Requirement for c-ras proteins during viral oncogene transformation

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Many retroviral oncogenes have been classified into one of several categories based on structure, enzymology and cellular localization. These genes originated from host cells and are probably derived from genes normally involved in the control of cell proliferation. The cellular counterparts of three oncogenes have been identified as a growth factor or growth factor receptor-6; related oncogenes include receptor-like membrane proteins which often express tyrosine kinase activity. These growth factor-related oncogenes are structurally and biochemically distinct from the membrane-associated ras gene family, which bind and hydrolyse GTP-9. Oncogenes localized primarily in the cytoplasm which probably have serine kinase activity, have also been identified-10. Although the structure and biochemistry of many oncogenes have been extensively studied, relatively little is known about the functional relationships of oncogene proteins within the cell. An opportunity to study such interaction is provided by the identification of a monoclonal antibody that neutralizes cellular ras proteins when microinjected into cells. It has been shown previously that the injected antibody inhibits the initiation of S-phase in NIH 3T3 cells. In the present study we injected this monoclonal antibody into NIH 3T3 cells transformed by a variety of oncogenes. The results show that transformation by three growth factor receptor-like oncogenes depends on c-ras proteins, while transformation by two cytoplasmic oncogenes appears to be independent of c-ras protein.

Anti-ras monoclonal antibodies originally prepared by Furth et al.12 have been analysed extensively. Monoclonal antibody Y13-259 (which binds the c-ras proteins of a variety of species) neutralized the activity of co-injected, purified ras protein, and

![Fig. 1 Morphological reversion of src-transformed cells following injection of anti-ras antibody. These NIH 3T3 cells were transformed by the Rous sarcoma viral genome (see Table 1). Neutralizing anti-ras antibody 259 was injected into the cells adjacent to or within a circular mark on the underside of the coverslip to which the cells were attached. A small section of this circular mark is visible, allowing identification of the injected area in the lower left half of this phase-contrast photomicrograph (x52). After 16 h, cells in the injected area had reverted to the flattened, non-refractile appearance of untransformed cells. The nuclei of most injected cells, for example, are clearly visible. Uninjected cells (or cells injected with control antibody 238; data not shown) remained refractile, with the spherical or spindle shape of transformed cells.](image-url)
Fig. 2. 3H-thymidine incorporation into NIH 3T3-transformed cells after antibody injection. NIH 3T3 cells transformed by the fes (a, b) or fms (c, d) oncogenes (see Fig. 1), the bovine papillomavirus (e, f, from D. R. Lowy), or the mos oncogene (g, h) were grown as a uniform monolayer. Cells within a small circle marked on the back of the coverslips were injected with antibody 259. After 18 h the cells were pulsed with 3H-thymidine and stained with a fluorescent antibody that recognizes injected antibody within the cells. Fluorescence micrographs (left) indicate that almost all the cells in the centre of the photographs were successfully injected. Few fms or fes-transformed cells incorporated thymidine after injection, while a definite reduction with BPV-transformed cells was observed. No reduction in thymidine incorporation was observed in mos-transformed cells.

induced a morphological reversion to the normal phenotype in ras-transformed NIH 3T3 cells. Antibody Y13-238 neither neutralized co-injected protein nor affected ras-transformed cells and was used as a negative control. When neutralizing antibody 259 was microinjected into untransformed NIH 3T3 cells, their rate of proliferation was decreased by approximately 90% compared with either uninjected cells or cells injected with control antibody 238. The antibody inhibited only the initiation of a new round of DNA synthesis. Inhibition was observed until just before the initiation of S-phase but did not affect a cycle of DNA synthesis once it had begun. Cell lines tested in this study were therefore pulsed for 3 h with labelled thymidine, beginning 15–21 h after antibody injection to ensure that any cycle of DNA synthesis in progress at the time of injection would have ended prior to labelling of the cells. Autoradiography would, therefore, reveal thymidine uptake only in cells able to initiate DNA synthesis following antibody injection.

To test the involvement of c-ras proteins in transformation, antibody was injected into NIH 3T3 cells transformed by several oncogenes. An attempt was made to inject all transformed cells within a circular area marked on a coverslip; fluorescent antibodies that could recognize the injected immunoglobulin within the cells were used at the end of each experiment to determine which cells had been successfully injected. Fluorescence staining also ensured that the injected antibody remained within the cell for the duration of the experiment. The proportion of injected cells able to incorporate thymidine was determined by comparing the results of fluorescent staining with those of autoradiography; this number was divided by the proportion of uninjected cells in S-phase on the same coverslip to give values for labelling efficiency. The latter values indicate the percentage of injected cells which incorporated thymidine compared with the number expected to have been labelled if no injection had occurred. In all cells injected with control antibody 238 the labelling efficiency was between 85 and 115%, with average values near 100%; this was the case with each cell line tested here (data not shown) as well as with numerous cell lines tested in other studies. A labelling efficiency close to 100% indicates that injected and uninjected cells enter S-phase with equal efficiency after injection of control antibody.

NIH 3T3 cells were transformed with membrane-associated, receptor-like proteins (src, fms and fes) by transfection of cloned viral DNA. Approximately 12–15 h after microinjection of antibody 259, a distinct morphological reversion from the transformed phenotype was observed in cells that had been transformed by each of the three viral oncogenes. Transformed
cells that had a rounded or spindle-shaped morphology with a refractile appearance reverted to the flattened shape of untransformed NIH 3T3 cells. Figure 1 shows such reversion after injection into src-transformed cells. Morphological reversion continued until 30–50 h after injection (at which time injected antibody had disappeared from the cells); then the cells became morphologically transformed once again (data not shown)\(^3\). In addition to its morphological effects, antibody 259 decreased dramatically the thymidine incorporation of transformed cells labelled during a 3-h pulse between 15 and 24 h after antibody injection. In Fig. 2a–d the decrease in thymidine incorporation following injection of antibody into fms- and fes-transformed cells is clearly visible. Several different clones transformed by each oncogene were analysed. In each of the three transformants labelling efficiencies varied from 10 to 30% (Table 1a). For comparison, ras-transformed NIH 3T3 cells and untransformed NIH 3T3 cells had labelling efficiencies of 10–15% (Table 1). It is unknown whether the small difference in labelling efficiency between NIH 3T3 cells transformed by ras and those transformed by other oncogenes is significant.

Next, we analysed two representatives of the cytoplasmic class of retroviral oncogenes (mos and ras\(^6,21,22\)). NIH 3T3 clones transformed by mos (from G. F. Vande Woude, R. B. Arlinghaus and R. H. Bassin) and cells infected with ras-containing murine sarcoma virus 3611 (aided by two separate helper viruses; prepared by U. R. Rapp) were injected with antibody as before. In no instance of repeated analysis was there any evidence that anti-ras antibody 259 altered the transformed morphology of these cells (data not shown). In addition, labelling efficiencies of mos- and ras-transformed cells were between 90 and 96% (Table 1a); this indicates that proliferation was inhibited little, if at all, by injected antibody, as shown in Fig. 2g, h, where a direct comparison can be made with cells transformed by other oncogenes. The average labelling efficiency obtained with control antibody 238 was consistently within a few percent of 100% (data not shown\(^14,16\)). It is unknown how these oncogenes overcome the normal requirement for c-ras in cellular proliferation but it is clear that both representatives of this oncogene class are able to do so. The group of oncogenes that we have shown to depend on c-ras proteins during transformation is similar, but not identical, to those reported to be suppressed following transformation (ref. 16 and our unpublished data).

In addition to retroviruses, several DNA viruses are able to transform cells. The transforming genes of these viruses are not closely related to the sequences of cellular genes or retroviral oncogenes but they may function similarly. These viruses commonly possess multiple transforming genes\(^1\). Transformation by these viruses displays differences in dependence on c-ras, just as observed with retroviral oncogenes. NIH 3T3 cells transformed by one such virus, bovine papillomavirus (BPV; from D. R. Lowy\(^24\)), were tested as described above, together with WI38 and BALB 3T3 cells transformed by simian virus 40 (SV40). Antibody 259 clearly induced morphological reversion in BPV-transformed NIH 3T3 cells (Fig. 2e, f) and reduced thymidine incorporation. While the antibody clearly inhibited entry into S-phase in cells transformed by BPV, the labelling efficiencies were normally between 26 and 45% (Table 1b). It is unknown whether the difference in labelling efficiencies between cells transformed by this virus and those transformed by the membrane-associated oncogenes is significant. In cells transformed by SV40, on the other hand, the anti-ras antibody inhibited proliferation little if at all. While both cell types tested were efficiently inhibited by the antibody prior to transformation\(^16\), labelling efficiencies after SV40 transformation were 86 and 93%, similar to those observed with the mos and ras oncogenes. It may not be possible at present to make a thorough comparison between the oncogenes of DNA viruses and the retroviral oncogenes tested here\(^1\), but these data indicate some similarities in their action.

Any conclusion drawn from the present study depends on the specificity of the cellular proteins neutralized by antibody 259. As described above, this antibody neutralizes ras protein within the injected NIH 3T3 cell\(^1\). However, it is possible that another cellular protein is also neutralized by the antibody. To ascertain that it is in fact c-ras proteins which are neutralized and responsible for the results described, we tested numerous tumour cell lines. In these tumour lines the phenotype of antibody inhibition strongly correlates with the presence of a mutant ras oncogene\(^16\). Furthermore, deletion mutants of the ras gene which are biologi-
cally active but which do not bind antibody 259 have been prepared recently; cells transformed by such mutants were not altered morphologically, nor did they show greatly inhibited thymidine incorporation, after injection of antibody 259. It is assumed that while viral oncogenes function without normal control, their mechanism of action is similar to that of related cellular genes. Here we have presented evidence to support this idea in the case of growth factor receptor-like molecules and related oncogenes. It is therefore possible that these data might aid in understanding the way in which cellular genes interact in the control of normal proliferation. Our results indicate that some receptor-like oncogenes depend on ras proteins for their signal transduction, whereas other cytoplasmic oncogenes do not. Therefore, numerous oncogenes which we have not yet tested which might behave differently from those described here. With this in mind and on the basis of our present data, we propose that an important class of proliferative signals are received at the cell surface by receptor molecules such as growth factor receptors, and the c-ras proteins are essential in the transfer of these signals to cytoplasmic effectors having serine kinase activity; the effectors then modify target molecules which are directly involved in initiating a proliferative cycle. Accordingly, if the cytoplasmic effector were mutated such that it functioned without activation, proliferation would continue independently of c-ras proteins. Receptor molecules, on the other hand, would always require c-ras to stimulate proliferation.

While our data are consistent with the above scheme, they do not exclude many other possibilities involving multiple metabolic pathways and more complex interactions. For example, we have not reported results with nuclear oncogenes owing to their difficulty in transforming NIH 3T3 cells. The proposed scheme is primarily attractive because of its similarity to the carefully studied mechanism of signal transduction involving cAMP. While it is unlikely that cyclic AMP itself regulates proliferation, G-regulatory proteins with enzymatic similarities to c-ras proteins are involved. These regulatory proteins control signal transduction from cell-surface receptors to cytoplasmic serine kinase effector molecules by regulating adenylyl cyclase activity.

While the present study has examined only one aspect of what is likely to be a highly complex system for regulating proliferation, it does provide a means of functionally comparing separate viral oncogenes. Injection of antibody has been used in other studies to characterize the types of molecules responsible for tumour cell proliferation. Like NIH 3T3 cells transformed by mos or raf genes, many tumour cells show no inhibition of proliferation when injected with anti-ras antibody. In this way their proliferation is distinct from that of the normal cell types studied, each of which was efficiently inhibited by the injected antibody.

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Viral particles induce Ia antigen expression on astrocytes

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Recent studies have shown that γ-interferon (IFN-γ) induces the expression of Ia antigen on astrocytes. This observation is of immunological significance because such activated astrocytes can act as antigen-presenting cells, as demonstrated with myelin basic protein for antigen-specific encephalitogenic T-cell lines. However, the lack of lymphatic drainage in brain and the presence of the so-called blood–brain barrier restricting traffic of cells and macromolecules suggests that IFN-γ may not be readily available, at least during the initial phases of viral infections. The question therefore arises as to whether astrocytes can be induced to express Ia antigens by other signals directly related to viral infection and possibly independent of IFN-γ. In the present report we demonstrate that a neutrotropic murine hepatitis virus induces expression of Ia antigen on astrocytes in tissue culture without infection, rendering these brain cells competent to participate directly in the immune response to a viral infection.

The murine coronaviruses are a group of agents causing acute, subacute or chronic infections in mice or rats accompanied by different disease processes. The JHM strain of this group is neutrotropic and has been shown to induce acute or subacute encephalomyelitis which depend on virus as well as host factors. One important factor in the case of subacute encephalomyelitis in Lewis rats is the immune response, which is directed not only against the virus but also against brain tissue. As various types of central nervous system (CNS) disease are associated with this neutrotropic murine coronavirus strain, we chose this virus to define its interaction with rat brain cells in culture with respect to the induction of Ia antigen on astrocytes.

As a baseline for our study, we analysed the response of Lewis primary glial cell cultures consisting of macrophages carrying Fc receptors (Fc receptor) and astrocytes expressing glial fibrillary acidic protein (GFAP) to recombinant rat IFN-γ (10 U ml⁻¹; Fig. 1a). Recombinant rat IFN-γ induced the expression of Ia on numerous cells in the primary cultures. Fluorescence-activated cell sorting showed that 3-10% of the cells were induced to express Ia with the control after 18 h treatment with 10 U ml⁻¹ IFN-γ, reaching a maximum at 48 h, when 20% of all cells were induced (Fig. 1e). By immunofluorescence microscopy, Ia⁺ cells also became apparent after 18 h of treatment whereas control cultures showed no Ia⁺ cells. Double immunofluorescence microscopy revealed that