ation of the fatty acids of phosphatidylglycerol by the introduction of an appropriate acyltransferase. They also define the *in vivo* function of acyltransferases. Factors apart from the extent of unsaturation of the fatty acids in phosphatidylglycerol may affect chilling sensitivity, but our experiments have shown that it is an important contributor. □

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Aberrant regulation of *ras* proteins in malignant tumour cells from type 1 neurofibromatosis patients

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DEFECTS in the *NF1* gene have been implicated in the inherited disorder neurofibromatosis type 1, which is characterized by several developmental abnormalities including an increased frequency of benign and malignant tumours of neural crest origin (neurofibromas and neurofibrosarcomas respectively)[1]. The *NF1* gene encodes a ubiquitous protein homologous to $p120^{GAP}$, the GTPase-activating protein (GAP) for the products of the *ras* proto-oncogenes[2-6]. When expressed in non-mammalian systems, the region of the *NF1* gene homologous to $p120^{GAP}$ produces a protein with GAP-like activity[7-9]. Here we present evidence that the *ras* proteins in malignant tumour cell lines from patients with type 1 neurofibromatosis are in a constitutively activated state, as judged by the guanine nucleotide bound to them, and are necessary for cellular proliferation. These cells contain $p21^{ras}$ and $p120^{GAP}$ that are both functionally wild type, but barely any functional NF1 protein. Our results show that the NF1 protein is normally essential for correct negative regulation of *ras* proteins in the cell, even in the presence of normal $p120^{GAP}$, and they support the hypothesis that *NF1* is a tumour-suppressor gene whose product acts upstream of *ras*.

The activation state of $p21^{ras}$ within a cell can be determined by measuring the ratio of GTP (active) to GDP (inactive) bound to it. Transforming mutants of $p21^{ras}$ bind large amounts of GTP, whereas wild-type $p21^{ras}$ is almost entirely GDP-bound unless the cell has received one of several extracellular stimuli (reviewed in ref. 10). The proportion of GTP to total guanine nucleotide bound to $p21^{ras}$ in a number of cell lines is shown in Fig. 1*a*. Normal Rat-1 fibroblasts, HeLa cells, primary rat Schwann cells, schwannoma cells and quiescent human T cells all have very small amounts of GTP bound to $p21^{ras}$ (less than 5% in the absence of serum). By contrast, Rat-1 cells transformed by an activated *ras* mutant encoding leucine at position 61 (Leu-61 *ras*) have ≥50% of GTP-bound $p21^{ras}$. A similar ratio was found for $p21^{ras}$ from phytohaemagglutinin-activated T cells[11]. This ratio was measured for three cell lines, probably of Schwann-cell origin, derived from neurofibrosarcomas (malignant schwannomas) from neurofibromatosis type 1 patients (NF 90-8, NF 88-3 and ST 88-14): each had high levels of GTP on $p21^{ras}$ (up to 50% in the case of ST 88-14).

The increased activation state of $p21^{ras}$ in the neurofibrosarcoma cells could either be due to a defect in a protein normally involved in the regulation of $p21^{ras}$ or to a mutation in a *ras* gene itself. To investigate the possibility of a *ras* mutation, $p21^{ras}$ was immunoprecipitated from [^{32}P]orthophosphate-labelled cells using the monoclonal antibody Y13-259. This antibody inhibits the biological function of $p21^{ras}$ and greatly reduces the ability of GAP to stimulate GTP hydrolysis on $p21^{ras}$, but it does not completely block the interaction of bound $p21^{ras}$ with GTPase-activating proteins. We then exposed the washed immunoprecipitates to recombinant GAP or NF1 catalytic domains. The amount of GTP bound to $p21^{ras}$ from Rat-1 cells transformed by Leu-61 *ras* was not affected by this treatment as activated mutants of $p21^{ras}$ are insensitive to the GTPase-activating activity of GAP or NF1 (see Fig. 1*b*), whereas the GTP levels on the endogenous normal $p21^{ras}$ from phytohaemagglutinin-activated peripheral blood T-lymphoblasts decreased significantly. The $p21^{ras}$ from the neurofibrosarcoma cell lines was also sensitive to GAP and to NF1 proteins in this assay (see Fig. 1*b*). Therefore $p21^{ras}$ in the neurofibrosarcoma cells behaves normally and shows no evidence of having undergone activating mutation, although some weakly activating mutations may not be evident in this assay.

If there are no functional defects in the *ras* proteins themselves, it is likely that the *ras* regulatory mechanisms are defective in neurofibrosarcoma cells. We therefore measured the enzymic activity of the GTPase-activating proteins $p120^{GAP}$ and NF1 GAP-related protein (GRP) in lysates and immunoprecipitates from the neurofibrosarcoma cell lines, HeLa cells, NIH3T3 cells and a transformed Schwann cell line from rat (Fig. 2). Lysates from all the neurofibrosarcoma cell lines have high levels of overall GAP-like activity, albeit less than from other cell types. Immunoprecipitates of $p120^{GAP}$ with an antiserum (PW6) raised against a non-catalytic region of the molecule also have GAP-like activity which is comparable to that in similar samples from other cell types. By contrast, immunoprecipitates of NF1 GRP using an antiserum against the carboxyl terminus of the protein (D1) do not have this GAP-like activity when they are produced from lysates of the neurofibrosarcoma cells, but are very active when derived from the other cell lines.

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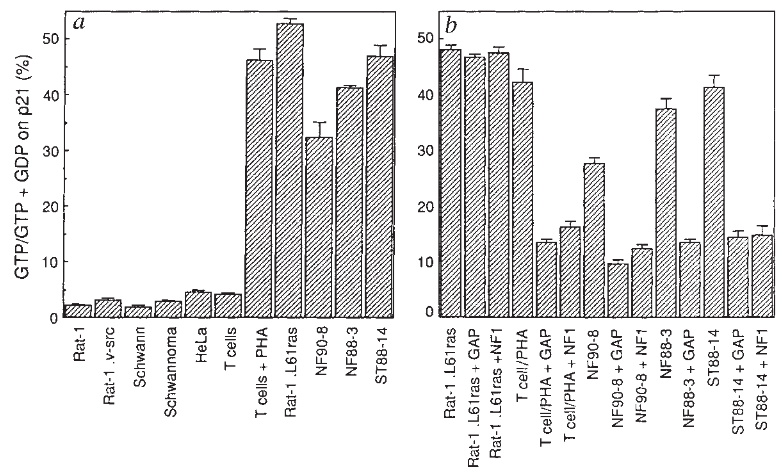

FIG. 1 Analysis of the guanine nucleotide bound to endogenous $p21^{ras}$ and its sensitivity to GAP in neurofibrosarcoma cells. Cells were labelled with [^{32}P]orthophosphate, lysed and $p21^{ras}$ was immunoprecipitated using anti-Ras monoclonal antibody Y13-259. *a*, Amount of GTP as a proportion of total nucleotide recovered on $p21^{ras}$ in various cell types. The T cells were peripheral blood human T lymphoblasts which were either quiescent or treated with 10 μg ml^{-1} phytohaemagglutinin for 10 min. *b*, $p21^{ras}$ immunoprecipitates were exposed as indicated to 20 μg ml^{-1} purified bacterially expressed human GAP or NF1 catalytic domain for 100 min at 30 °C before analysis of bound nucleotide.

METHODS. Cells (5×10^6) were labelled for 16 h in the absence of serum, lysed and $p21^{ras}$ immunoprecipitated using the procedure described in ref. 11, with the following modifications. Cells were lysed in buffer containing 1% Triton X-114 instead of Triton X-100; after removal of cell debris by centrifugation, phases were partitioned in the presence of 0.5 M NaCl for 2 min at 37 °C. The detergent gel containing $p21^{ras}$ was recovered by spinning down for 2 min at 14,000*g* at 20 °C and redissolved in lysis buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulphate and 0.5 M NaCl. Washed immunoprecipitates were incubated in 10 μl 50 mM HEPES buffer, pH 7.5, plus 5 mM $MgCl_2$ and 1 mg ml^{-1} bovine serum albumin, with or without 0.2 μg GAP or NF1 protein. GAP protein was made as a fusion protein in pGEX-2T (Pharmacia) using the product of a polymerase chain reaction designed to insert specific cloning sites. The fusion protein contained amino acids 702–1,044 of the human GAP sequence. NF1 catalytic domain was expressed in the same vector as described in ref. 7. The Rat 1-derived cell lines have been described[19]. Rat Schwann cells were grown as primary cultures[17]. T cells were prepared as in ref. 20. The rat schwannoma is line 33B. All determinations are the mean of three experiments with standard errors indicated.

Neurofibrosarcoma cells therefore seem to contain functional $p120^{GAP}$ but no functional NF1 GRP, whereas the other cell types contain both. These data also indicate that the full-length NF1 GRP is a GTPase-activating protein for $p21^{ras}$; previously it has been shown that antibodies raised against the carboxy-terminal sequence of NF1 GRP can liberate GAP activity from a detergent-insoluble fraction of bovine brain[12].

To analyse the $p120^{GAP}$ and NF1 GRP present in these cell types, we ran immunoprecipitates similar to those used to assay GAP activity on SDS–polyacrylamide gels. In the case of $p120^{GAP}$, [^{35}S]methionine-labelled lysates were immunoprecipitated with antibody PW6; we found that all the neurofibrosarcoma cell lines expressed $p120^{GAP}$ of the expected molecular weight and to the same extent as HeLa cells (Fig. 3*a*). For NF1 GRP, we raised a second antibody, G1, against the catalytic domain of NF1 GRP which did not crossreact with $p120^{GAP}$, and used it to probe immunoblots of D1 immunoprecipitates (Fig. 3*b*). In HeLa cells this combination of the two antisera D1 and G1 reveals an NF1 GRP with an apparent relative molecular mass (M_r) of ~250,000 (250K)[13]. In the neurofibrosarcoma cells we could not detect this protein initially, although when five times as much ST 88-14 lysate was used and the immunoblots were overexposed, a faint band of the correct M_r could be detected (Fig. 3*c*). The level of expression is ~2% of that in HeLa cells. No expression of NF1 GRP could be found in NF 90-8 or NF 88-3 cells, nor in whole lysates of any neurofibrosarcoma cells immunoblotted directly with G1 (data not shown).

The neurofibrosarcoma cell lines used here are all derived from malignancies from patients with neurofibromatosis type 1. ST 88-14 cells are transformed, as established by their ability to form small colonies in soft agar and to grow in low serum concentrations (data not shown). ST 88-14 was derived from a tumour containing a translocation at chromosome 17 band q11.2, close to the site of *NF1*; this neoplasm contained no normal chromosome 17 (case 7 in ref. 14). There is, however, no evidence for disruption of the *NF1* coding sequence in ST 88-14 (D. Marchuk and F.C., unpublished data). NF 88-3 has undergone

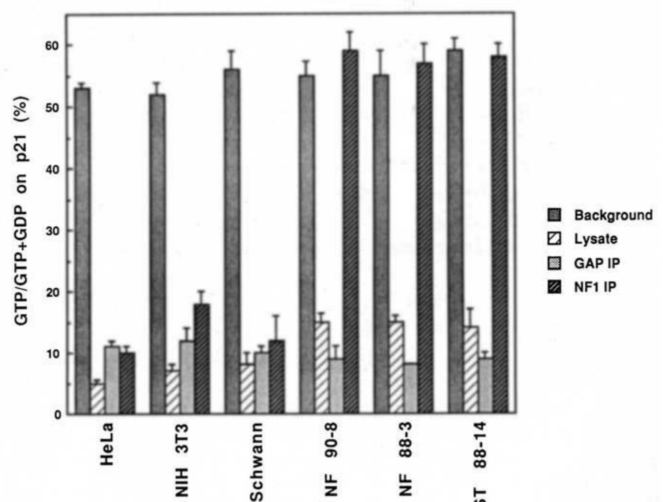

FIG. 2 GAP-type activity of $p120^{GAP}$ and NF1 GRP in immunoprecipitates (IP) from neurofibrosarcoma and control cells. The $p120^{GAP}$-specific antiserum PW6 and the NF1 GRP-specific antiserum D1 were used to immunoprecipitate these proteins from cell lysates. Washed immunoprecipitates were tested for their ability to stimulate the hydrolysis of [α-^{32}P]GTP bound to added $p21^{ras}$. The proportion of GTP to total guanine nucleotide bound at the end of the assay is shown.

METHODS. Cells (2×10^7) were lysed in 2 ml 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EGTA, 10 mM benzamidine, 10 μg ml^{-1} trypsin inhibitor, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} leupeptin, 1 mM dithiothreitol, 1 mg ml^{-1} BSA. After centrifugation (for 5 min at 14,000*g* and 4 °C), 300 μl lysate were immunoprecipitated for 90 min at 0 °C in duplicate with 5 μg of the antibody indicated or with non-specific IgG (background). Protein A–agarose (10 μl) was then added and the mixture tumbled for 30 min at 4 °C. Immune complexes were washed four times with 1 ml 50 mM HEPES, pH 7.5, 0.5 M NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 0.005% SDS, and once with 1 ml 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$. To the immune complex was added 0.25 ng $p21^{Ha\text{-}ras}$ bound to 1 μCi of [α-^{32}P]GTP. The mixture was incubated for 30 min at 30 °C and the reaction stopped by addition of 0.3 ml cold 50 mM HEPES, pH 7.5, 0.5 M NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 0.005% SDS, 1 mg ml^{-1} BSA. The $p21^{ras}$ was immunoprecipitated from the supernatant using antibody Y13-259. Other procedures have been described[11]. In addition, 50 μl lysate was assayed directly.

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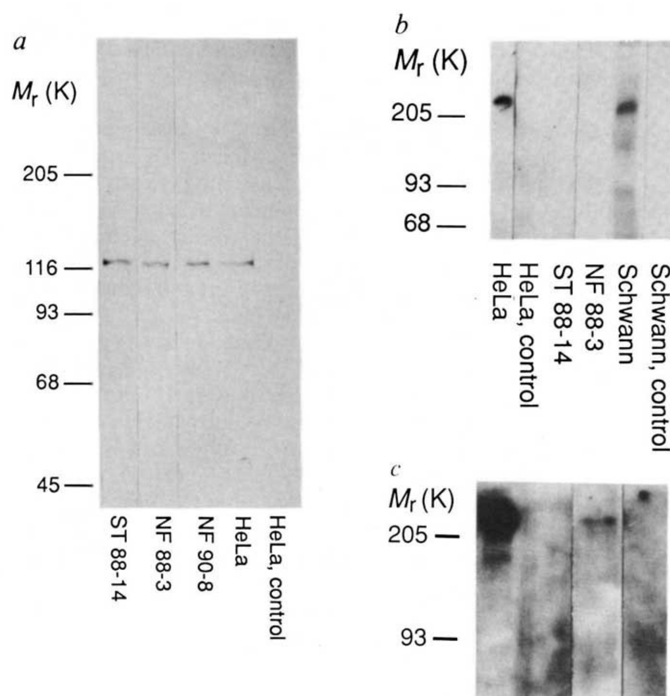

FIG. 3 Characterization by immunoprecipitation and western blotting of p120[GAP] and NF1 GRP in neurofibrosarcoma cells. *a*, Lysates from cells labelled with [^{35}S]methionine were immunoprecipitated using antibodies against p120[GAP] (PW6). *b*, Lysates from unlabelled cells were immunoprecipitated with antibodies against the carboxyl terminus of NF1 GRP (D1) and then immunoblotted using antibodies against the catalytic domain of NF1 GRP (G1). *c*, Longer exposure of immunoblot of NF1 GRP immunoprecipitates from HeLa cells and fivefold excess of ST 88-14 cells.
METHODS. Labelling and immunoprecipitation are described in ref. 19. Each lane in *a* and *b* represents lysates from 10^7 cells. PW6 was an affinity-purified rabbit antiserum raised against a pGEX fusion protein containing residues 350–702 of human p120[GAP] sequence (C. Allbright and R. Weinberg, personal communication). Antiserum D1 is described in ref. 13. Antiserum G1 was raised against a peptide from the GAP catalytic domain of NF1 GRP (residues 1,070–1,087, using the numbering in ref. 5) and immunoblotting was as described in ref. 21, using ^{125}I-labelled donkey antirabbit IgG. Lanes marked 'control' were immunoprecipitated with nonspecific rabbit IgG (*a*) or preimmune D1 IgG (*b*, *c*) In *c*, each track represents 10^7 HeLa cells or 5×10^7 ST 88-14 cells; exposure time was five times longer than in *b*. Migration of M_r standards is shown on the left of each blot.

allele loss on 17q (ref. 15) and has a mutation in exon 4 of the p53 gene[16]. The defects in the *NF1* genes of these cells are unknown.

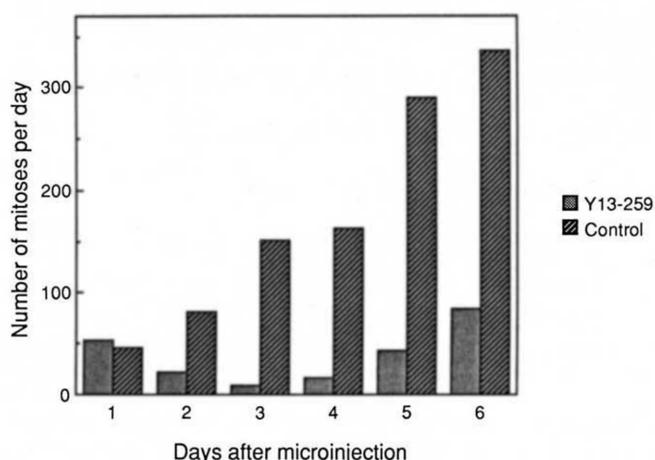

FIG. 4 Inhibition of neurofibrosarcoma cell growth by microinjection of antibodies against p21[ras]. ST 88-14 cells grown in the presence of 15% fetal calf serum were microinjected with either 1 mg ml^{-1} Y13-259 anti-p21[ras] monoclonal antibody or 1 mg ml^{-1} control immunoglobulin[22]. Mitotic events in a field of ~50 injected cells were followed using time-lapse video recording. The number of mitoses in each 24-h period is plotted as a proportion of the number of cells injected.

It has been proposed that p21[ras] acts upstream of NF1 GRP because *ras* oncogenes can act alone to inhibit proliferation of Schwann cells, although in combination with nuclear oncogenes they are transforming[17]. Because there are frequent mutations in p53 in neurofibrosarcomas from NF1 patients[18], including at least one of the three studied here, a model in which NF1 GRP is a downstream target of a growth-inhibitory p21[ras] cannot explain the malignancies that are features of NF1. Furthermore, microinjection of the p21[ras]-neutralizing monoclonal antibody Y13-259 strongly inhibits growth of ST 88-14 cells (Fig. 4); p21[ras] therefore has a positive effect on proliferation in this system and the absent NF1 GRP cannot be an essential downstream effector (barring the unlikely possibility that the extremely low residual levels of NF1 GRP are sufficient to carry such a signal).

Our data show that these malignant neurofibrosarcoma cells do not produce functional full-length NF1 GRP, or only very low levels of it. The loss of NF1 GRP causes a constitutive activation of p21[ras], even in the presence of normal levels of fully functional p120[GAP]. This suggests that NF1 GRP is the primary negative regulator of p21[ras], at least in neural crest-derived cells, possibly owing to the higher affinity of p21[ras] for NF1 GRP than for p120[GAP] (ref. 8), and to our knowledge is the first indication that NF1 GRP is involved in the negative control of *ras* proteins in whole cells and hence in the inhibition of cell proliferation. □

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