs used but not shown were platelet-derived GF, prostaglandin $F_2\alpha$ and PGE_1, vasopressin, phosphatidic acid L-α-dipalmitoyl, and fibroblast growth factor (FGF). For all these GFs, no differences were observed in the induction of [^{3}H]thymidine incorporation when R1$^+$ and HE$^+$ cells were compared. 0 stands for no additives, i.e., medium change only (control value). As far as tested in this and other experiments, there is no difference between HE cells grown in the absence of dexamethasone and rat-1 cells.

of EJ$^+$ cells. At lower plating densities (less than 2×10^5 cells), 8-bromo-cAMP also inhibited growth of EJ$^+$ cells. It is noteworthy that the ability of v-K-*ras*-transformed NRK cells to grow in serum-free medium has been reported to depend on the initial plating densities (19). This suggests that certain in vitro characteristics of *ras*-transformed cells depend on cell density. On the other hand, both R1$^+$ and HE$^+$ cells were inhibited in their growth independent of the initial plating density.

8-Bromo-cAMP inhibited the growth of HE$^+$ and EJ$^+$ cells in soft agar under all conditions tested (Table 1). Even when EJ$^+$ cells were seeded at high densities and in the presence of high concentrations of serum, virtually no colonies were seen. This clearly indicates that the growth of H-*ras*-transformed cells is unaffected by 8-bromo-cAMP only under certain defined conditions.

Effect of insulin/IGF-1 on growth of H-*ras*-transformed cells. As already discussed, HE$^+$ cells do not grow in absolutely serum-free medium. Therefore, if insulin/IGF-1 is primarily responsible for the ligand-induced transformed phenotype of HE$^+$ cells, one would expect that addition of insulin/IGF-1 to serum-free medium is sufficient to restore growth of HE$^+$ cells. This appeared to be the case (Fig. 2). The addition of insulin to serum-free medium was indeed effective. Again, as with EJ$^+$ cells, HE$^+$ cells still benefited from other components present in FCS, although insulin/IGF-1 alone was sufficient. These other components are most likely attachment factors and not GFs since combinations of GFs (i.e., insulin plus EGF) had no additional effect and, especially in absolutely serum-free medium, *ras*-transformed cells tended to detach from the culture dish.

Gene induction. An important characteristic of GFs that provide quiescent cells with signals necessary for the progression through the cell cycle is the increase in mRNA level of genes most commonly referred to as competence genes. The induction of these genes, such as c-*myc* and c-*fos*, precedes DNA synthesis. Therefore, to extend the observations of the [^{3}H]thymidine assay, we also investigated the induction of c-*myc* and c-*fos* expression in R1$^+$, HE$^+$, and EJ$^+$ cells.

Normally, insulin/IGF-1 does not induce the expression of either c-*myc* or c-*fos* in untransformed cell lines. This also held for R1$^+$ cells (Fig. 6). Under the same experimental conditions, other GFs, as represented by FCS, were capable of inducing both c-*myc* and c-*fos*. In contrast to R1$^+$ cells, insulin/IGF-1 induced c-*myc* and c-*fos* expression in HE$^+$ cells in a manner largely identical to the response obtained after the induction with FCS, although c-*myc* induction was variable.

Since EJ$^+$ cells do not respond to insulin in a [^{3}H]thymidine assay, one would not expect to observe induction of either *c-myc* or *c-fos* expression. Indeed, al-

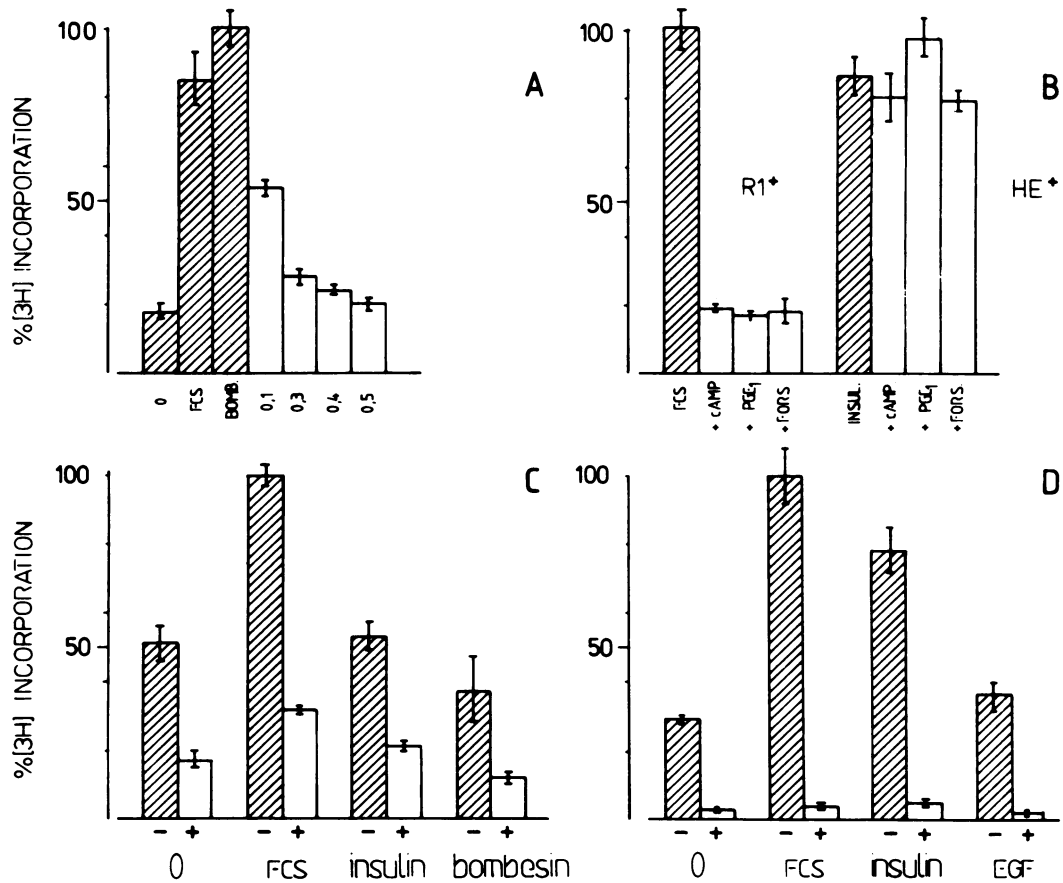

FIG. 4. Specificity of the insulin/IGF-1 and cAMP effects. All experimental conditions were identical to those described in the legend to Fig. 3. (A) Dose-response curve for 8-bromo-cAMP-mediated inhibition of [^{3}H]thymidine incorporation. HE$^+$ cells stimulated with bombesin (BOMB.) were chosen as an example since this combination gives the maximal difference between [^{3}H]thymidine incorporation in the absence or presence of 8-bromo-cAMP. The increasing amounts (millimolar) of 8-bromo-cAMP added together with bombesin are indicated by the values 0.1 to 0.5. (B) Effect of other agents elevating intracellular levels of cAMP on R1$^+$ and HE$^+$ cells. Concentrations used were as follows: IBMX, 100 μM; forskolin (FORS), 50 μM; PGE_1, 30 μM; 8-bromo-cAMP, 0.5 mM. PGE_1 and forskolin were added in combination with IBMX. IBMX is an inhibitor of cAMP phosphodiesterase, and PGE_1 and forskolin are agonists of adenylate cyclase. (C) Effect of 8-bromo-cAMP on induction of [^{3}H]thymidine incorporation in Rous sarcoma virus-transformed rat-1 cells. Rous sarcoma virus-transformed rat-1 cells received the same treatment as *ras*-transformed rat-1 cells and were stimulated with the indicated agents in the presence (+) or absence (−) of 8-bromo-cAMP. (D) Effect of arresting R1$^+$ cells in the presence of 20 ng of EGF per ml on the 8-bromo-cAMP-mediated inhibition of [^{3}H]thymidine incorporation. Cells were grown to subconfluency, and the medium was changed for medium containing 0.1% bovine serum albumin, 10 μg of transferrin per ml, and 20 ng of EGF per ml. After 48 h, cells were stimulated with the indicated agents in either the presence (+) or absence (−) of 8-bromo-cAMP. Medium change only is indicated by 0 (i.e., no additives).

though insulin stimulated both *c-myc* and *c-fos* expression in HE$^+$ cells, insulin did not induce either in EJ$^+$ cells (Fig. 6). FCS still induced *c-fos* and slightly induced *c-myc* expression, so other serum components are able to induce the expression of competence genes in mutant-*ras*-transformed cells. These data support the idea that normal p21 H-*ras* is functionally linked to the insulin/IGF-1 receptor, while mutant p21 H-*ras* is functionally uncoupled from this receptor.

Insulin and IGF-1 receptor numbers and receptor autophosphorylation. In *ras*-transformed cells, the expression of cellular genes, including genes coding for GF receptors, can be altered (12; Downward et al., in press). Consequently, altered insulin/IGF-1 responsiveness of HE$^+$ cells relative to that of R1$^+$ cells might be due to changes in receptor number. To test for this possibility, we measured insulin and IGF-1 receptor numbers on R1$^+$ and HE$^+$ cells. The results (Table 2) showed no major differences in high-affinity binding sites for insulin/IGF-1. Hence, a change in receptor number cannot account for the effects of insulin and IGF-1 seen on HE$^+$ cells.

The altered response to insulin/IGF-1 could also be due to an increased efficiency of transmembrane signalling by the insulin/IGF-1 receptor. To analyze this aspect, we also investigated the ligand-induced autophosphorylation activity of both the insulin and the IGF-1 receptor β-chains (Fig. 7). There were no significant differences between untransformed R1$^+$ cells and HE$^+$ or EJ$^+$ cells with respect to IGF-1- and/or insulin-induced autophosphorylation. The presence of two major phosphorylated bands after stimulation with insulin or IGF-1 is intriguing. The lower band migrated at the position of the β-chains of the receptors for IGF-1 and insulin. The upper band (approximately 105 kilodaltons) most likely represents a novel or differently glycosylated species of the IGF-1 receptor β-chain. The presence of two different IGF-1 receptor β-chains has previously been reported only in a neuroblastoma cell line (45). It is not clear how this presumed extra IGF-1 receptor β-chain relates to the effects seen in our system.

Both the insulin/IGF-1 and the cAMP effects are specific. A cause of major concern for all experiments with 8-bromo-

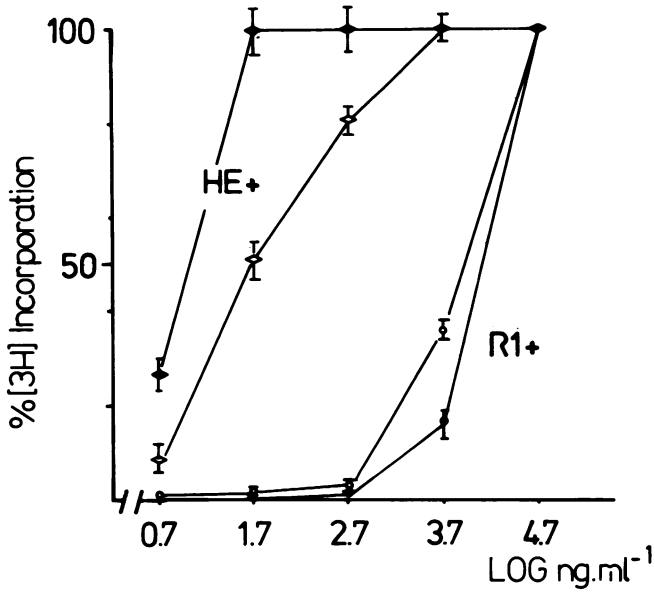

FIG. 5. Dose-response curves of the mitogenic action of phosphatidic acids on $R1^+$ and HE^+ cells. Phosphatidic acids (50-mg/ml stock solution) were dissolved in water and briefly sonicated under an N_2 atmosphere to prevent oxidation. Increasing amounts of phosphatidic acids were added to serum-deprived cultures, and [^{3}H]thymidine incorporation into cells was quantitated as described in Materials and Methods. Symbols: ◇, HE^+ cells treated with phosphatidic acid from egg lecithin; ○, $R1^+$ cells treated with phosphatidic acid from egg lecithin; ◆, HE^+ cells treated with L-α-dipalmitoyl phosphatidic acid; ●, $R1^+$ cells treated with L-α-dipalmitoyl phosphatidic acid.

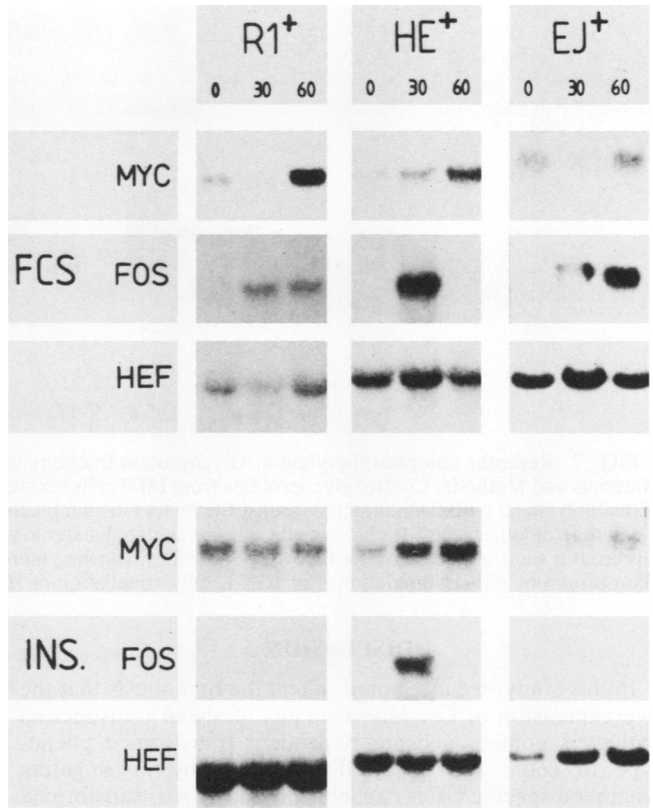

FIG. 6. Induction of c-*myc* and c-*fos* mRNA expression after GF stimulation. Northern blot analysis of c-*myc* and c-*fos* RNA expression after the addition of GFs to serum-starved $R1^+$, HE^+, and EJ^+ cells. Total RNA was isolated at the indicated time points and probed for the expression of the genes indicated. Hybridizations with the human elongation factor 1 (HEF) were performed as a control to indicate that equivalent amounts of RNA were loaded. Control inductions with serum-free medium alone did not show an increase in either c-*fos* or c-*myc* mRNA (data not shown). INS, Insulin; FCS, fetal calf serum.

cAMP is the physiological significance of the results obtained with this analog. We therefore performed several control experiments. A dose-response curve revealed a half-maximal value of 0.11 mM for cAMP-mediated inhibition of [^{3}H]thymidine incorporation (Fig. 4A). Since this is still a rather high concentration, we also tested other agents that are known to increase intracellular cAMP levels (Fig. 4B). Both the combinations of forskolin plus 3-isobutyl-1-methylxanthine (IBMX) and of prostaglandin E_1 (PGE_1) plus IMBX gave results identical to those obtained with 0.5 mM 8-bromo-cAMP. These findings confirm that the observed effects of 8-bromo-cAMP are due to changes in intracellular levels of cAMP.

To establish whether the lack of cAMP blockade on the induction of DNA synthesis is a trait specific of *ras* transformation and not a general feature of transformation, Rous sarcoma virus-transformed rat-1 fibroblasts were used as control cells. The effect of v-*src* is highly reminiscent of that of the *ras* oncogenes, but [^{3}H]thymidine incorporation of Rous sarcoma virus-transformed rat-1 cells was blocked at 8-bromo-cAMP concentrations that were ineffective for *ras*-transformed cells (Fig. 4C). Neither insulin nor bombesin could abolish this inhibition by 8-bromo-cAMP. This latter observation also excludes trivial explanations such as restoration of amino acid transport by insulin after serum deprivation (27).

Although the HE cell line could be repeatedly induced with dexamethasone resulting in a GF-dependent transformed phenotype as described above, we extended our analysis using two other rat-1-derived cell lines (H-*ras*9 and H-*ras*13 [Downward et al., in press]) expressing elevated levels of normal H-*ras*. Similar to HE^+ cells, these cells were GF dependent and responded strongly to insulin/IGF-1 in a [^{3}H]thymidine assay. Furthermore, the FCS- and insulin/IGF-1-induced responses are resistant to the inhibitory effect of 8-bromo-cAMP (data not shown). This resistance is not so strong as found for the HE^+ cell line (40 to 50% versus 0 to 10% inhibition, respectively). However, the level of H-*ras* in HE^+ cells is two- to threefold higher than that in H-*ras*9 and H-*ras*13 cells.

Taken together, these results suggest to us that in our system both the insulin/IGF-1 and the cAMP effects are specific to *ras*-transformed cells.

TABLE 2. Binding of insulin and IGF-1 to $R1^+$ and HE^+ cells[a]

Cell line	fmol/10^6 cells	
	^{125}I-insulin	^{125}I-IGF-1
$R1^+$	0.12 ± 0.01	1.26 ± 0.02
HE^+	0.09 ± 0.01	0.99 ± 0.01

[a] High-affinity binding was determined by performing duplicate experiments with tracer amounts of ^{125}I-labeled insulin or IGF-1. Background values in the presence of unlabeled insulin or IGF-1 were subtracted to yield specific binding. Cell numbers were determined by counting identical plates.

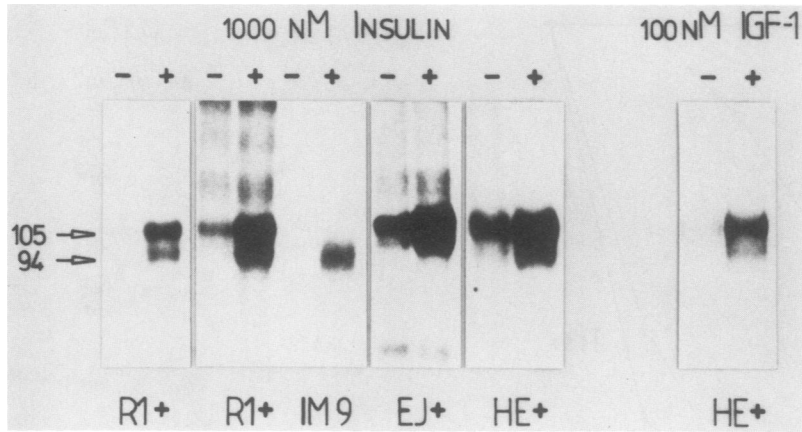

FIG. 7. Receptor autophosphorylation. Glycoprotein fractions of the different cell lines were prepared and stimulated as described in Materials and Methods. Control glycoproteins from IM-9 cells (expressing no IGF-1 receptors) were also stimulated and immunoprecipitated with a polyclonal antiserum directed against the human insulin receptor (21, 22). This revealed the migration position of the insulin receptor β-chain. In all other lanes, trichloroacetic acid-precipitated material was directly layered onto the gel. The left-most two lanes of the $R1^+$ cells represent a short exposure of the following $R1^+$ lanes, showing more clearly the two phosphorylated bands (105 and 94 kilodaltons) arising upon insulin or IGF-1 stimulation. For IGF-1, only stimulation of HE^+ glycoproteins is shown.

DISCUSSION

In this study, we attempted to test the hypothesis that the overexpression of key intermediates of signal transduction pathways confers a ligand-dependent transformed phenotype to cells. We tested this concept by investigating whether a specific GF is capable of inducing *ras*-transformed properties in cells expressing high levels of normal p21 H-*ras*. The cellular event measured was the induction of DNA synthesis. In mutant-H-*ras*-transformed (EJ^+) cells, DNA synthesis was mainly independent of exogenous (added) GFs. Cells transformed by overexpression of normal H-*ras* (HE^+) responded strongly to GFs, indicating that for full transformation of these cells GFs are required. Comparison of the GF responsiveness of HE^+ cells with that of the parental untransformed rat-1 cells ($R1^+$) showed that HE^+ cells display an elevated responsiveness toward IGF-1, insulin, and bombesin and an increased sensitivity for the action of phosphatidic acids. It appeared that 8-bromo-cAMP was a useful agent to discriminate between these different responses. The induction of DNA synthesis was inhibited in $R1^+$ cells but not in EJ^+ cells. Note that what we measured was the immediate induction of DNA synthesis. Usually, elevated intracellular levels of cAMP will potentiate GF action, but this is only seen after a lag period or after treating cells with cAMP several hours prior to GF addition (36, 40). The striking observation was that in HE^+ cells 8-bromo-cAMP inhibited DNA synthesis induced by all GFs except insulin and IGF-1. Half-maximal responses for insulin and IGF-1 were found at approximately 100 and 5 to 10 ng/ml, respectively (data not shown). Since cross-binding of insulin to the IGF-1 receptor occurs at high concentrations, it seems likely that the response to insulin, which was used at nonphysiological concentrations, is mediated by the IGF-1 receptor. Thus, IGF-1 in combination with the overexpression of normal p21 H-*ras* can prevent the 8-bromo-cAMP block on the induction of DNA synthesis. This synergism is functionally similar to mutant H-*ras* expression since mutant H-*ras* by itself can surmount the 8-bromo-cAMP block. These results strongly suggest that IGF-1 is the ligand that induces the transforming properties of cells overexpressing normal H-*ras*. However, this does not necessarily imply that insulin/IGF-1 is the only ligand that is functionally coupled to normal H-*ras*. Indeed, in the presence of FCS and 8-bromo-cAMP, HE^+ cells did not grow, whereas EJ^+ cells did, indicating that the combination of insulin/IGF-1 and an overexpressed normal p21 H-*ras* protein is not sufficient for the induction of all transformed properties.

Several different explanations could be given for the observed effects. We tried to exclude some of them. First, although the elevated responsiveness to insulin/IGF-1 probably is the result of a synergism between these GFs and TGFα, it is evident that the lack of inhibition of 8-bromo-cAMP is not due to this synergism. If, in $R1^+$ cells, TGFα secretion is simulated through the continuous presence of EGF, the elevated insulin response is still inhibited by 8-bromo-cAMP. Second, as shown by Downward and coworkers (in press), aberrant H-*ras* expression may lead to elevated GF receptor numbers and therefore to augmentation of subsequent cellular events, such as the generation of second messengers. However, we showed that there is no substantial difference in either high-affinity binding sites or signal transduction efficiency for insulin or IGF-1. Third, we also compared IGF-1 mRNA levels of the cell lines used in this study. Although these were hardly detectable, we did not find them to vary (data not shown). Hence, an IGF-1 autocrine mechanism for EJ^+ cells but not for HE^+ cells does not constitute a likely explanation. Finally, insulin did not induce c-*fos* expression in mutant-*ras*-transformed cells. Since these cells might be expected to secrete all factors archetypical to *ras* transformation, this observation supports our notion that in HE^+ cells the different response to insulin/IGF-1 results not necessarily only from a synergism with any secreted factor but also from a linkage between H-*ras* and the insulin/IGF-1 pathway.

A functional linkage between insulin/IGF-1 and normal p21 H-*ras* is further supported by our observations that, in contrast to $R1^+$ cells, in HE^+ cells both the *c-fos* and the *c-myc* genes were induced by insulin. The induction of these genes by insulin has previously been reported only for cases in which insulin serves as a strong mitogen (54). Moreover, cells expressing high levels of the insulin receptor respond to insulin with a substantial induction of c-*myc* and c-*fos* expression (50). This latter case illustrates that GFs such as insulin that normally do not induce c-*myc* or c-*fos* may

induce the expression of these genes in cells overexpressing a component of their signal transduction pathway. If, as we propose, insulin/IGF-1 induces c-*myc* and c-*fos* via the overexpressed H-*ras* protein, we would not expect to find any effect of insulin/IGF-1 on the induction of these genes in EJ^+ cells, since mutant *ras* is postulated to act autonomously. Indeed, whereas FCS can induce c-*myc* and c-*fos*, insulin does not induce the expression of these genes in EJ^+ cells.

A linkage between normal p21 H-*ras* and the insulin/IGF-1 signal transduction pathway is supported by several other observations. First, both insulin/IGF-1 and p21 behave pleiotropically and are very similar in their effects on cells. Both induce membrane ruffling (1, 14) and elevated glucose transport (12, 61) and decrease cAMP levels (6, 41). Furthermore, *ras*-transformed cells often contain elevated levels of proteases (23), whereas certain proteases have also been implicated in the early events following insulin stimulation (44). Second, coprecipitation of the insulin receptor with p21 (29) and phosphorylation of p21 by the insulin receptor (17) have been reported. Third, a functional linkage between p21 and insulin action has already been shown in the *Xenopus* oocyte system (20). *Xenopus* oocytes can be induced to mature in vitro by injecting p21 or by incubation with either insulin or progesterone. Unlike progesterone, this p21- as well as the insulin-induced maturation is not accompanied by changes in the level of cAMP (3). Microinjection with neutralizing α-ras monoclonal antibodies inhibited the maturation induced by insulin but not that induced by progesterone (20).

It has been reported that insulin stimulates de novo synthesis of phosphatidic acid(s) (10). The mechanism of GF action on phosphatidic acid(s) itself has just recently been investigated (26, 28). One possible mechanism is suggested by the observation that the effect of GTPase-activating protein upon *ras* is inhibited by mitogenically responsive lipids, including phosphatidic acid(s) (56). Our observation that the HE^+ cells are more sensitive toward the mitogenic action of phosphatidic acid(s) might be explained by such a mechanism. Although suggestive, the precise biochemical relationship between insulin/IGF-1, phosphatidic acid(s), and normal p21 H-*ras* remains to be established.

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