RESEARCH COMMUNICATION

Rac1 is required for cell proliferation and G2/M progression

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We have transiently expressed a dominant negative form of rac1 (N17rac1) using adenoviral-mediated gene transfer. The level of N17rac1 expression is demonstrated to be proportional to the multiplicity of infection. Expression of N17rac1 in Rat 2 fibroblasts results in cytostatic growth arrest. Cell-cycle analysis demonstrates that cells expressing N17rac1 accumulate in G2/M. These results suggest that rac1 is required for cell proliferation and provide the first demonstration in mammalian cells of a role for small GTP-binding proteins in the G2/M transition.

INTRODUCTION

Rac1 is a member of the ras superfamily of small GTP-binding proteins. Members of this family appear to regulate a diverse array of cellular events, including the control of cell growth, cytoskeletal reorganization and the activation of protein kinases (reviewed in [1,2]). In particular, rac1 appears to function in the regulation of actin filaments at the plasma membrane resulting in the production of lamellipodia and ruffles [3], the generation of reactive oxygen species in phagocytic [4,5] and non-phagocytic cells [6–8], and activation of the family of stress-activated protein kinases (JNKs/SAPKs) [9–11].

The ras-related family of proteins also function to regulate cell cycle progression. In mammalian cells, microinjection of neutralizing antibodies to ras proteins has demonstrated a role for these proteins in the transition from quiescence (G0) to S phase [12]. Recent evidence suggests that in quiescent Swiss 3T3 fibroblasts, rac1 is also required for entrance into S phase [11]. Although small GTP-binding proteins have been implicated in the G0/G1–S transition in mammalian cells, in yeast this family of proteins also plays a role in the G2/M transition [13]. To date, some support exists for a role of ras-related proteins in G2/M transition in other species [14–18]. Efforts to elucidate such a role in mammalian cells have been hampered, except in certain specialized situations [19], by the difficulty in transiently or stably expressing dominant negative forms of ras or rac.

Another line of evidence suggesting a role for rac-related proteins in growth control derives from the observation that these proteins appear to co-operate with ras in transformation [20]. This suggests that, in addition to the well characterized pathway of ras/Raf/MEK/MAPK leading to growth and transformation, other potential ras-dependent but Raf-independent pathways exist. This notion is further supported by the observation that mutants of ras that lack the ability to activate Raf are still capable of transforming NIH 3T3 cells [21–23]. Although such studies suggest a potential role for rac-related proteins in the maintenance of the transformed state, relatively little is known about the role of rac proteins in the growth of normal cells. In this study, we demonstrate an essential role of rac1 in normal cell proliferation and a requirement for rac proteins in the G2/M transition.

MATERIALS AND METHODS

The rat fibroblast cell line Rat 2 (ATCC) was maintained in Dulbecco's modified essential media supplemented with 5% (v/v) fetal bovine serum (Gibco–BRL). For proliferation assays, cells were infected at an approximate density of 1.5×10^4 cells/cm^2. Triplicate cultures were harvested 2 and 4 days later and the number of viable cells determined by Trypan Blue exclusion.

The myc-epitope tagged dominant negative rac1 cDNA containing a Ser → Asp substitution at position 17 (N17rac1) has been described previously [3]. A recombinant E1-deleted adenovirus was constructed by homologous recombination in 293 cells [7]. The E1$^-$ adenovirus Ad.d1312 [24], which lacks a recombinant transgene, was used as a control. Adenoviral stock were amplified in 293 cells and purified by double caesium gradient as described previously [25].

For Western-blot analysis, Rat 2 cells were infected with 20 or 200 multiplicity of infection (moi) and were harvested in RIPA buffer [50 mM Tris/HCl, pH 7.5,/150 mM NaCl containing 1% (v/v) NP-40, 0.5% (v/v) sodium deoxycholate and 0.1% (w/v) SDS]. The protein lysate (30 μg) was resolved by SDS/PAGE (12% gels) and then transferred to a nitrocellulose filter. The filters were probed with an antibody recognizing the myc-epitope tag (9E10, Santa Cruz) at a final concentration of 2 μg/ml. Immunocomplexes were visualized using enhanced chemiluminescence (Tropix).

Analysis of cell-cycle distribution was performed using propidium iodide stained nuclei according to the manufacturer's recommendation (Cycle Test™, Becton Dickinson). Cell nuclei were analysed using an EPICS Elite-ESP cytometer (Coulter) and the distribution was determined with Multi-cycle software (Phoenix Flow System). For assessment of nuclear morphology, cells were briefly fixed in an acetic acid/methanol (3:1, v/v) solution and were subsequently stained with propidium iodide (100 μg/ml). Fluorescent images were obtained with a Leica scanning confocal microscope (model TC-D4).

For analysis of the growth-inhibitory effects of other small GTP-binding proteins, dominant-negative forms of cdc42 or rhoA were inserted into the mammalian expression vector pCEV [26], which also encodes for neomycin resistance. Rat 2 cells were transfected with lipofectamine (Gibco–BRL) and 5 μg of DNA

Abbreviations used: Ad.dl312, a control Ad5 serotype adenovirus; Ad.N17rac1, an adenovirus encoding an epitope-tagged form of a N17rac1; moi, multiplicity of infection; N17rac1, a dominant negative form of rac1.

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containing the expression vector alone or the same vector containing N17cdc42, N19rhoA (a gift from S. Gutkind, National Institute of Dental Research, NIH, Bethesda, MD, U.S.A.), or with an expression vector encoding N17ras described previously [27]. Three days after transfection, cells were re-seeded in G418-containing media (500 μg/ml). Ten to 14 days after selection was initiated, the cells were fixed, stained and the number of viable colonies were counted.

RESULTS

Western-blot analysis of Rat 2 fibroblasts previously infected with an adenovirus encoding an epitope-tagged form of a N17rac1 (Ad.N17rac1) resulted in the detection of high level, transient expression of the gene product (Figure 1). The level of expression of N17rac1 correlated with the moi, with a moi of 200 resulting in considerably higher expression than a moi of 20 (Figure 1, compare lanes 2 and 3). Infection of Rat 2 cells with an adenovirus encoding the LacZ marker gene revealed that at the higher moi more than 90% of the cells were transduced (results not shown).

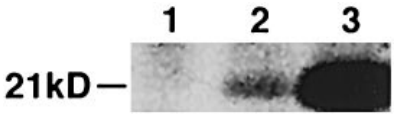

Figure 1 Expression of N17rac1 in Rat 2 cells

Lysates from uninfected Rat 2 cells (lane 1), or Rat 2 cells infected 24 h earlier with 20 moi (lane 2) or 200 moi (lane 3) of Ad.N17rac1. Lysate protein (30 μg/lane) was applied and filters were probed with an antibody recognizing the myc-epitope tag.

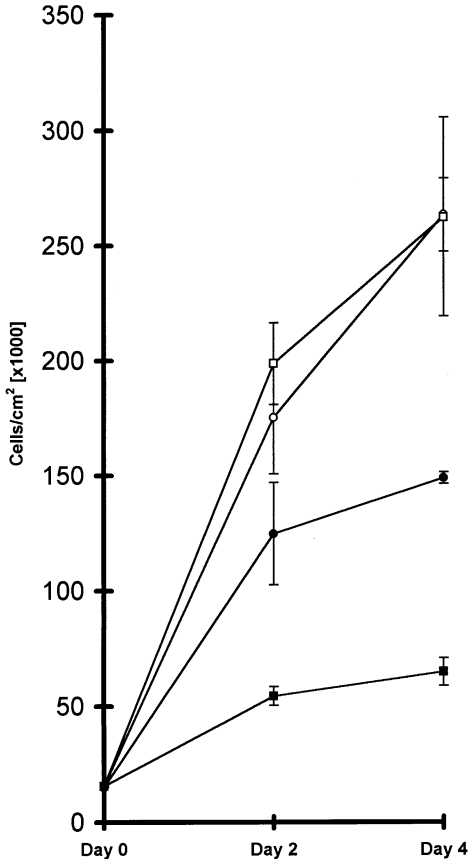

Figure 2 Growth of Rat 2 cells following N17rac1 expression

Rat 2 cells were infected with Ad.d1312, 20 moi (○); 200 moi (□) or with Ad.N17rac1, 20 moi (●); 200 moi (■). The number of viable cells, determined by Trypan Blue exclusion, was determined from triplicate cultures on days 2 and 4 following infection.

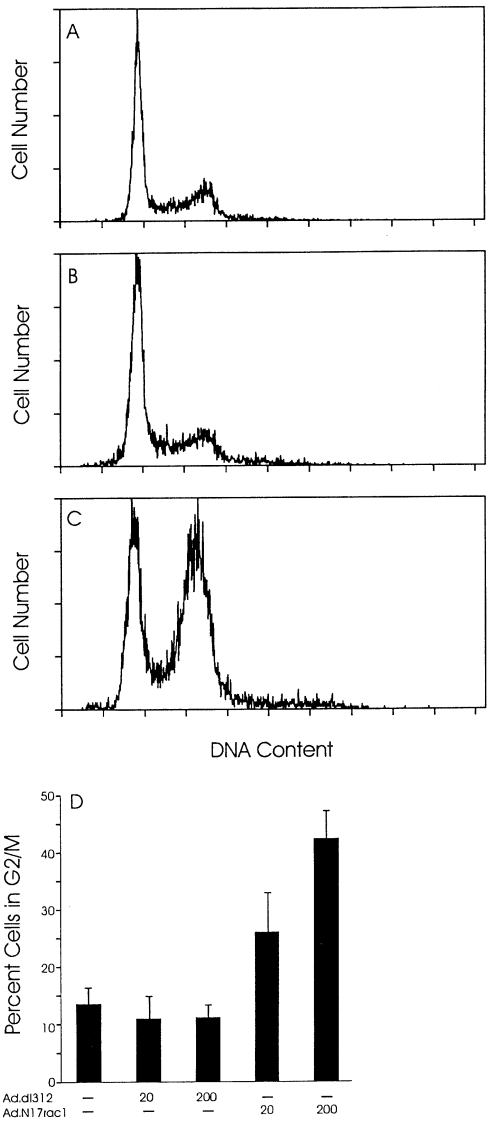

Figure 3 Cell-cycle distribution following N17rac1 expression

Analysis of propidium iodine stained nuclei from (**A**) uninfected Rat 2 cells, (**B**) Rat 2 cells infected 72 h earlier with 200 moi of Ad.d1312, or (**C**) Rat 2 cells infected with 200 moi of Ad.N17rac1. (**D**) The percentage of cells in G2/M determined from triplicate cultures (means ± SD) as a function of N17rac1 expression.

We next sought to assess the effects on cell proliferation following expression of N17rac1. Cell cultures were infected with 20 or 200 moi of either Ad.N17rac1, or with a control Ad5 serotype adenovirus, Ad.dl312. As seen in Figure 2, compared with cells infected with Ad.d1312, cells infected with Ad.N17rac1 had a considerably slower growth rate. After 2 days, cells infected with Ad.N17rac1 at the higher moi (200), growth arrest appeared almost complete. Over this time period the effects of N17rac1 appeared be cytostatic rather than cytotoxic, since the percentage of Trypan Blue positive cells was not significantly

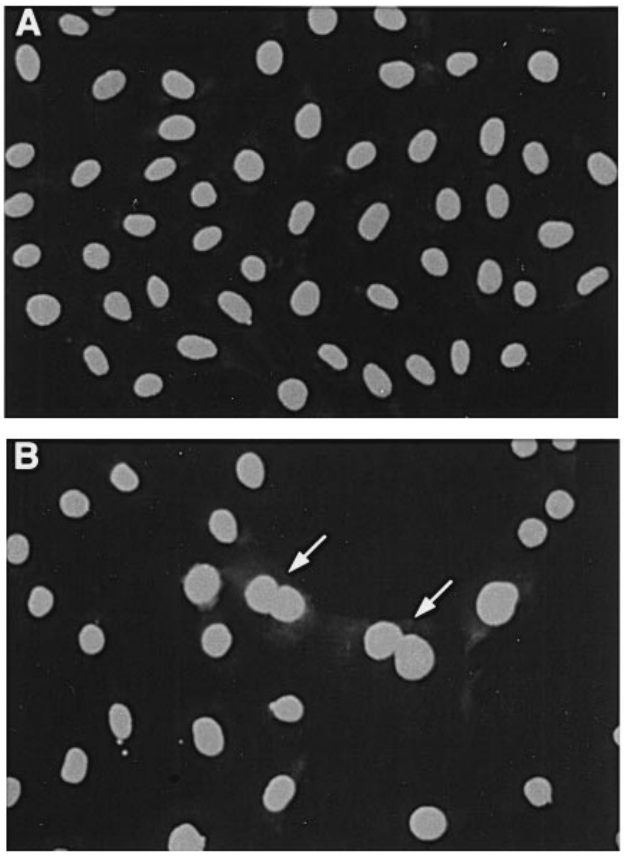

Figure 4 Nuclear morphology of Rat 2 infected cells

Fluorescent micrographs of propidium iodide stained nuclei 72 h after infection with 200 moi of (**A**) Ad.d1312 or (**B**) Ad.N17rac1. The level of binucleated cells (arrows) was slightly increased in Ad.N17rac1 infected cells.

increased in Ad.N17rac1- compared with Ad.d1312-infected cells (results not shown).

Since the expression of N17rac1 appears to result in a cytostatic growth arrest we sought to determine if this was accompanied by an accumulation in a specific phase of the cell cycle. Uninfected cells, or cells infected 72 h earlier with either Ad.d1312 or Ad.N17rac1, were assessed by flow cytometry. As seen in Figure 3(A) and (B), uninfected cells and cells infected with Ad.d1312 had a similar distribution of cells in each phase of the cell cycle. In contrast, cells infected with Ad.N17rac1 (Figure 3C) accumulate in the G2/M phase. The percentage of cells in the G2/M phase correlated with the level of N17rac1 expression (Figure 3D).

Direct assessment of propidium iodide stained nuclei of cells infected 72 h earlier with Ad.N17rac1 revealed predominantly round, homogenous, single nuclei (Figure 4). This suggests that, consistent with assessment of cell viability using Trypan Blue exclusion, N17rac1 expression does not result in significant apoptosis. Infection with Ad.N17rac1 resulted in a slight increase in the ratio of binucleated cells to cells with single nuclei [Ad.d1312 (200 moi), 12:435; Ad.N17rac1 (200 moi), 23:328]. However, since cell cycle analysis showed that 72 h after infection with Ad.N17rac1 approx. 50% of the cells were in G2/M phase, this suggests that the block with N17rac1 expression occurs before the onset of cytokinesis.

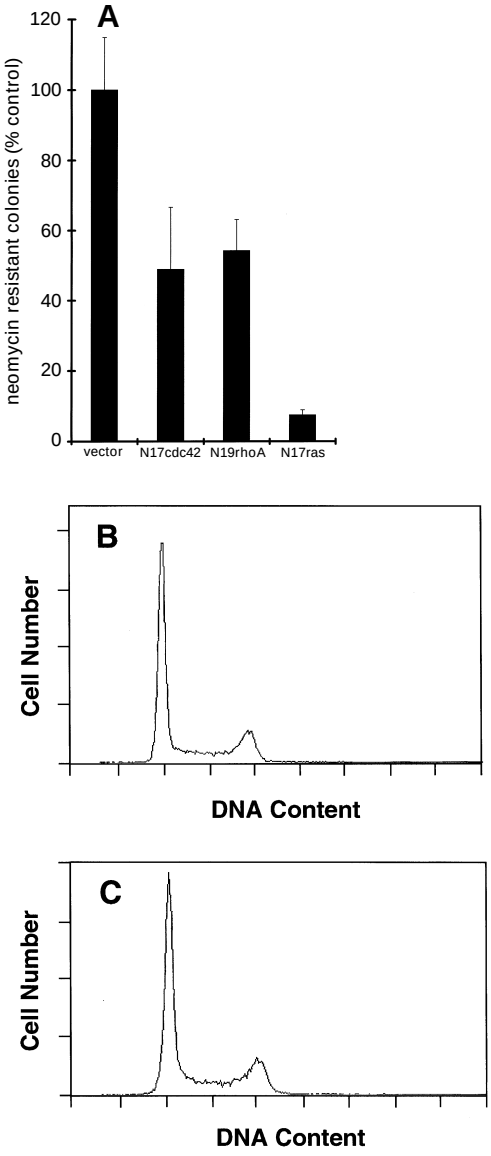

Figure 5 Cell-cycle and growth-inhibitory effects of N17cdc42, N19rhoA and N17ras

(**A**) Rat 2 cells were transfected with 5 μg of an expression vector encoding for neomycin resistance alone or the same vector including the dominant-negative small GTP-binding proteins. The number of neomycin-resistant colonies was assessed 10–14 days after selection in G418-containing media. The results are the number of colonies (means $\pm$ S.D.) expressed as a percentage of control (neomycin-resistance plasmid alone) and were obtained from one of two similar experiments, each performed in triplicate. Cell-cycle analysis from pooled G418-resistant colonies following transfection with (**B**) N17cdc42 or (**C**) N19rhoA.

Finally, we sought to address the specificity of the N17rac1-induced growth arrest. In particular, we wanted to know whether expression of dominant-negative forms of the related small GTP-binding proteins ras, cdc42 or rhoA would produce similar effects. Rat 2 cells were transfected with an empty expression vector encoding for neomycin resistance alone or with vectors encoding N17ras, N17cdc42 or N19rhoA. Compared with control cells transfected with the expression vector alone, expression of the mutant small GTP-binding proteins led to a reduction in the number of neomycin-resistant colonies consistent with a growth-inhibitory effect (Figure 5A). Of the three constructs tested, N17ras had the most potent growth-inhibitory effect.

Cell-cycle analysis of pooled colonies transfected with N17cdc42 or N19rhoA revealed no significant increase in the percentage of cells in G2/M phase (Figures 5B and 5C).

DISCUSSION

Our results suggest an essential role for rac1 in cell proliferation and G2/M progression. Recent evidence from other organisms suggests a role for the family of small GTP-binding proteins in G2/M, and, in particular, cytokinesis. Injection of *Clostridium botulinum* C3 exoenzyme, which inhibits rho activity, has been shown to inhibit the formation of the contractile ring in both sandollar [16] and *Xenopus* embryos [17]. Similarly, a novel protein, racE, appears to regulate cytokinesis in *Dictyostelium* [18].

In mammalian cells, examination of the cell-cycle perturbations of dominant-negative forms of the small GTP-binding proteins has focused predominantly on the G0/G1–S transition. This, most likely, is a reflection of the predominant methodology (microinjection) used to introduce the interfering mutants. One limitation of microinjection techniques is that a large enough sample size is not produced to permit flow-cytometric analysis and thereby examination of other phases of the cell cycle. As such, the use of a recombinant adenovirus encoding a dominant negative form of a small GTP-binding protein represents a useful strategy to examine the cell-cycle effects of these proteins in a wide variety of cell types. Such flexibility may be essential, given recent evidence that the effects of an activated form of the ras oncogene varies considerably depending on the content of its expression. In particular, an activated ras gene can induce transformation in immortalized rodent fibroblasts but, surprisingly, leads to growth arrest and senescence in primary cultures of rodent fibroblasts [28].

The ability of N17rac1 to inhibit cell growth appears to be shared by dominant-negative forms of ras, cdc42 and rhoA. All three small GTP-binding proteins produced a reduction in the number of neomycin-resistant colonies consistent with a growth-inhibitory phenotype. Cell-cycle analysis of neomycin-resistant colonies expressing dominant-negative forms of cdc42 and rhoA revealed no increase in the percentage of cells in G2/M. It should be noted however, that growth under these conditions selects for cells with low expression of N17cdc42 or N19rhoA. As such, it is conceivable that higher, transient expression of these small GTP-binding proteins would lead to a corresponding inhibition at G2/M. To date, for reasons which are not clear, we have been unsuccessful in the construction of recombinant adenoviruses encoding dominant-negative forms of cdc42 or rhoA. We therefore, at this time, are unsure whether the block at G2/M is a unique property of N17rac1 or whether other small GTP-binding proteins, when expressed at equivalent amounts, would lead to a similar cell-cycle arrest.

It is presently unclear precisely how rac1 or, for that matter, other small GTP-binding proteins regulate cell-cycle progression (for a review see [29]). It is tempting to speculate that the myriad of cytoskeletal alterations that need to occur before mitosis and cytokinesis require the participation of rac proteins. Alternatively, rac has recently been linked to the regulation of calcium influx [30], which is known to be required for the G2/M transition [31,32]. In addition, recently the generation of reactive oxygen species by ras and rac have been linked to cell growth and cell-cycle progression [8]. Finally, the rac-regulated activation of the JNK/SAPK protein kinases, which at least in certain cell types have been linked to cell growth, may play an essential role in cell division [33].

Further studies are therefore essential to determine which of the downstream effector(s) of rac1 mediate its ability to regulate cell growth. Nonetheless, although the mechanism remains undefined, our results provide, to our knowledge, the first demonstration in mammalian cells of the requirement of small GTP-binding proteins in the G2/M phase of the cell cycle.

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