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Differential Regulation of Cellular Activities by GTPase-Activating Protein and NF1

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The regulation of the GTPase activity of the Ras proteins is thought to be a key element of signal transduction. Ras proteins have intrinsic GTPase activity and are active in signal transduction when bound to GTP but not following hydrolysis of GTP to GDP. Three cellular Ras GTPase-activating proteins (Ras-gaps) which increase the GTPase activity of wild-type (wt) Ras but not activated Ras in vitro have been identified: type I and type II GAP and type I NF1. Mutations of wt Ras resulting in lowered intrinsic GTPase activity or loss of response to cellular Ras-gap proteins are thought to be the primary reason for the transforming properties of the Ras proteins. In vitro assays show type I and type II GAP and the GAP-related domain of type I NF1 to have similar biochemical properties with respect to activation of the wt Ras GTPase, and it appears as though both type I GAP and NF1 can modulate the GTPase function of Ras in cells. Here we report the assembling of a full-length coding clone for type I NF1 and the biological effects of microinjection of Ras and Ras-gap proteins into fibroblasts. We have found that type I GAP, type II GAP, and type I NF1 show markedly different biological activities in vivo. Coinjection of type I GAP or type I NF1, but not type II GAP, with wt Ras abolished the ability of wt Ras to induce expression from an AP-1-controlled reporter gene. We also found that serum-stimulated DNA synthesis was reduced by prior injection of cells with type I GAP but not type II GAP or type I NF1. These results suggest that type I GAP, type II GAP, and type I NF1 may have different activities in vivo and support the hypothesis that while type I forms of GAP and NF1 may act as negative regulators of wt Ras, they may do so with differential efficiencies.

Ras proteins are membrane-associated guanine nucleotide-binding proteins with intrinsic GTPase activity which are thought to be an integral part of many regulated signal transduction systems (7, 17). Regulation of the GTPase activity of Ras appears crucial to these processes, as only the GTP-bound form of Ras is thought to be active. Recently, several Ras GTPase-activating proteins (Ras-gaps) which form a complex with both activated and wild-type (wt) Ras but increase the in vitro GTPase activity of only wt Ras have been identified (12). Type I GAP is ubiquitously expressed in all tissues, while type II GAP, an alternative splice product of type I GAP lacking the type I-form hydrophobic domain, appears restricted to placental trophoblasts (35). Recently another cellular Ras-gap has been identified as the product of the neurofibromatosis gene, NF1 (21). Two alternative splice products of NF1, type I and II, have been identified (25, 32). Type I NF1 appears to be more highly expressed in proliferative cells, while type II NF1, which contains an insert within the GTPase-activating domain (the GAP-related domain) of the type I protein, is more prominent in differentiated cells.

In vitro assays show type I and II GAP and the GAP-related domain of type I NF1 to have similar biochemical properties with respect to activation of the wt Ras GTPase (21). Similarly, both type I GAP and the GAP-related domain of type I NF1 can rescue yeast (*Saccharomyces cerevisiae*) strains which are deficient in the yeast Ras-gap homologs (the IRA proteins) (2, 3, 33, 38). Transfection of type I GAP into cells transformed by overexpression of wt Ras results in the reversion of the transformed phenotype of the cells (40). While these data suggest that type I GAP may be negatively regulating wt Ras, they do not fully address the function of type I GAP activity toward wt Ras activity in untransformed cells. Furthermore, little is known about the respective roles of type I and II GAP and of type I and II NF1 in cellular signal transduction.

The introduction of neutralizing anti-Ras antibodies or dominant interfering Ras proteins into quiescent fibroblasts blocks the mitogenic action of serum as well as selected growth factors (24, 29). In accordance with this finding, mutations which constitutively activate wt Ras are frequently associated with several human cancers. The introduction of either activated or wt Ras into cells induces membrane ruffling, fluid-phase pinocytosis, and the induction of the proto-oncogene c-*fos*, all of which can be hallmarks of growth factor action (4, 31). The introduction of Ras proteins has also been shown to induce gene expression from reporter genes containing serum response elements and AP-1 enhancer elements in their promoters (1a). When activated Ras is introduced into cells, these effects persist for many hours, whereas for wt Ras, these effects recede within several hours. Activated Ras also induces DNA synthesis and cell division, whereas wt Ras appears not to have these effects when introduced into cells at similar levels (30). The difference in activity between wt and activated Ras is thought to be due to the reduced GTPase activity of activated Ras and the loss of regulation of this form of Ras by intrinsic Ras-gaps (7, 17).

Here, we report the cloning of the full-length *NF1* coding

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sequence and demonstrate for the first time a biological activity for full-length NF1 protein. In addition we have found differential regulation of wt Ras activities by type I and II GAP and type I NF1. Specifically, through microinjection of full-length proteins into living cells, we found that type I GAP and type I NF1, but not type II GAP, suppressed Ras-induced expression of an AP-1-controlled reporter gene with different efficiencies. Moreover, we found that of the three Ras-gaps examined, only type I GAP was able to reduce serum stimulated DNA synthesis.

MATERIALS AND METHODS

Construction of the *NF1* full-length coding clone. Six overlapping cDNA plasmids in the vector of pBluescript SK- (Stratagene), FR34, FB52, FB50, FB15, FB9, and FB8, were released in vivo from their corresponding lambda-ZAP phage clones. All of these phages had been isolated from a human fetal brain cDNA library as previously described (37) except for FR34, which came from a fetal retina cDNA library (Stratagene no. 937202) (39). The 1.0-kb *Bgl*II-*Kpn*I fragment from FB9 was ligated to the double-digested FB15 to generate clone pNFL1. The 3.2-kb *Xho*I-*Kpn*I fragment released from pNFL1 was joined to FB50 to form clone pNFL2. pNFL5 was obtained by releasing the 1.4-kb *Sph*I-*Spe*I fragment from FB52 and inserting it into double-digested FR34. pNFL2 and pNFL5 were ligated at the unique site of *Spe*I, forming a new clone, pNFL8. The 6.8-kb insert of pNFL8 was then released with *Sac*II and *Kpn*I and cloned directionally into the same two sites of a plasmid (pBGX1) constructed by inserting several unique cloning sites at the *Eco*RI site of pBR322. The resultant clone was then joined to FB8 at the *Kpn*I site to create the first full-length clone, pNFL19.

Each cloning step was verified by restriction site mapping and by sequencing through the ligation sites. The full-length clone, pNFL19, was sequenced either manually or with an Applied Biosystems model 373A automatic sequencer. The sequences obtained in this way were merged and compared with the *NF1* cDNA consensus sequence (20, 37).

The *Xho*I-*Kpn*I fragment of pNFL19 was replaced with the corresponding fragment from pNFL1, to repair the one-base deletion identified in pNFL19. The resultant clone, pNFL22, was confirmed by sequencing through the ligation sites as well as through the region where the deletion occurred in pNFL19.

Cell culture and injections. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) response element (TRE) indicator cells were created by stably transfecting Rat-2 cells with a reporter plasmid containing a neomycin resistance gene and the reporter gene *lacZ* under the regulation of four AP-1 promoter elements (TRE plasmid) (22). The indicator cells were grown in the presence of G418 (300 μg/ml) for selection purposes. The TRE indicator cell line, rat REF52 cells, and human WI38 cells were grown on etched coverslips in 10% fetal calf serum-supplemented Dulbecco's modified Eagle medium (Cell Grow). When the cells reached 50% confluency, they were rendered quiescent by 36-h starvation in 0.05% fetal calf serum-supplemented medium. Cells were subsequently injected with an Eppendorf automatic microinjector. Approximately 100 cells were injected per coverslip per experiment. Type I and II GAP and NF1 proteins were produced in insect cells by using baculovirus expression constructs (6a, 16). They were purified under nondenaturing conditions to homogeneity, as judged by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gels. Unless indicated otherwise, TRE indicator cells and REF52 cells were injected with proteins at the following concentrations: 0.3 μM the respective purified Ras-gap, 95 μM wt H-Ras, and 48 μM activated T24 H-Ras (following expression and purification from *Escherichia coli* [14]). Alternatively, REF52 cells were directly injected with 0.2 mg of the reporter plasmid and various proteins per ml, as indicated. For these experiments, nuclear and cytoplasmic compartments were injected, while for all other experiments, only the cytoplasm was injected. WI38 cells were injected with 0.4 μM the respective Ras-gap. All of the cells were coinjected with 2 mg of mouse immunoglobulin G (IgG) per ml.

Detection of reporter gene expression. The TRE indicator cells and REF52 cells were fixed with 3.7% formaldehyde 12 and 2 h, respectively, after microinjection. The cells were rinsed briefly in phosphate-buffered saline (PBS) and subsequently incubated overnight to stain for the production of β-galactosidase (β-Gal) in a PBS solution containing 1 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml, 5 mM ferrocyanide, 5 mM ferricyanide, and 2 mM $MgCl_2$. The coverslips were then rinsed with PBS, stained with fluorescein-conjugated goat anti-mouse antibody to detect the coinjected marker in injected cells, and then rinsed and mounted onto slides. The coverslips were allowed to dry before examination for injected cells (indicated by fluorescein-stained cytoplasm) and the production of β-Gal, using a fluorescence microscope. The number of fluorescent cells which stained dark blue was calculated for each experiment and compared with the number of cells injected. Typically, quiescent cells injected with the reporter construct along with only the marker IgG showed little expression of β-Gal, while cells coinjected with the same reporter and wt H-Ras showed about 50% of cells expressing β-Gal.

Detection of serum-stimulated DNA synthesis. WI38 cells were rendered quiescent by serum deprivation prior to injection. One hour after microinjection, starvation medium of the WI38 cells was replaced with medium containing 10% fetal calf serum and the nucleotide analog bromodeoxyuridine (BrdU; Amersham cell proliferation kit, used according to instructions); 24 h after the addition of serum, the cells were rinsed and fixed with 3.7% formaldehyde. The cells were subsequently rinsed with PBS and stained with a monoclonal rat antibody against BrdU. Nuclei incorporating BrdU were stained with a rhodamine-conjugated donkey anti-rat antibody after a brief rinse with PBS. Injected cells were then detected with a fluorescein-conjugated goat anti-mouse antibody. The coverslips were then rinsed with PBS and mounted onto slides. The coverslips were allowed to dry before examination for injected cells (indicated by fluorescein-stained cytoplasm) and the incorporation of BrdU (indicated by rhodamine-stained nuclei). The number of fluorescein-stained cells which contained rhodamine-stained nuclei was calculated for each coverslip as an indication of the number of injected cells which synthesized DNA.

RESULTS

The strategy that assembled two other large full-length clones, *CFTR* (11, 13) and *DMD* (18), was used to clone an *NF1* full-length coding sequence. Six well-characterized overlapping cDNA clones were selected, and six unique restriction sites were identified along the open reading frame (Fig. 1). The clones were joined step by step as detailed in Materials and Methods.

Initial efforts to obtain a full-length clone by connecting pNFL8 (6.8 kb) and FB8 (2.0 kb) resulted in clones with

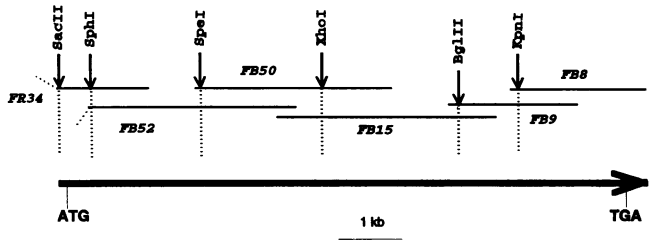

FIG. 1. Strategy for constructing the *NF1* full-length coding clone. The thick horizontal arrow, with translation initiation (ATG) and termination (TGA) indicated, represents the full-length coding sequence and some flanking noncoding sequence of the *NF1* gene. Solid lines above this arrow show the relative sizes and positions of the six overlapping cDNA clones, which were used to assemble the full-length clone; the slanted, dashed lines at the 5′ ends of FR34 and FB52 indicate the unspliced intronic sequences of these clones. Clone names are indicated near their corresponding lines. Vertical arrows extended by dotted lines indicate positions of six unique restriction sites in this series of clones.

TABLE 1. Inhibition of wt H-Ras induction of AP-1 promoter elements by type I GAP and type I NF1 but not type II GAP

Injected protein	% of positive cells[a]	
	TRE/*lacZ* indicator cell line	TRE/*lacZ* reporter plasmid injection
wt Ras alone	36 ± 6 (334)	49 ± 5 (475)
wt Ras + type I GAP	7 ± 3 (199)	5 ± 1 (378)
wt Ras + type II GAP	47 ± 14 (124)	ND
wt Ras + type I NF1	ND	6 ± 4 (557)
T24 Ras	33 ± 5 (199)	44 ± 1 (85)
T24 Ras + type I GAP	27 ± 5 (121)	ND
T24 Ras + type II GAP	ND	55 ± 4 (169)
T24 Ras + type I NF1	ND	44 ± 9 (132)
Control injections	2 ± 2 (228)	8 ± 4 (483)

[a] Percentage of injected cells which stained positively for production of β-Gal. Errors indicate the standard errors of the means of two or more separate experiments. Numbers in parentheses indicate the number of injected cells. ND, not determined.

extensive deletions. The transfected bacteria might have been selecting against the full-length construct because of toxicity; if so, reducing the number of cDNA copies might circumvent the rearrangement problem. In fact, a medium-copy-number vector, pBR322, did yield a stable full-length clone, pNFL19, after the cDNA inserts of pNFL8 and FB8 were joined at the *Kpn*I site.

Sequencing of pNFL19, however, revealed a single-base-pair deletion (Fig. 2). This deletion, near the center of the GAP-related domain, would result in a premature translation termination just a few codons downstream. We sequenced all of the intermediate constructs and found that this deletion first appeared when FB50 was ligated to the insert of pNFL1, the recombinant clone of FB15 and FB8, which contained no such alteration. Therefore, we repaired the deletion in pNFL19 by replacing the *Xho*I-*Kpn*I (3.2-kb) fragment with the corresponding fragment from pNFL1.

Type I GAP and type I NF1, but not type II GAP, inhibit wt H-Ras induction of AP-1 promoter elements. Microinjection of wt H-Ras into the TRE indicator cell line resulted in the induction of expression of the *lacZ* gene in an average of 36% of the injected cells (Table 1). Corresponding control injections, on the other hand, led to expression of β-Gal in only 2% of the cells. Coinjection of wt H-Ras with type I GAP completely abolished the β-Gal induction seen in cells injected with wt H-Ras alone. Surprisingly, coinjection of type II GAP at similar levels with wt H-Ras had no inhibitory effect on wt H-Ras induced β-Gal expression (Table 1) even though biochemical assays indicated that preparations of type I and II GAP have identical abilities to activate the wt Ras GTPase. In similar experiments with T24 Ras, it was found that this protein induced β-Gal expression in 33% of the injected cells. As expected, coinjection of type I GAP with activated Ras did not diminish the induction of β-Gal by T24 Ras.

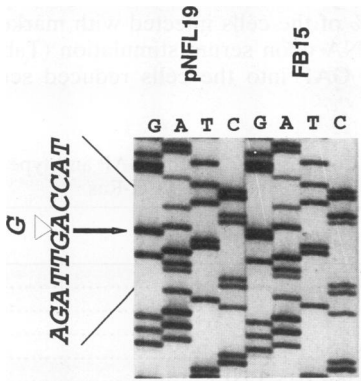

FIG. 2. Sequence comparison between cDNA FB15 and the first full-length construct, pNFL19. Shown is part of the sequencing gel, with each lane indicated. The expanded sequence at the left corresponds to the middle part of the gel, and the triangle indicates the site of the one-base deletion identified in pNFL19. The arrow marks the point where the aberration in pNFL19 is revealed in the gel.

Coinjection of wt H-Ras with the TRE/*lacZ* reporter plasmid into REF52 cells resulted in a Ras-dependent expression of the *lacZ* gene, similar to that observed following injection of Ras protein into the TRE indicator cell line. Because this assay method proved to be more sensitive (i.e., more rapid responses and lower background) than use of the transfected cell line, it was also used to assess the role of GAPs and type I NF1 on Ras-induced gene expression. The type I form of GAP or full-length NF1 was coinjected with wt H-Ras or T24 Ras, and the reporter plasmid was injected into quiescent REF52 cells. Two hours after injection, the cells were stained for injected protein and expression of β-Gal. Expression of β-Gal was induced in an average of 49% of the cells injected with wt H-Ras (Table 1). β-Gal was not induced by injection of type I GAP, type I NF1, or control proteins (data not shown). Coinjection of wt H-Ras with type I GAP completely abolished the β-Gal induction seen when wt H-Ras was injected alone with the reporter plasmid. Similarly, it was found that type I NF1 also inhibited wt H-Ras-induced gene expression (Table 1; Fig. 3). As was observed previously, type II GAP injections with the reporter plasmid had no inhibitory effect on wt H-Ras-induced β-Gal expression (data not shown). As expected from in vitro biochemical data as well as the data from injection into the TRE cell line, none of the Ras-gaps had any effect on induction of the reporter gene by T24 Ras. To test that the inhibition of TRE-regulated gene expression by the type I forms of GAP and NF1 was not due to a nonspecific inhibition of the reporter construct, cells were injected with type I GAP or NF1 and the reporter plasmid, stimulated with TPA, and treated as described above. This was done be-

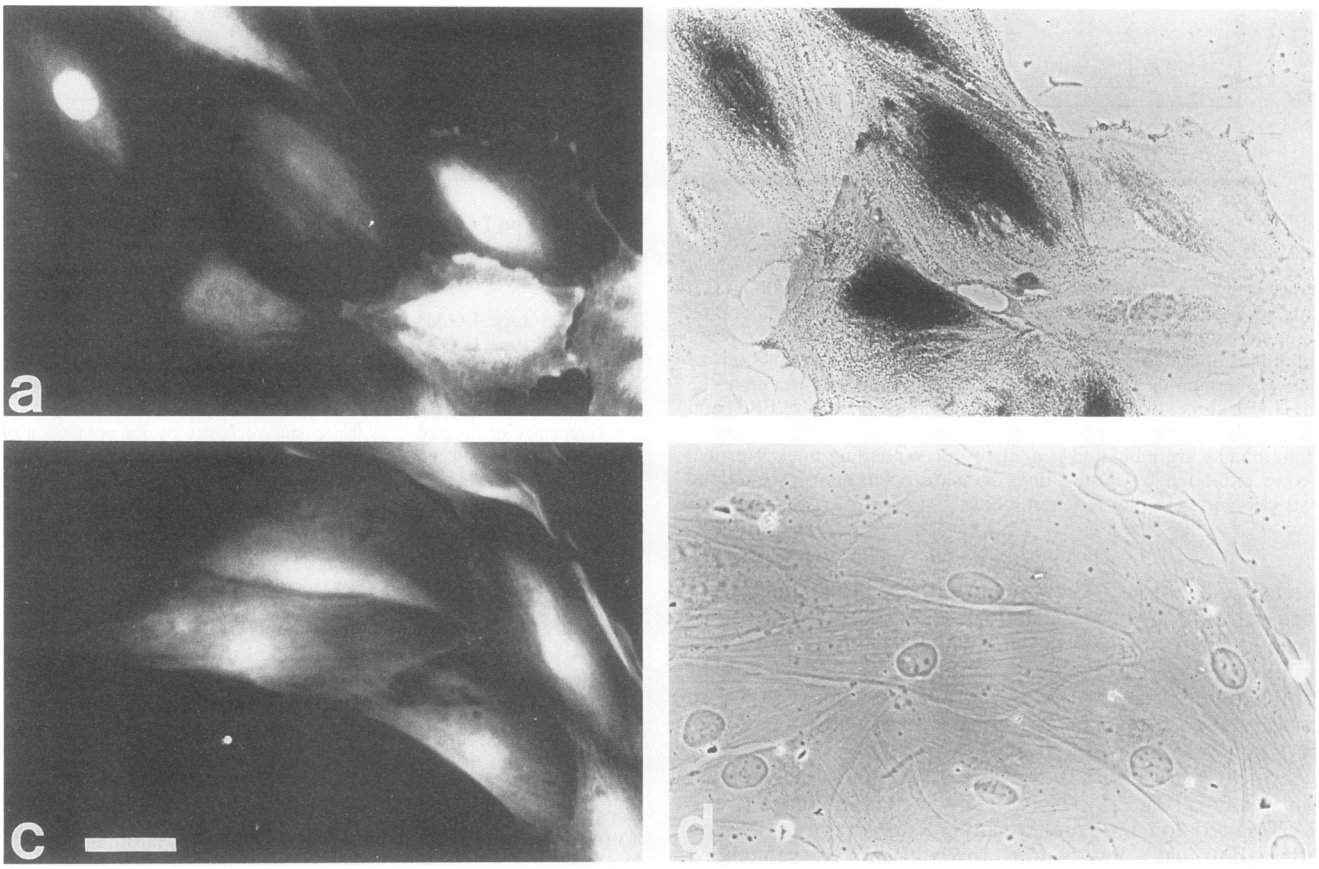

FIG. 3. Photomicrographs of wt Ras-injected cells (a and b) and wt Ras/type I NF1-coinjected cells (c and d) showing that coinjection of type I NF1 inhibits wt Ras induction of AP-1-controlled genes. Fluorescence photomicrographs (a and c) indicate brightly stained injected cells, and corresponding phase-contrast photomicrographs (b and d) show β-Gal production as darkly stained cells. The bar represents 1 μm.

cause TPA also induces expression of the TRE/*lacZ* construct. Neither type I GAP nor type I NF1 had any effect on TPA-stimulated β-Gal expression (data not shown), indicating that these proteins did not nonspecifically inhibit the reporter gene.

Type I GAP appears to be a more effective inhibitor of wt H-Ras than type I NF1. The foregoing results suggested that the type I forms of GAP and NF1 may have similar activities in vivo with respect to inhibition of wt Ras signal transduction. In vitro analysis of the GAP-related domain of NF1 shows it to have a 30-fold-lower specific activity for wt Ras than does type I GAP with respect to activation of Ras GTPase (21). To further compare the activities of type I GAP and NF1 in living cells, serial dilutions of each Ras-gap were prepared and coinjected with wt H-Ras and the reporter plasmid, and effects upon wt H-Ras-induced gene expression were assessed. The ability of type I NF1 to inhibit wt H-Ras gene expression was lost fully at 1/100 of its original concentration (Table 2). Type I GAP, however, appeared to be a more potent inhibitor of wt H-Ras-induced gene expression. Type I GAP activity was only partially lost at 1/100 of its original concentration. Its full activity was lost at 1/1,000 of its original concentration.

Type I GAP, but not type II GAP or type I NF1, reduces serum-stimulated DNA synthesis. While these results indicate that type I GAP and type I NF1 are capable of inhibiting gene expression in response to coinjected wt H-Ras, they do not address the possible function of these Ras-gaps with respect to endogenous cellular Ras activity. Microinjection of dominant interfering Ras protein or antibodies to Ras into cells reduces serum-stimulated DNA synthesis, suggesting that cellular Ras activity is necessary for serum-induced DNA synthesis (24, 29). To examine the effects of Ras-gaps on serum-stimulated DNA synthesis, quiescent human WI38 cells were injected with purified GAP proteins or type I NF1 and subsequently stimulated with serum. As expected, an average of 53% of the cells injected with marker IgG alone synthesized DNA upon serum stimulation (Table 3). Injection of type I GAP into the cells reduced serum-induced

TABLE 2. Comparison of type I GAP and type I NF1 as inhibitors of wt H-Ras

Injected protein	% of positive cells[a]
wt H-Ras alone	49 ± 5 (475)
wt H-Ras + 280 nM type I GAP	5 ± 2 (183)
wt H-Ras + 2.8 nM type I GAP	15 ± 4 (229)
wt H-Ras + 0.28 nM type I GAP	42 ± 7 (265)
wt H-Ras + 280 nM type I NF1	8 ± 6 (182)
wt H-Ras + 2.8 nM type I NF1	38 ± 3 (254)
wt H-Ras + 0.28 nM type I NF1	32 ± 0 (264)

[a] Percentage of injected cells which stained positively for production of β-Gal. Errors represent the standard errors of the means of two or more separate experiments. Numbers in parentheses indicate the number of injected cells.

TABLE 3. Inhibition of serum-stimulated DNA synthesis by type I GAP but not type II GAP or type I NF1

Injected protein	% of cells synthesizing DNA upon serum stimulation[a]
Type I GAP	20 ± 5 (284)
Type II GAP	52 ± 2 (196)
Type I NF1	53 ± 3 (246)
Mouse IgG	53 ± 1 (232)
Uninjected	68 ± 7 (622)

[a] Errors indicate the standard errors of the means of two or more experiments. Numbers in parentheses indicate the number of injected cells.

DNA synthesis to an average of 20% (Fig. 4). Similar to the effects on gene expression, injection of type II GAP had no effect on serum-stimulated DNA synthesis. Surprisingly, despite having activity similar to that of type I GAP with respect to Ras-induced gene expression, injection of type I NF1 had no effect on serum-stimulated DNA synthesis. To test whether the injected GAP or NF1 remained active for a prolonged period of time in the cells (i.e., the time required for mitogenesis assays), GAP or NF1 was injected into cells; 10 h later, the same cells were reinjected with wt Ras and the TRE/*lacZ* constructs. Two hours later, the cells were assessed for β-Gal expression. It was found in both GAP- and NF1-preinjected cells that wt Ras was unable to induce the TRE/*lacZ* construct (data not shown). These results suggest that the reason for a lack of inhibitory effect of NF1 on DNA synthesis (and therefore the difference between NF1 and GAP in this assay) was not simply due to a rapid loss of NF1 activity following injection into cells, in keeping with its apparently long half-life of 36 h (8).

DISCUSSION

These results show that injection of an excess of type I GAP or NF1 into living fibroblasts inhibited signaling mechanisms initiated by wt H-Ras, whereas type II GAP did not inhibit Ras at all. Type I GAP inhibited the wt H-Ras induction of a reporter gene regulated by AP-1 promoter elements and also reduced serum-stimulated DNA synthesis, a process which is thought to depend upon endogenous wt Ras (24, 29). These data support the hypothesis that type I GAP can act as a negative regulator of wt Ras, although it may also act as an effector of Ras (12, 17, 27). These results are consistent with earlier observations that transfection of type I GAP into wt Ras-transformed cells reversed their transformed phenotype (40) as well as with experiments showing that an increase in Ras activity occurred upon T-cell activation concomitant with a decrease in GAP activity (presumably type I GAP) (10) and those showing a diminished mitogenic response in fibroblasts transfected with GAP (25a). It has been shown that type I GAP associates with the putative wt Ras effector domain (1). This finding has led to the suggestion that type I GAP may also be a Ras effector. Similar results have been observed with respect to the receptor-coupled G protein $G_{q/11}$ and phospholipase C-β. Phospholipase C-β has been shown to be both an effector of the $G_{q/11}$ protein (28, 34) and a negative regulator of G_q by activating the G_q GTPase (6, 26). It is possible that GAP acts on Ras in a similar fashion, acting as both an effector and a down-regulator of wt Ras. The present results cannot distinguish between these activities but suggest that perhaps the balance between Ras and GAP is crucial to the normal signaling process and that excess GAP inhibits the action of cellular Ras.

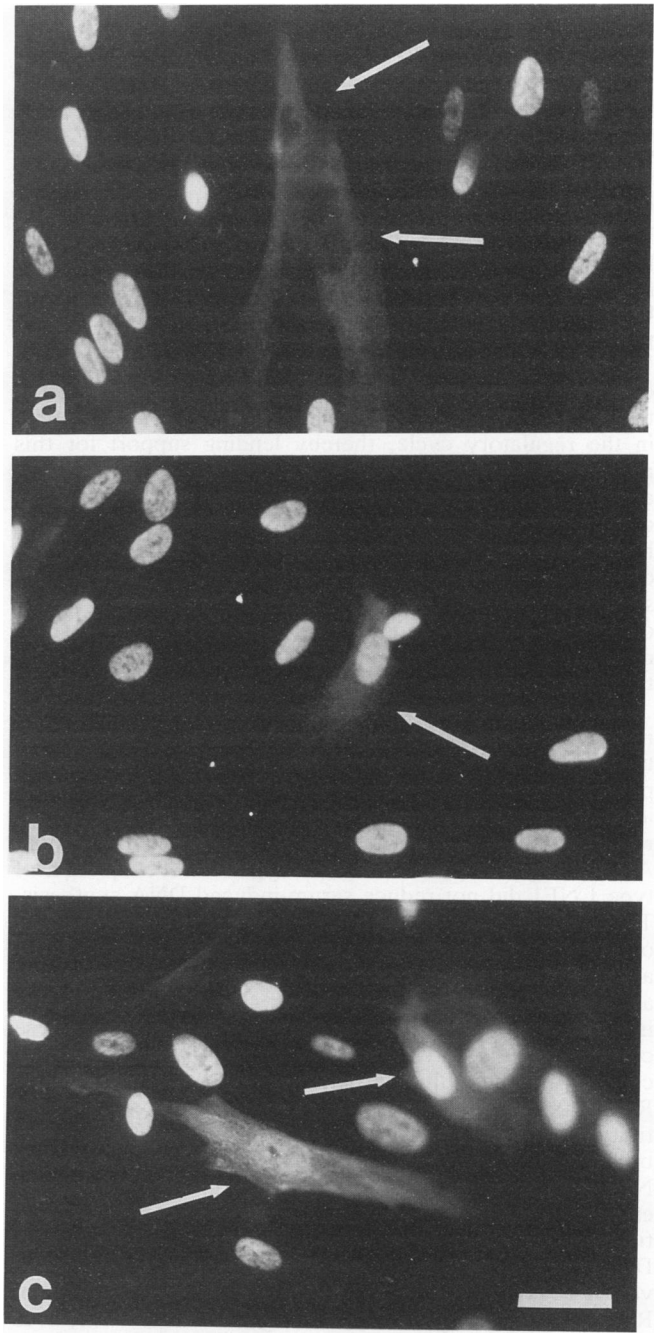

FIG. 4. Fluorescence photomicrographs of cells injected with type I GAP (a), type II GAP (b), and type I NF1 (c), showing that serum-stimulated DNA synthesis was reduced only in type I GAP-injected cells. Brightly stained cytoplasm indicates injected cells, and brightly stained nuclei indicate DNA synthesis (the incorporation of BrdU into DNA). Arrows indicate injected cells. The bar represents 1 μm.

The present studies also show that despite having the same in vitro activity, type I and type II GAP did not behave similarly with respect to inhibiting wt Ras in living cells. Unlike type I GAP, type II GAP did not inhibit wt H-Ras induction of AP-1 promoter elements, nor did it reduce

serum-induced DNA synthesis. Type I and II GAP were assayed in vitro for biochemical activity with respect to activation of the wt Ras GTPase under the same buffer conditions used for the microinjection experiments and, as reported previously (35), were found to contain identical catalytic activities. Thus, the observed difference in their in vivo activities cannot be attributed to a biochemical difference in activity. Because type II GAP lacks the amino-terminal hydrophobic domain found in type I GAP, it is possible that this domain is required for inhibition of wt Ras activity in vivo. The amino-terminal hydrophobic domain of type I GAP may allow the protein to associate with the plasma membrane, which is the site of localization of wt Ras. While type I GAP has been isolated primarily from the cytosolic fraction of quiescent cells (23), it can be speculated that GAP associates with Ras at the membrane at some time in the regulatory cycle, thereby lending support for this possibility. Alternatively, the two forms of GAP may have different half-lives in cells, which could also give rise to the observed cellular activities. Likewise, the amino-terminal hydrophobic domain could also be involved in the specificity of interaction with GTP-binding proteins and hence in its specificity in cells. Should this prove to be the case, it will be of importance to determine whether other proteins, which may include Ras-related molecules, serve as the target of type II GAP activity in the placenta.

Our data also show that type I NF1 has the ability to inhibit Ras activity. As was found for type I GAP, full-length type I NF1 inhibited wt H-Ras induction of AP-1-regulated promoter activity. Type I NF1, however, exhibited a significantly less potent phenotype than did type I GAP with regard to inhibition of wt H-Ras-induced gene expression. Additionally, unlike the case for type I GAP, injection of type I NF1 did not reduce serum-induced DNA synthesis. This finding may reflect the fact that the GAP-related domain of NF1 constitutes only 10% of the full-length NF1 protein and may indicate that NF1 is performing a function(s) in addition to down-regulation of wt Ras activity. These results indicate that type I GAP and NF1 are not equivalent in these cells in terms of presumably lowering Ras-GTP levels in cells. Given the multitude of cellular effects attributable to Ras function (e.g., altered gene expression, membrane activity, and cytoskeleton structure), a bifurcation of signal transduction pathways may occur at the level of GAP and NF1. The idea that GAP or NF1 may in addition provide effector functions for Ras remains plausible. The observation that NF1 injection does not block serum-stimulated DNA synthesis may indicate that serum leads to an inactivation of NF1 but not GAP with respect to stimulation of Ras GTPase activity. This possibility, while attractive, does not seem likely, as preliminary experiments in cells that respond mitogenically to injected wt Ras also show that GAP but not NF1 coinjection inhibits the Ras-dependent DNA synthesis in the absence of serum. Alternatively, because the GAP-related domain of type I NF1 has a 20-fold-higher affinity for wt Ras yet a 30-fold-lower specific activity for activation of the wt Ras GTPase than does type I GAP (21, 36), the inability of NF1 to inhibit serum-stimulated DNA synthesis may simply reflect its lower specific activity, particularly in light of the fact that endogenous Ras levels are reported to increase upon mitogenic stimulation of cells (19). In any case, the ability to abolish the function of GAP or NF1 in living cells and examination of the resultant phenotypes will be important for exploration of these possibilities.

For NF1, the results presented in this report may also reflect the fact that fibroblasts express predominantly type II NF1 and relatively little type I NF1 (25); hence, type I NF1 injected into fibroblasts may not be as effective in inhibiting endogenous Ras signal transduction. Further studies comparing directly the activities of type I and II NF1 proteins on signal pathways in cells will be important. Other possibilities come to light when these results are compared with those of previous studies comparing NF1 and GAP. Type I NF1 is associated primarily with the plasma membrane, while type I GAP is mainly cytosolic in quiescent cells (8, 15). Also, neural cells deficient in type I NF1 but containing type I GAP exhibit transformed growth properties and an activation of endogenous Ras (5, 9). These results may suggest that GAPs and NF1 proteins play different regulatory roles in signaling pathways involving Ras and that the multitude of Ras-gaps found thus far may reflect this difference. It will be important to further compare the functions of these molecules as well as that of type II NF1 in living cells to determine their normal roles in the regulation of Ras and hence in cell growth and differentiation.

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