current inhibition by the impermeant anion substitute. On the other hand, changes in the intracellular Cl^- concentration produced by the exchange of solutions in the pipette (9) failed to alter the magnitude of the shrinking-induced current or the reversal potential ($n = 5$). These results suggest the presence of an external anion binding site that may act as a modulator for the cation conductance.

We have characterized in airway epithelial cells a nonselective cation conductance that is activated during shrinkage of cells and is dependent on the concentration of external Cl^-. Although nonselective cation channels have been characterized in a number of epithelial tissues, including those of rat pancreatic cells (*10*) and human nasal and sweat gland epithelial cells (*11*), the physiological role of the conductance has not been elucidated experimentally. The nonselective cation channels previously described were characterized by a variable Ca^{2+} dependence; the nonselective cation conductance observed in our study was not Ca^{2+}-dependent. In our experiments, the concentration of intracellular free Ca^{2+} was buffered to 20 nM, which is several orders of magnitude lower than the concentration used in the earlier studies. Activation of the shrinking-induced currents was also observed when cells were bathed in Ca^{2+}-free solutions ($n = 4$). Thus, the Cl^--dependent cation conductance may be different from the nonselective cation conductances described in earlier studies.

The Cl^- efflux induced during cell swelling (*2, 3*) and the Na^+ influx through the nonselective cation conductance activated during shrinkage appear to be part of a volume regulatory response that offsets anisosmotically induced swelling and shrinkage of cells, respectively. The anion dependence of the nonselective cation conductance provides a means for the regulation of Na^+ entry or the inward movement of osmolytes during RVD. When cells are exposed to a hypotonic environment (low external Cl^-), the cation conductance is inactivated and the anion conductance becomes maximally activated, thereby facilitating an efflux of osmotically active particles and consequently a reduction in cell volume. Conversely, when cells are exposed to a hypertonic environment, maximal activation of the cation conductance and the reciprocal inactivation of the anion conductance would facilitate an influx of osmotically active particles and the accompanying increase in cell volume. Thus, the two conductances seem to have complementary functions in cell volume regulation (*12*). The unusual dependence of the nonselective cation conductance on the extracellular Cl^- concentration may be a mechanism whereby cells under osmotic stress control Na^+ entry in such a way that would allow for the tight regulation of Cl^- secretion and Na^+ reabsorption.

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6. Both standard pipette and bath solutions had an osmolarity of 280 mOsM. A bath solution with an osmolarity greater than or less than 280 mOsM was considered either hypertonic or hypotonic with reference to the standard pipette solution, respectively.
7. The pipette solution contained 40 mM NaCl, 100 mM NMDG-aspartate, 10 mM Hepes, 5 mM EGTA, 0.5 mM $CaCl_2$ (20 nM free Ca^{2+}), and 1 mM $MgCl_2$, pH 7.2. The bath solution contained 140 mM NaCl, 10 mM Hepes, 2 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.2.
8. H. C. Chan *et al.* (*3*). Bumetanide and furosemide were dissolved in dimethyl sulfoxide (DMSO) stock solutions and used at a final DMSO concentration of 0.1%.
9. Cells were dialyzed with intracellular (pipette) solutions containing 20, 40, or 80 mM Cl^-. Pipette solutions were exchanged according to the method of M. Soejima and A. Noma [*Pfluegers Arch.* **400**, 424 (1984)].
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12. Our studies do not address the role of K^+ conductances in volume regulation. Because the role of K^+ channel activation has been characterized previously (*2, 3*), we omitted K^+ from our solutions to simplify the present investigation.

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Mechanistic Aspects of Signaling Through Ras in NIH 3T3 Cells

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Serum and growth factors can increase the proportion of Ras in the active guanosine triphosphate (GTP)-bound form. Growth factors might stimulate guanine nucleotide exchange or decrease the activity of the guanosine triphosphatase–activating proteins GAP and neurofibromin (NF1). In NIH 3T3 cells that overexpress the mutant Ras protein His^{116}, which releases bound guanine nucleotide at a constitutively high rate and retains sensitivity to GAP and NF1, the proportion of GTP bound to the His^{116} protein was not altered by serum or platelet-derived growth factor. However, these mitogens increased the proportion of Ras in the GTP-bound form in cells that overexpressed control Ras proteins with a normal intrinsic rate of guanine nucleotide release. The amount of GTP-bound His^{116} or control Ras proteins was higher in cells at low density than in cells at high density, which have more GAP-like activity. The lower proportion of GTP-bound Ras in NIH 3T3 cells at high density may result from increased GAP-like activity. By contrast, serum and platelet-derived growth factors appear to stimulate guanine nucleotide exchange.

The proteins encoded by the *ras* genes are essential for the transduction of diverse extracellular signals to intracellular targets (*1–3*). The Ras proteins bind guanine nucleotides with high affinity, have intrinsic guanosine triphosphatase (GTPase) activity, and cycle between an active, GTP-bound state and an inactive, guanosine diphosphate (GDP)-bound state (*4, 5*). The proportion of GTP-Ras appears to be determined by two reactions: (i) the rate of guanine nucleotide exchange, which because of the much higher intracellular concentration of GTP than GDP will tend to activate the protein by favoring the GTP-bound form, and (ii) the rate of GTP hydrolysis, which converts GTP-bound Ras to the inactive, GDP-bound form.

Both reactions appear to be enzymatically regulated. GAP (GTPase activating protein) and neurofibromin, the product of *NF1* (the gene that is affected in von Recklinghausen's neurofibromatosis), ac-

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celerate the intrinsic GTPase activity of normal Ras in vitro and negatively regulate Ras in vivo (*6–10*). The *CDC25* gene product in *Saccharomyces cerevisiae* activates Ras by stimulating guanine nucleotide exchange (*11*), and in *Drosophila* the *sos* gene, which is similar to *CDC25*, is required for *ras* function (*3*). Extracts from mammalian cells contain activities that stimulate dissociation of guanine nucleotides from Ras (*12–14*), but their physiological relevance remains to be determined. Mutant Ras proteins that have enhanced biological activity are resistant to negative regulation by GAP and NF1 or have an increased intrinsic rate of guanine nucleotide release, or both (*6, 9, 15*). In T cells, the amount of GTP-Ras increases in response to the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) or activation of the C3 receptor. This response is associated with a decrease in GAP-like activity without detectable alteration in the rate of guanine nucleotide exchange (*16*).

The NIH 3T3 fibroblast system has been used extensively to study signaling and morphologic transformation. These cells are susceptible to cellular transformation by mutationally activated *ras* or by overexpression of normal *ras*, and Ras protein activity is required for mitogenic stimulation of these cells by serum or growth factors such as platelet-derived growth factor (PDGF) (*1, 17*). Treatment of cells with these and other mitogens increases the proportion of GTP-Ras (*18–20*), but the mechanism by which extracellular signals activate Ras is unclear. PDGF induces phosphorylation of GAP and its localization in the membrane as well as its association with the PDGF receptor and other cellular proteins (*21–23*). However, it is unclear whether the increased amount of GTP-Ras results from a decrease in negative regulation by GAP or NF1, from an increase in the rate of guanine nucleotide release, or from a combination of these activities.

To distinguish between these possibilities, we analyzed NIH 3T3 cells expressing a Ras protein that carries a single amino acid substitution, at amino acid 116 (*24*), which is a residue that participates in guanine nucleotide binding. The mutant protein has normal in vitro sensitivity to negative regulation by GAP (*24*) and NF1. However, it is partially activating, inducing about 20 times as many foci per microgram of DNA as normal c-*ras* in an NIH 3T3 focal transformation assay. Substitution of His^{116} for the normal Asn creates a protein with an intrinsic guanine nucleotide exchange rate that is about ten times that of normal Ras, which should result in a higher proportion of GTP-Ras relative to GDP-Ras. If inhibition of negative regulation by GAP or NF1 is the predominant mechanism by which mitogens induce the change in GTP-Ras in NIH 3T3 cells, then treatment of cells with mitogens should increase the amount of His^{116} protein in the GTP-bound form. On the other hand, if stimulation of guanine nucleotide release is the predominant mechanism by which mitogens increase the amount of GTP-bound normal Ras, then the proportion of His^{116} protein in the GTP-bound form should undergo little or no change in response to mitogens because the intrinsic rate of release from His^{116} is much greater than normal.

We determined whether, in confluent NIH 3T3 cells transformed by overexpression of c-*ras*, the serum- and PDGF-induced changes in the amount of GTP-Ras were similar to those induced in untransformed cells, because the c-*ras* transformants are more appropriate than untransformed cells as controls for mutant *ras* transformants. Cells were grown to confluence, deprived of serum overnight, and metabolically labeled with [^{32}P]orthophosphate. The cells were then stimulated with serum or PDGF and the amount of GTP- and GDP-bound Ras was assayed 30 min later. Serum or PDGF treatment of untransformed cells induced a twofold to fourfold increase in the concentration of GTP-Ras (*18–20*) (Figs. 1 and 2A). The proportion of Ras in the GTP-bound form was also increased twofold to threefold in c-*ras* overproducers treated with serum or PDGF, although the actual percentages of Ras in the GTP-bound form in both untreated and treated cells were lower than those of endogenous Ras in the parental cells (Figs. 1 and 2A). These results indicate that the response to serum and PDGF is qualitatively similar in untransformed parental cells and c-*ras* transformants.

In cells overexpressing the His^{116} mutant, the proportion of Ras in the GTP-

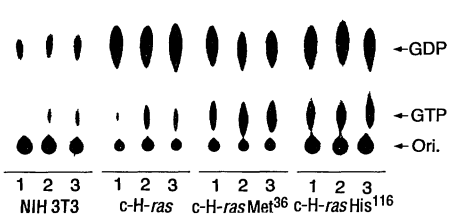

Fig. 1. Binding guanine nucleotides to Ras proteins in transformed cells. Untransformed cells (NIH 3T3), cells transformed by the normal *ras* gene (c-H-*ras*), the Met^{36} mutant, or the His^{116} mutant were inoculated in growth medium [Dulbecco's MEM with FCS (10%)] at 40,000 cells per centimeter squared (high cell density). GTP- and GDP-bound Ras were determined as described (*18–20*), with minor modifications. One day later the serum was removed and the cells were incubated overnight with [^{32}P]orthophosphate (350 μCi/ml) in serum-free medium. The serum-starved cells were then placed in medium containing FCS (10%) or PDGF (50 ng/ml) (Boehringer Mannheim) for 30 min and extracted in lysis buffer [50 mM tris (pH 7.5), 150 mM NaCl, 20 mM $MgCl_2$, NP-40 0.5%, and trasylol (16 μg/ml)]. Ras proteins were immunoprecipitated with a monoclonal antibody to Ras, Y13-259 (*35*), and protein A Sepharose (Sigma). The beads were washed and proteins solubilized in SDS (1%). The bound guanine nucleotides were chromatographed on polyethyleneimine (PEI) cellulose plates in 1.3 M LiCl and subjected to autoradiography. Ori., sample applied at origin.

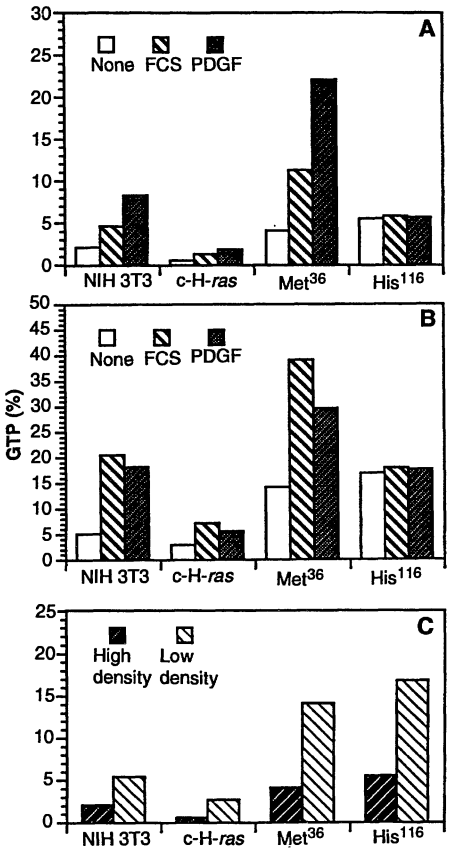

Fig. 2. Effects of PDGF, serum, and cell density on the proportion of Ras in the GTP-bound form. (**A**) Cells at high density. (**B**) Cells at low density. (**C**) Comparison of unstimulated (serum-starved) cells at high and low densities. Cell lines are as designated in Fig. 1. The results are the averages from two experiments. Chromatograms were scanned and quantitated on an AMBIS radioanalytic imaging system (Automated Microbiology Systems, San Diego). The proportion of Ras in the GTP-bound form was determined from the amount of radioactivity associated with GTP and GDP. We assumed that each phosphate in the nucleotides was labeled equally, and we corrected for the number of phosphates in each nucleotide. The results in (A) include those shown in Fig. 1 and a second experiment. Cells at low density were inoculated at 4000 cells per centimeter squared and grown and processed as described in Fig. 1.

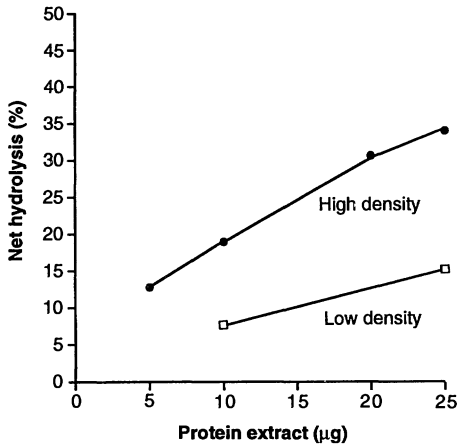

Fig. 3. Activity of GAP in cells at high and low density. Cells overexpressing His^{116} were inoculated in growth medium at 4,000 (low density) and 40,000 (high density) cells per centimeter squared, grown for 1 day in growth medium, incubated overnight in serum-free medium, washed once, scraped into phosphate-buffered saline, centrifuged at low speed, and resuspended, at a protein concentration of approximately 2 mg/ml (determined by the Bradford technique), in 20 mM tris-HCl (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, NP-40 (0.5%), and 1 mM dithiothreitol (DTT). The GTPase accelerating activity (GAP-like activity) was determined, in the above buffer, in 50-μl reactions containing various amounts of extract and bacterially expressed normal Ras bound to [α-^{32}P]GTP. The mixtures were incubated at room temperature for 15 min, and proteins were immunoprecipitated with monoclonal antibody to Ras Y13-259 and protein A Sepharose. The beads were washed, and bound proteins were solubilized in SDS (1%). The guanine nucleotides were separated by chromatography on PEI cellulose plates in 1.3 M LiCl. Chromatograms were scanned and quantitated on an AMBIS radioanalytic imaging system. Net hydrolysis was calculated by subtracting the percentage of Ras on the GTP-bound form in the reactions with the cell extract from that in a control reaction without extract.

bound form in unstimulated cells was greater than that in the line that overexpressed c-*ras*, as expected for a partially activating *ras* mutant (Figs. 1 and 2A). In contrast to the c-*ras* overexpressors, confluent His^{116} overexpressors did not contain increased amounts of GTP-Ras after treatment with serum or PDGF. This failure to respond to PDGF was not due to a lack of PDGF receptor activation, because phosphorylation of PDGF receptors on tyrosine was detected in response to PDGF (as above). The lack of response therefore suggests that there was no decrease in GAP-NF1 activity, because decreased GAP-NF1 activity should lead to an increase in the proportion of mutant protein encoded by His^{116} in the GTP-bound state. This result implies that another mechanism, most likely that of stimulated guanine nucleotide exchange, accounts for most of the increase in the amount of GTP bound to normal Ras after stimulation of the cells with serum and PDGF.

To rule out the possibility that the lack of response might occur because a higher proportion of the His^{116} protein is in the GTP-bound form, we tested cells transformed by another partially activating *ras* substitution mutant that induces about ten times as many foci per microgram of DNA as normal c-*ras*. The protein encoded by this mutant, which specifies Met^{36} in place of Ile, releases guanine nucleotide at the normal intrinsic rate, but the mutant protein is partially resistant in vitro to negative regulation by GAP and NF1 (*25*). In cells transformed by the Met^{36} mutant, the proportion of GTP-bound Ras was similar to that of the cells transformed by His^{116} (Figs. 1 and 2A). Unlike the His^{116} overexpressors, the Met^{36} overexpressors responded with a threefold to fourfold increase in the amount of GTP bound to the mutant Ras protein when treated with serum or PDGF.

We also examined these cell lines for increases in the amount of GTP-Ras under conditions of low cell density. Cells were deprived of serum overnight, metabolically labeled with [^{32}P]orthophosphate, and then treated with serum or PDGF for 30 min (Fig. 2B). The changes in the amount of GTP-Ras in cells at low density were qualitatively similar to those obtained at high density (Fig. 2, A and B). In particular, treatment with PDGF or serum increased the amount of GTP-Ras in the Met^{36} line but not the His^{116} line.

The proportion of Ras in the GTP-bound form was also compared for each cell line at high and low cell densities. Under each condition (unstimulated, PDGF-treated, or serum-treated), the amount of GTP-Ras for each line was greater in cells at low density than at high density (Fig. 2C). For cells grown continuously in serum, the percentage of Ras in the GTP-bound form in each line was about twice as high at low cell density as at high cell density (*26*). The proportion of Ras in the active form therefore depends on both cell density and growth factor stimulation. Because these two effects are additive, their mechanisms may be independent.

In BALB 3T3 cells, GAP-like activity is higher in extracts from confluent cells than in those from subconfluent cells (*27*). To determine whether the density-dependent change in the proportion of Ras in the GTP-bound form might be caused by alterations in the activity of GAP or NF1, we measured GAP-like activity in cells at high and at low density. As measured by the ability of a crude cell extract (which contains both GAP and NF1) to accelerate the conversion of bacterially expressed GTP-bound Ras to the GDP-bound form, the extract from cells at high density contained about four times as much GAP-like activity as the extract from cells at low density (Fig. 3). Thus, the lower GAP-like activity noted for cells at low density correlates with the higher amount of GTP-Ras in cells at low density.

The density-dependent difference in the amount of GTP-bound Ras in cells overexpressing the His^{116} mutant is consistent with the finding that this mutant is sensitive in vitro to negative regulation by GAP and NF1 (*24*). This observation reinforces the conclusion, based on the lack of response of the His^{116} line to stimulation with PDGF or serum, that such stimulation does not induce a major change in GAP or NF1 activity (*20*).

Our observations suggest that, in NIH 3T3 cells, the increase in the amount of GTP-Ras that occurs when serum-starved cells are treated with serum or PDGF may result from stimulation of guanine nucleotide dissociation rather than from changes in GAP-like activity. On the other hand, the lower concentration of GTP-Ras found in cells at high density apparently results in large part from increased GAP-like activity. Thus, the proportion of Ras in the GTP-bound form in NIH 3T3 cells is determined, at least in part, by the balance of guanine nucleotide exchange and GAP-like activities. Overexpression of GAP can reduce the amount of GTP-Ras in cells (*20*) and can antagonize transformation of cells induced by overexpression of c-*ras* (*7*) or of other genes that transform cells by mechanisms that require the activity of endogenous c-*ras* (*28, 29*).

The results obtained with serum and PDGF appear to be analogous to those from experiments with yeast, in which induction of Ras activity by glucose requires guanine nucleotide exchange stimulated by *CDC25* (*11, 30, 31*). However, our results appear to differ from those reported in T cells (*16*). In that system, the signaling-dependent increases in the amount of GTP-Ras are correlated with a reduction in GAP-like activity and are independent of changes in guanine nucleotide exchange on Ras, which was measured after permeabilization of cells and appeared to be constitutively fast. Another apparent difference is that treatment of T cells with TPA, which is a potent inducer of protein kinase C, induced increased amounts of GTP-bound Ras. In NIH 3T3 cells, protein kinase C may be downstream in the signaling pathway from Ras (*32, 33*), and treatment of these cells with TPA does not induce changes in the amount of GTP-Ras (*34*). The relative contributions of guanine nucleotide disso-

ciation and GAP-like activity to changes in amounts of GTP-Ras may depend on the system under study and the physiological context in which it is examined.

It is likely that the lower proportion of Ras in the GTP-bound form in confluent cells is relevant to the phenomenon of contact inhibition. Ras is required for serum-induced growth of NIH 3T3 cells, and high activity of Ras, which results in cell transformation, can overcome contact inhibition. Our observations raise the possibility that the activity of Ras may help determine whether a cell will commit to continued proliferation. Normal concentrations of serum may be sufficient to induce mitogenic amounts of GTP-bound active Ras in subconfluent cells but not in confluent, contact-inhibited cells.

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Repression of the Insulin-Like Growth Factor II Gene by the Wilms Tumor Suppressor WT1

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The Wilms tumor suppressor gene *wt1* encodes a zinc finger DNA binding protein, WT1, that functions as a transcriptional repressor. The fetal mitogen insulin-like growth factor II (IGF-II) is overexpressed in Wilms tumors and may have autocrine effects in tumor progression. The major fetal IGF-II promoter was defined in transient transfection assays as a region spanning from nucleotides −295 to +135, relative to the transcription start site. WT1 bound to multiple sites in this region and functioned as a potent repressor of IGF-II transcription in vivo. Maximal repression was dependent on the presence of WT1 binding sites on each side of the transcriptional initiation site. These findings provide a molecular basis for overexpression of IGF-II in Wilms tumors and suggest that WT1 negatively regulates blastemal cell proliferation by limiting the production of a fetal growth factor in the developing vertebrate kidney.

Wilms tumor is a pediatric malignancy thought to arise when multipotent kidney blastemal cells fail to differentiate and instead continue to proliferate after birth (*1*). The occurrence of both sporadic and hereditary forms of Wilms tumor and the early age of bilateral kidney tumor onset suggest that Wilms tumors result when a predisposing germ line mutation is accompanied by a second mutation or loss of heterozygosity at the disease locus (*2*). Fine mapping of deletions in the chromosomal locus 11p13, which are associated with Wilms tumors, has culminated in the cloning of a potential tumor suppressor gene *wt1* (*3*). The *wt1* gene encodes a DNA binding protein with a serine- and proline-rich NH_2-terminus and four Zn^{2+} fingers (*3, 4*). In the kidney, *wt1* mRNA is first detectable in the early stages of epithelial differentiation in the condensing mesenchymal cells or renal vesicle (*5*). The WT1 protein binds the DNA sequence GCGGGGGCG, a recognition element common to the early growth response (EGR) family of Zn^{2+} finger transcriptional activators (*4, 6*). However, in contrast to the EGR transcription factors, WT1 behaves as a transcriptional repressor in transient transfection assays with synthetic promoter constructs (*7*). The biological significance of DNA binding and transcriptional regulation by WT1 is underscored by the observation that small deletions and point mutations in the WT1 Zn^{2+} fingers that abolish DNA binding have been detected in a number of Wilms tumors, especially in tumors associated with the Denys-Drash syndrome (*8*). The early expression of *wt1* during kidney development and WT1's capacity to function as a repressor of transcription suggest that the protein plays a key role in halting blastemal cell proliferation and in initiating a program of epithelial differentiation in the kidney.

Several lines of evidence suggest the involvement of the fetal mitogen insulin-like growth factor II (IGF-II) in the genesis of Wilms tumors. IGF-II is overexpressed in all Wilms tumors examined thus far (*9*). The Beckwith-Weidemann syndrome, a condition characterized cytogenetically by paternal duplications of the 11p15.5 chromosomal region, is associated with a predisposition for the development of Wilms tumors (*10*). This region contains the gene for IGF-II (*11*). Also, studies of Wilms tumors heterotypically transplanted in nude mice have shown that the tumor cells express the IGF type I receptor (*12*) and that antibodies that block this receptor inhibit tumor growth (*13*). These results suggest that IGF-II may function as an autocrine growth factor in Wilms tumor. Develop-

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