

Interference with Endogenous Ras Function Inhibits Cellular Responses to Wounding

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Abstract. Wounding of tissue induces cellular responses that ultimately result in wound repair. Studies in tissue culture model systems indicate that these responses include induction of AP-1 regulated genes, cell migration and mitogenesis which are also characteristic of cellular responses to growth factors. Investigations have identified cellular ras proteins as critical components of growth factor-stimulated signal transduction pathways, however their role in the wounding response is less clear. Investigation of the potential in-

volvement of c-Ras in this process utilized quiescent living bovine corneal endothelium cells (BCE) which were microinjected with ras dominant interfering mutant protein (N17) and subsequently stimulated by mechanical wounding. Analysis of these cells demonstrated that microinjection of dominant-interfering ras protein, but not control proteins, inhibited the wounding response as evidenced by diminished Fos expression, lack of cell migration and a block in DNA synthesis.

THE cellular response to wounding is a complex process. Cells change from a contact-inhibited state to one of high motility and growth. Cellular responses in this process include induced expression of differentiation signals (i.e., autocrine factors) and increased expression of genes responsible for cell division. Tissue responses to wounding can lead to scarring and increased tumorigenesis (Bailleul et al., 1990; Schuh et al., 1990). An understanding of the wounding process at the molecular level may lead to increased knowledge of growth regulation of cells and tissue and therefore is an important goal.

Wound response in an organism occurs in contact-inhibited or quiescent cells that are likely in a "G₀" phase of the cell cycle. Model systems consisting of a monolayer of cultured cells amenable to experimental induction of a G₀ state have been widely used to study the molecular basis of signal transduction of the wound stimulus. Some of the documented cellular responses to wounding in related systems include increased expression of fos proteins (Verrier, 1986), enhanced cell motility, and stimulation of DNA synthesis (Dulbecco and Stoker, 1970; Todaro et al., 1965). Previous work in our lab has shown that Fos induction occurs primarily at the edges of a linear wound created by a scalpel incision across a monolayer of serum-deprived quiescent bovine corneal endothelium cells (BCE's)¹ (Feldman et al., 1992).

Several observations suggest the association of Ras function with signal transduction pathways responsible for the cellular wound response. Tyrosine kinases, which are likely

intracellular initiators of growth signal transduction, can be activated by the interaction of the extracellular matrix (ECM) with integrins (Guan and Shalloway, 1992). An argument supporting Ras involvement in this interaction is plausible since upstream and downstream modulation of Ras function by tyrosine kinases appears likely (Hunter, 1991). Additional examples implicating Ras function with the wounding response are found in independent studies of a wound-related growth factor, basic FGF (bFGF) (Sato and Rifkin, 1988), where evidence supports ras (Chiao et al., 1991) or ECM protein (Ruoslahti and Yamaguchi, 1991) involvement in bFGF-mediated cellular stimulation. Also, changes in cell shape and enhanced motility observed in wounded cells (Thurston et al., 1988; Dulbecco and Stoker, 1970) may be ras protein regulated (Trahey et al., 1987; Barsagi and Feramisco, 1986) and thereby provide an additional potential connection between Ras and wounding responses. Further, the observation that fibronectin fragments (likely produced by wounding) induce collagenase I expression through a potentially Ras-regulated AP-1-dependent transcription event (Werb et al., 1989) implies a link between Ras and the wounding response. In the present studies, we have tested the possible role of ras protein function in regulating cellular responses to wounding and the results provide direct evidence for such a role.

Materials and Methods

Cell Culture and Microinjection

Passages of primary cells derived from BCE as described (Feldman et al., 1992) were grown to ~80% confluency on etched coverslips (Bellco Vineland, NJ). Coverslips were pretreated in a 2 µg/ml solution of laminin

1. *Abbreviations used in this paper:* BCE, bovine corneal endothelium cell; bFGF, basic FGF; BrdU, bromodeoxyuridine; ECM, extracellular matrix.

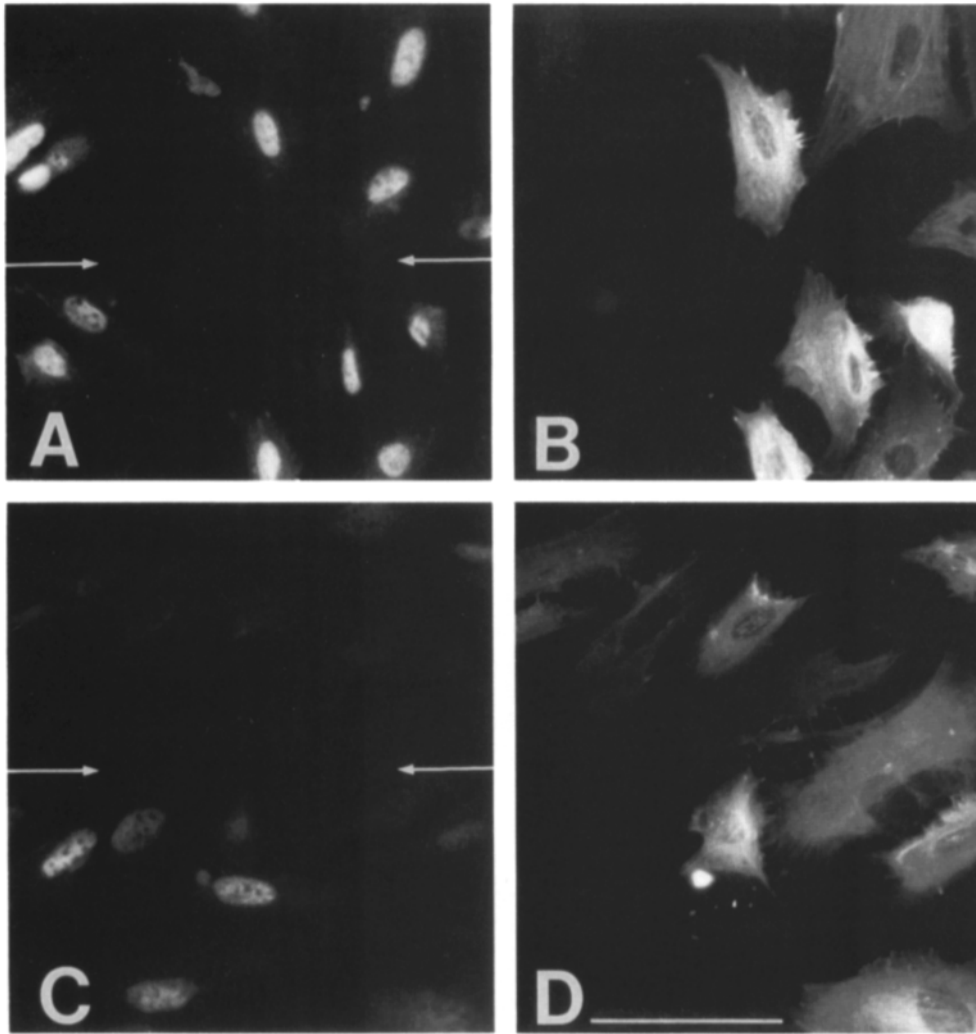


Figure 1. Inhibition of wound-induced Fos expression by dominant negative ras N17 protein. Immunofluorescent micrographs of BCE cells stained for Fos (A and C) or marker protein (B and D). Starved cells were injected with either rat IgG (A and B) or N17 plus marker rat IgG (C and D). Cells were wounded along a line indicated by arrows (A and C). Cells were fixed and stained 2 h after wounding. Bar, 40 μm .

in F99 medium for several hours. F99 medium is a 1:1 mixture of Ham's F12 and Medium 199. Cells were grown in F99 supplemented with 10% FCS, penicillin, and streptomycin. Cells were starved in F99 containing 0.05% serum for 24 h before treatment. Microinjections were carried out using a Zeiss Axiovert microscope (Carl Zeiss, Inc., Thornwood, NY) and Eppendorf 5170 micromanipulator and 5242 microinjector (Eppendorf North America, Inc., Madison, Wis.). Cells were injected in the cytoplasm with either rat IgG (7 mg/ml) or purified N17 protein (3 mg/ml) plus rat IgG (4 mg/ml). Lower concentrations of N17 (0.5 mg/ml) were biologically active, however due to the extended temporal aspect of these assays (cells were fixed up to 40 h after injection) and to insure reliable inhibition of ras in different experiments, the higher concentration of N17 was used in this work. No additional phenotypes were observed with the higher concentration of N17. Cells were injected along the bottom edge of a labeled box of the etched coverslips. Serum-stimulated cells were treated by changing medium from F99 with 0.05% FCS to F99 with 20% FCS. N17 protein was purified from bacterial cultures transfected with N17 expression vectors (Gross et al., 1985). N17 protein and marker IgG (Sigma Immunochemicals, St. Louis, MO) were injected in 20 mM Tris, pH 7.4, 2 mM MgCl_2 , 0.1 mM EDTA, 20 mM NaCl.

Wounding

Initial experiments indicated that wound responses were limited to 3–4 cell diameters from the wound edge, therefore cells were injected only that distance from the bottom of the etched box. After 6–16 h cells were wounded with a glass needle touching the coverslip and pushed along the etched line marking injected cells. This location was easily identified since each etched box has a unique alphanumeric designation. For cell motility and DNA synthesis experiments, a 0.6 μm^2 area below the injected cells was denuded in addition to wounding.

Fixing and Staining

Cells were fixed and stained as previously described (Feldman et al., 1992). Briefly, cells were fixed in 3.7% formaldehyde/PBS, solubilized in 0.3% Triton X-100/PBS, and stained with antibodies diluted in 0.1% Tween 20/PBS. Fos antibody was rabbit polyclonal (Oncogene Science Inc., Uniondale, NY) diluted 1:100 followed by biotinylated anti-rabbit and Texas red streptavidin. FITC-conjugated donkey anti-rat was used to stain marker protein. To measure DNA synthesis in cells a Cell Proliferation Kit (Amersham, UK) was modified by labeling the bromodeoxyuridine (BrdU) antibodies with FITC-conjugated anti-mouse (La Morte et al., 1992). Cells were incubated with BrdU for 24 h before fixation and staining.

Results

To establish a tissue culture model for cell wounding suitable for microinjection and single cell analysis of responses, we used cell monolayers of BCE. Cells were grown to ~70% confluency on etched glass coverslips then serum starved for 24 h to synchronize the cell population in the G_0 stage of the cell cycle. In this state cells had minimal levels of Fos expression and DNA synthesis. A mutant of Ras which acts as a dominant-negative inhibitor protein, N17 (Feig and Cooper, 1988; Cai et al., 1990) or control proteins (nonrelevant IgG) were microinjected into the BCE cells to inhibit the function of the endogenous cellular ras protein. N17 may inhibit endogenous ras protein function by binding guanine

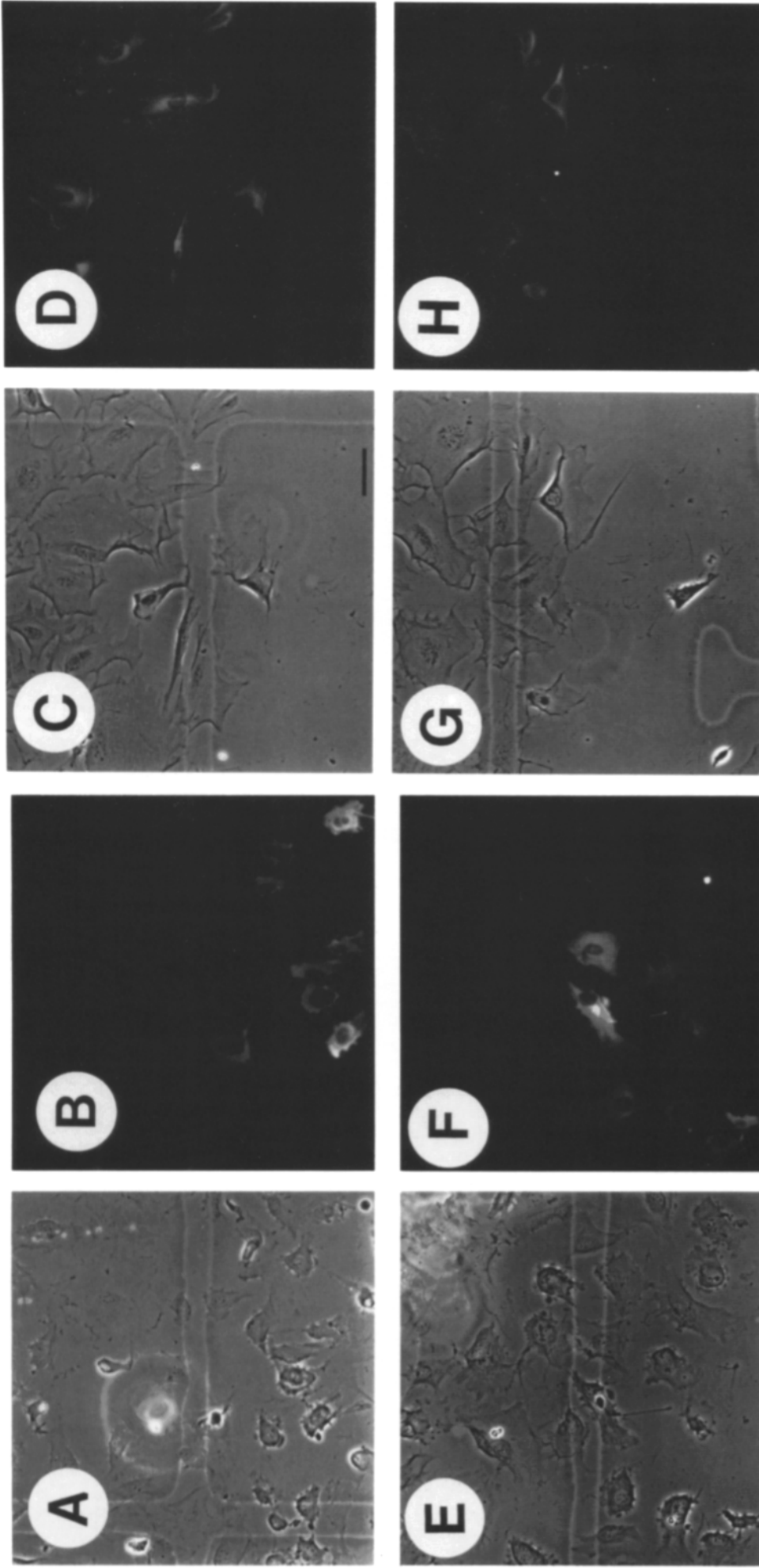


Figure 2. Inhibition of wound-induced cell motility by ras N17. Phase (A, C, E, and G) and immunofluorescent (B, D, F, and H) photomicrographs of BCE cells. (B and F) IgG injections. (D and H) N17 plus IgG injections. B, D, F and H were stained for injected marker IgG protein. Cells were injected within an area 120–140 mm above the etched horizontal line. 4–6 h later an area beneath the injected cells was scraped clear with a glass needle. Cells were fixed and stained 22 h after scraping. Bar, 40 μ m.

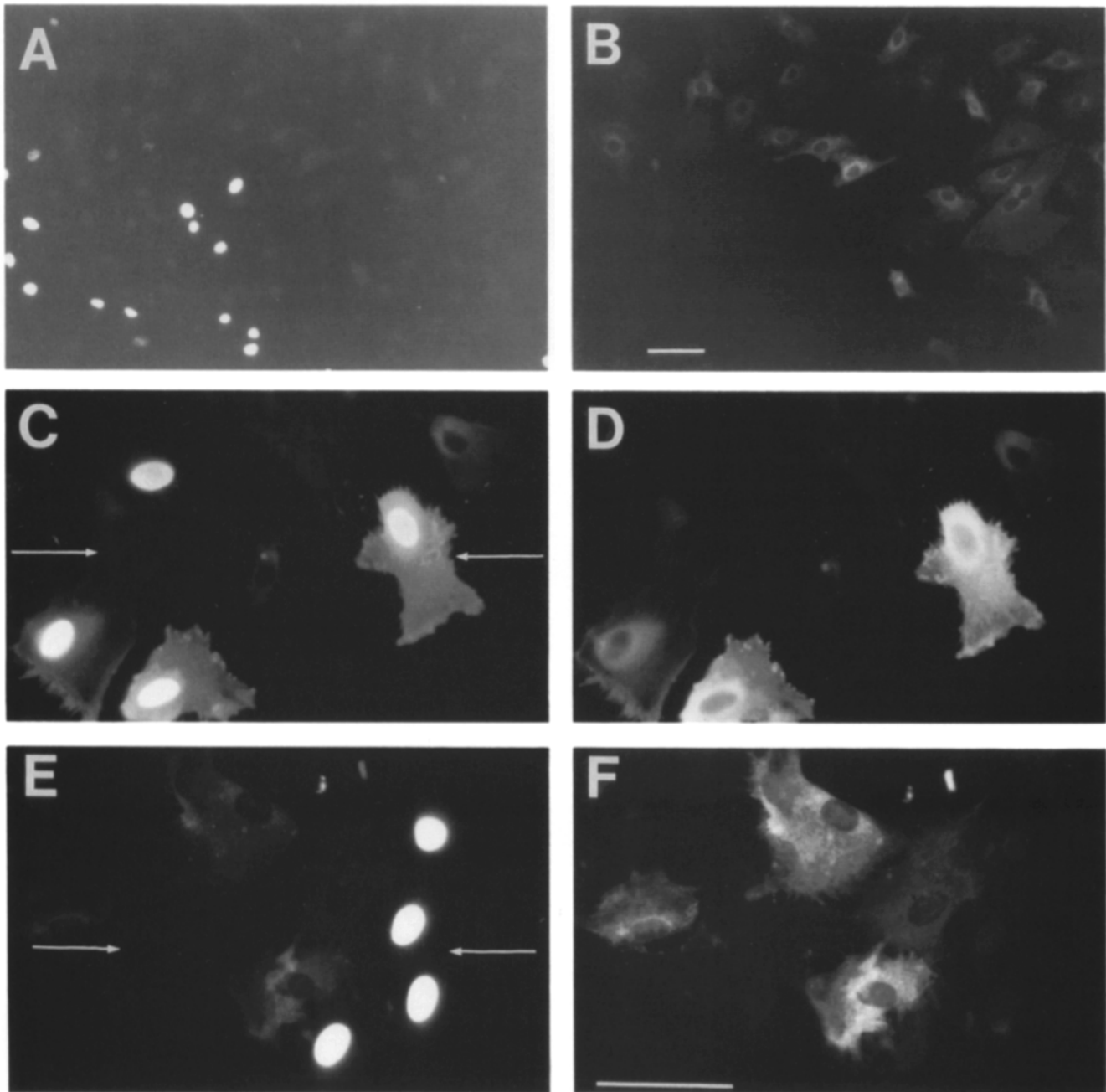


Figure 3. Inhibition of serum and wound-induced DNA synthesis by ras N17. Immunofluorescent micrographs of BCE cells stained for BrdU (*A*, *C*, and *E*) or marker protein (*B*, *D*, and *F*). Starved cells were injected with N17 (*A*, *B*, *E*, and *F*) or rat IgG (*C* and *D*). Cells were subsequently wounded (*C–F*) or stimulated by addition of FCS to 20% (*A* and *B*). Bars, 40 μ m.

nucleotide-releasing factor, thereby inhibiting GDP-GTP exchange and activation of endogenous Ras (Farnsworth and Feig, 1991).

Fos Protein Levels

The role of Ras in wound-induced Fos expression was examined by the above protocol. Cells injected with N17 or control protein were mechanically wounded and fixed after 90–120 min. Cells at or near the wound edge, either uninjected or injected with control proteins, responded by expressing Fos, as determined by immunofluorescence (Fig. 1). Cells as distant as 2–3 cell diameters from the wound

edge responded by expressing fos protein (Fig. 1, *A* and *C*). Investigation of Fos expression in cells injected with the N17 protein showed a significant reduction of the percentage of cells expressing Fos compared with cells injected with control protein (see Fig. 4 *A*). Injection of N17 ras protein did not block serum stimulation of Fos expression however, as discussed below.

Cell Motility

Increased cell migration is another hallmark of the wound response (Dulbecco and Stoker, 1970). To measure cell motility, an area of cells adjacent to a wound edge containing

microinjected cells was removed with a glass needle (Fig. 2). Visual monitoring of these areas continued for the subsequent 24 h. Uninjected cells or cells injected with control proteins showed migration of up to 300 μm from their original position and had a rounded morphology (Fig. 2, A, B, E, and F) while cells injected with the N17 protein did not migrate into the cleared space and maintained a flat morphology (Fig. 2, C, D, G, and H). Numerical evaluation of cell migration was obtained by measuring the distances injected cells migrated from the wound edge. Fig. 4 C shows that cells injected with N17 migrated with lower frequency and to lesser distances relative to control injected cells.

DNA Synthesis

We also examined increased DNA synthesis, a third cellular response to wounding, for potential involvement of cellular Ras function. For these experiments, cells in a quiescent monolayer were microinjected in defined areas of the coverslip and wounded. The addition to the medium of the thymidine analog, BrdU provided a single cell immunofluorescence based assay for DNA synthesis (La Morte et al., 1992). Fig. 3 shows cells injected with either control proteins or the N17 ras protein and their ability to synthesize DNA following wounding. Results summarized in Fig. 4 B demonstrate that interference with endogenous Ras function inhibits wound-induced DNA synthesis. Two independent experiments demonstrated that only 1 cell in 124 injected with N17 was able to synthesize DNA in response to wounding while 69% of cells injected with IgG made DNA in response to wounding.

Wound Induction versus Growth Factor Induction

Interesting results obtained in a comparison of the cellular growth responses to wounding versus serum stimulation prompted further studies. Analysis of the effect of microinjected N17 protein showed that while the Ras mutant blocked DNA synthesis induced by either serum or wounding, it could block increased Fos expression in wounded cells but not in serum-stimulated cells. These results, consistent with those previously reported for N17 and serum induction (Cai et al., 1990), indicate a distinction in the pathways regulating Fos expression by serum versus wounding. To further examine this hypothesis, bFGF, a compound thought to be important in the regulation of wounding (Sato and Rifkin, 1988), was tested for its ability to induce Fos and stimulate DNA synthesis in these cells. Fig. 4, A and B demonstrate that cells treated with only bFGF express Fos and incorporate BrdU and that injection of N17 significantly blocks these responses, supporting the hypothesis that wound induction of the Fos expression is less complex than serum induction of Fos.

Discussion

The data presented here suggest a requirement for ras protein function in the signal transduction pathways stimulated upon wounding monolayers of cultured BCE cells. This relationship has been established via the microinjection of a dominant negative ras protein inhibitor, N17, into living cells and the subsequent analysis of several cellular responses to wounding. N17 is well studied and its effects have been

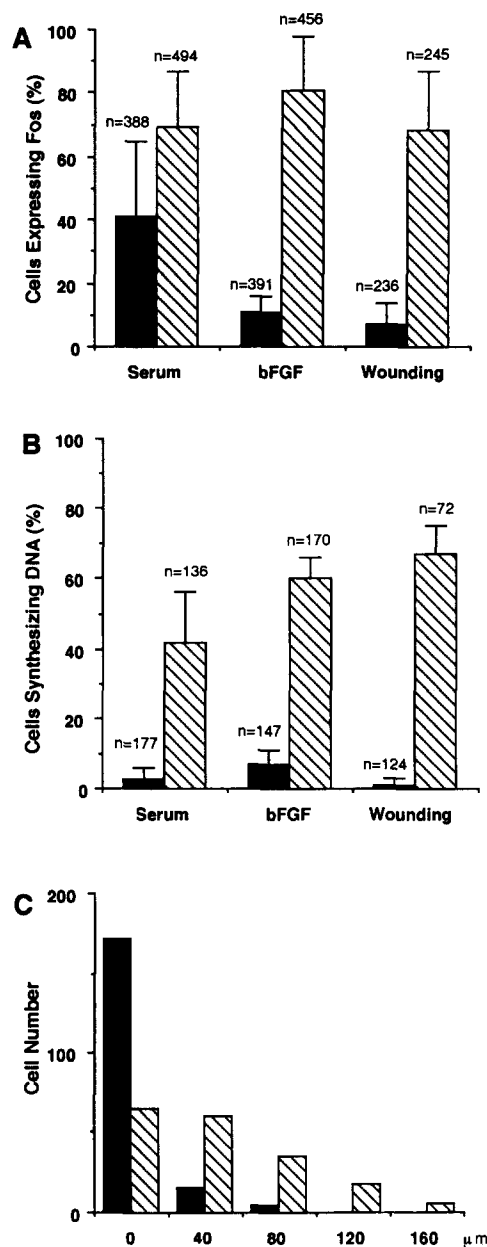


Figure 4. Histograms of N17 inhibition of mitogen and wound induced cellular responses. (A and B) To determine percentage of cells responding, the number of injected cells displaying Fos or BrdU staining were divided by the corresponding total number of injected cells and multiplied by 100. Error bars indicate a standard deviation from the mean. *n*, Total number of cells analyzed. (C) Motility of cells was calculated by measuring the distance cells migrated from the bottom of the injection area delineated by the etched line. C shows a frequency distribution of cells migrating to areas below the etched line at distances (μm) indicated on the x-axis. All experiments were repeated 2–4 times. (■) N17; (▨) IgG.

shown to be identical to the effects of functionally inhibitory p21 Ras antibodies and other mutants of endogenous cellular Ras (Stacey et al., 1991), thus the use of N17 to inhibit endogenous Ras function and the conclusions drawn from its use are likely to be valid.

The potential involvement of endogenous ras protein function in the wounding response provides insight into intracel-

lular signaling in this cellular function. Cells attached to an ECM (laminin and secreted cellular ECMs) likely incur mechanically induced disruption of that attachment when wounded. Previous work has shown cellular attachment to be mediated by integrins (Hynes, 1992). It has also been established that the wound response includes cellular functions typically associated with growth factor responses (i.e., Fos expression, motility, and DNA synthesis). A deductive hypothesis is now emerging which suggests that growth factor responses can be affected by the ECM and integrins (Hynes, 1992; Ruoslahti and Yamaguchi, 1991). As a wealth of literature exists associating Ras with growth factor mediated mitogenesis (Bourne et al., 1991) the potential involvement of Ras in the wounding response appears plausible. Since both Ras signaling and the integrins (Werb et al., 1989) stimulate AP-1 regulated gene expression, a potential exists for the intracellular convergence of these signal pathways.

The control by Ras of multiple responses to wounding suggests a network of intracellular signaling. At least two molecules which can interact with Ras, GAP-associated protein p190 and guanine nucleotide-releasing factor, have domains which could potentially regulate other ras family proteins and/or transmit signals to the nucleus (Shou et al., 1992; Settlemen et al., 1992). Recent studies have suggested that Ras may modulate Rac regulation of actin reorganization as it was shown that membrane ruffling and cell morphology were affected by interference with Ras function (Ridley et al., 1992). The data presented in this study along with previous work (Stacey et al., 1991) indicate that endogenous Ras can regulate cytoskeletal structure, however the relationship between Ras and Rac in signal transduction remains unclear.

It has been suggested that breaks in the integrity of the cell membrane may allow release of endogenous growth factors as bFGF into the wound area (McNeil et al., 1989). The observation that cells distant from the wound edge also have increased Fos expression, motility, and DNA synthesis support a mechanism involving a soluble factor in the induction of the wound response. The finding that bFGF-stimulated cells respond to N17 inhibition of Fos expression and DNA synthesis in a manner similar to wound stimulated cells (Fig. 4, A and B) permits a model incorporating a growth factor in the wound response. It is tempting to speculate that proteoglycans and growth factor complexes interact in response to wounding and that signal transduction initiated by this interaction is channeled through Ras to obtain the effects of wounding demonstrated here. Alternatively, Fos induction by mechanical loading without membrane rupture has been demonstrated and this induction can involve tyrosine kinases (Ingber, 1991) and, as suggested by our data potentially involve Ras.

The disparity between N17 inhibition of serum-stimulated Fos expression and wound or bFGF stimulation of Fos may reflect the plethora of stimuli present in serum and the complexities of the induced signal transduction pathways. This further suggests a limit to the complexities of these factors in the wound or bFGF-initiated responses. Numerous agents induce Fos, apparently through Ras independent pathways, without stimulating DNA synthesis (Sheng and Greenberg, 1990). The ability of N17 Ras to block DNA synthesis in serum-stimulated cells suggests that DNA synthesis is more dependent on Ras-regulated signal transduction than is Fos expression. Additionally, the lack of N17 inhibition of

serum-stimulated Fos expression indicates that N17 is acting in a specific fashion and not disrupting general cell function.

While the focus of the present work is on wound-induced cellular responses, cell motility and mitogenesis appear to be related to oncogenic potential and metastasis. Concerning oncogenesis, wounding may provide a "second hit" for cells already expressing mutant protooncogenes (Bailleul et al., 1990; Schuh et al., 1990). In relation to metastasis, in certain tumor types increased levels of ras protein or activating mutations in Ras are present in metastatic tissue but are not found in primary tumor tissue of the same individual (Deng et al., 1991; Kryprianou and Isaacs, 1990; McKenna et al., 1990). Numerous studies have suggested a link among integrins, metastasis and transformation (Hynes, 1992; Plante-faber and Hynes, 1989). Other work suggests a correlation between metastasis and bFGF (Yayon and Klagsbrun, 1990). Our data provide a possible link among these independent observations. Deregulation of cellular growth control by expression of oncogenic ras proteins may enhance metastasis by uncoupling the biochemical link between the ECM and signal transduction pathways in cells. Further investigations focusing on these interrelationships are in progress.

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