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The molecular basis of viral oncolysis: usurpation of the Ras signaling pathway by reovirus

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NIH-3T3 cells, which are resistant to reovirus infection, became susceptible when transformed with activated Sos or Ras. Restriction of reovirus proliferation in untransformed NIH-3T3 cells was not at the level of viral gene transcription, but rather at the level of viral protein synthesis. An analysis of cell lysates revealed that a 65 kDa protein was phosphorylated in untransformed NIH-3T3 cells, but only after infection with reovirus. This protein was not phosphorylated in infected or uninfected transformed cells. The 65 kDa protein was determined to be the double-stranded RNA-activated protein kinase (PKR), whose phosphorylation leads to translation inhibition. Inhibition of PKR phosphorylation by 2-aminopurine, or deletion of the *Pkr* gene, led to drastic enhancement of reovirus protein synthesis in untransformed cells. The emerging picture is one in which early viral transcripts trigger PKR phosphorylation in untransformed cells, which in turn leads to inhibition of translation of viral genes; this phosphorylation event is blocked by an element(s) in the Ras pathway in the transformed cells, allowing viral protein synthesis to ensue. The usurpation of the Ras signaling pathway therefore constitutes the basis of reovirus oncolysis.

Keywords: PKR activation and inactivation/Ras signaling pathway/reovirus infection

Introduction

Although the presence or absence of virus receptors on the cell surface remains a major determining factor of the susceptibility of a cell to virus infection, there is now increasing evidence that the intracellular environment plays an important role in dictating the outcome of viral invasion. In the case of the human reovirus, the receptor is the ubiquitous sialic acid (Gentsch and Pacitti, 1985, 1987; Paul *et al*., 1989; Choi *et al*., 1990), a fact accounting for the observation that reovirus binds to most mammalian cells. However, neither virus binding nor even internalization assures a productive outcome, suggesting that downstream events are required for reovirus infection. An interesting clue has come from earlier studies which showed that normal and transformed cells manifested differential sensitivity to reovirus infection. Hashiro *et al*. (1977) reported that certain virally and spontaneously transformed cell lines of murine origin were susceptible to reovirus infection, whereas normal human and subhuman primate cells, primary mouse cells, normal rat kidney cells and baby hamster kidney cells were not. Duncan *et al*. (1978) found that normal and SV40-transformed WI-38 cells exhibited different sensitivities to reovirus infection, with cytopathology observed only in the transformed cells and not in normal cells, which nonetheless produced virus for a sustained period. Collectively, these observations suggest that reovirus infection efficiency is somehow linked to the transformed state of the cell. However, the molecular basis of this correlation remains obscure.

We recently reported that two mouse cell lines (NR6 and B82) expressing no epidermal growth factor receptors (EGFRs) were relatively resistant to reovirus infection, whereas the same cell lines transfected with the gene encoding EGFR manifested significantly higher susceptibility (Strong *et al*., 1993). This enhancement of infection efficiency requires a functional EGFR, since it was not observed in cells expressing a mutated (kinase-inactive) EGFR. Thus, the reovirus infection process is closely coupled to the EGFR-mediated cell signal transduction pathway. Furthermore, we found that reovirus is capable of binding directly to the N-terminal extracellular domain of EGFRs (Tang *et al*., 1993). Taken together, these observations suggest two alternative explanations for the augmentation of reovirus infection by functional EGFRs. The first possibility is that reovirus plays an active role by first binding to EGFRs, thereby activating the tyrosine kinase activity of the latter and triggering a cell signaling cascade which is required for subsequent steps of the infection process. This mechanism would be similar to that proposed for *Salmonella typhimurium* invasion of mammalian cells (Galan *et al*., 1992; Pace *et al*., 1993), in which binding of the bacteria to cell surface structures stimulates the EGFR, leading to a signaling cascade that promotes *Salmonella* invasion. The second possibility is that reovirus takes advantage of an already activated signal transduction pathway conferred by the presence of functional EGFR on the host cell. In this case, the binding of the virus to EGFRs would represent a fortuitous event that is unrelated to the ensuing infection. The latter possibility is favored because of the following considerations. First, a single infectious reovirus particle is sufficient to initiate the infection process. Second, reovirus recognizes cell surface sialic acid residues and is therefore capable of interacting with a variety of cell surface sialoglycoproteins, rather than with a single species such as the EGFR (Choi *et al*., 1990; Tang *et al*., 1993). Third, even if this interaction (between a reovirion and an EGFR) occurs and results in the triggering of a signal, it is doubtful that the signal from a single bound virus is strong enough to generate an intracellular environment that is now conducive to the subsequent infection process.

More recently, we demonstrated that NIH-3T3 cells, which express a low number of EGFRs and do not respond mitogenically to EGF, are poorly infectible by reovirus. However, when transformed with the v-*erbB* oncogene, they become highly susceptible (Strong *et al*., 1996). This enhanced susceptibility is abrogated by treatment of the cells with genistein, an inhibitor of tyrosine protein kinases. Since v-erbB is essentially a truncated EGFR lacking the extracellular ligand-binding domain (residues 1–555) and possessing ligand-independent, constitutive tyrosine kinase activity (Fung *et al*., 1983; Ullrich *et al*., 1984; Miles and Robinson, 1985; Raines *et al*., 1985; Massoglia *et al*., 1990; Carter *et al*., 1995), our results strongly suggest that the mechanism of enhancement of infection efficiency conferred by EGFR and v-erbB is through the opportunistic utilization by the virus of an already activated signal transduction pathway.

In the present study, we probe the mechanism of reovirus oncolysis using NIH-3T3 cells transformed with intermediates in the EGFR and Ras signaling pathway. We show that the block in reovirus-resistant NIH-3T3 cells is at the level of translation of viral transcripts, and that activated intermediates in the Ras signaling pathway such as Son of Sevenless (Sos) or Ras are capable of releasing this block. Inhibition of viral mRNA translation in parental NIH-3T3 cells is accompanied by the phosphorylation of a 65 kDa cellular protein identified as the double-stranded RNA (dsRNA)-activated protein kinase (PKR). Phosphorylated PKR is known to catalyze the phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF-2α) on Ser51, leading to inhibition of the initiation of protein synthesis (for reviews, see Hershey, 1991; Redpath and Proud, 1994; Proud, 1995). PKR is not phosphorylated in the reovirus-infected Sos- or Ras-transformed cells. Inactivation of PKR by 2-aminopurine, or deletion of the *Pkr* gene in untransformed cells, results in enhanced translation of viral transcripts in these cells. Taken together, our results indicate that reovirus exploits the ability of an element(s) of the cellular Ras signaling pathway to down-regulate PKR. Transformed cells with an activated Ras signaling pathway are therefore particularly susceptible to reovirus infection.

Results

Activated intermediates in the Ras signaling pathway augment reovirus infection efficiency

Previously, we showed that NIH-3T3 cells and their derivatives lacking EGFRs are poorly infectible by reovirus, whereas the same cells transformed with either EGFR or v-erbB are highly susceptible as determined by cytopathic effects, viral protein synthesis and virus output (Strong *et al*., 1993; Strong and Lee, 1996). To determine which downstream mediators of the EGFR signal transduction pathway may be involved in this capacity, we took advantage of the availability of a number of different NIH-3T3-derived cell lines transformed with constitutively activated oncogenes downstream of the EGFR and assayed for their relative susceptibility to reovirus infection. Of particular interest were intermediates in the Ras signaling pathway (reviewed by Barbacid, 1987; Cahill *et al*., 1996). To this end, NIH-3T3 parental cell lines and NIH-3T3 lines transfected with activated versions of *sos* (Aronheim *et al*., 1994) or *ras* (Mundschau and Faller, 1992) oncogenes were exposed to reovirus and compared in terms of their capacity to promote viral protein synthesis.

Detection of viral proteins initially was carried out using indirect immunofluorescent microscopy (Figure 1A). On comparing the uninfected parental cell lines with the various transformed cell lines, it was apparent that the morphology of the cells was quite distinct upon transformation. Whereas the NIH-3T3 cells adopted a typically flattened, spread-out morphology with marked contact inhibition, the transformed cells often grew as spindle-shaped cells with much less contact inhibition. Upon challenge with reovirus, it became clear that the parental NIH-3T3 line was poorly infectible (<5%; this occurred regardless of the source of the parental NIH-3T3 line). The observation that a small proportion of cells were infectible was probably due to the fact that NIH-3T3 cells can sometimes undergo transformation spontaneously (Rubin *et al*., 1995). In contrast, cell lines transformed with Sos or Ras demonstrated relatively pronounced immunofluorescence by 48 h post-infection.

To demonstrate further that viral protein synthesis was more efficient in the Sos- or Ras-transformed cell lines, cells were labeled continuously with [^{35}S]methionine from 12 to 48 h post-infection and the proteins were analyzed by SDS–PAGE. The results (Figure 1B) show clearly that the levels of viral protein synthesis were significantly higher in the Sos- or Ras-transformed cells than in parental NIH-3T3 cells. The identities of the viral bands were confirmed by immunoprecipitation of the labeled proteins with polyclonal anti-reovirus antibodies. Since the uninfected NIH-3T3 cells and their transformed counterparts displayed comparable levels of cellular protein synthesis and doubling times (data not shown), the observed difference in the level of viral protein synthesis could not be due to intrinsic differences in growth rates or translation efficiencies for these cell lines.

The long-term fate of infected NIH-3T3 cells was followed by passaging these cells for at least 4 weeks. They grew normally and appeared healthy, with no sign of lytic or persistent infection; no virus could be detected in the medium after this time (data not shown).

Enhanced infectibility conferred by the activated oncogenes is not due to long-term transformation or the generalized transformed state of the cell

To determine whether the differences in susceptibility may be the result of some long-term effects of transformation, or just the result of the presence of the activated oncogene itself, a cell line expressing a Zn-inducible cellular Harvey-*ras* (c-H-ras) gene was tested for its susceptibility to reovirus as before. These cells (called 2H1) were derived from the murine fibroblast cell line C3H 10T1/2 which we have shown previously to be poorly infectible by reovirus (unpublished data), and carry the c-H-*ras* gene under the control of the mouse metallothionein-I promoter (Trimble *et al*., 1986). Cells were either mock treated or pre-treated with 50 μM $ZnSO_4$ 18 h prior to infection, followed by indirect immunofluorescent analysis of these cells at 48 h post-infection or mock infection. The results (Figure 2A) demonstrate that uninduced cells were poorly infectible (<5%) whereas those induced for only 18 h were much more susceptible (>40%). Enhanced viral

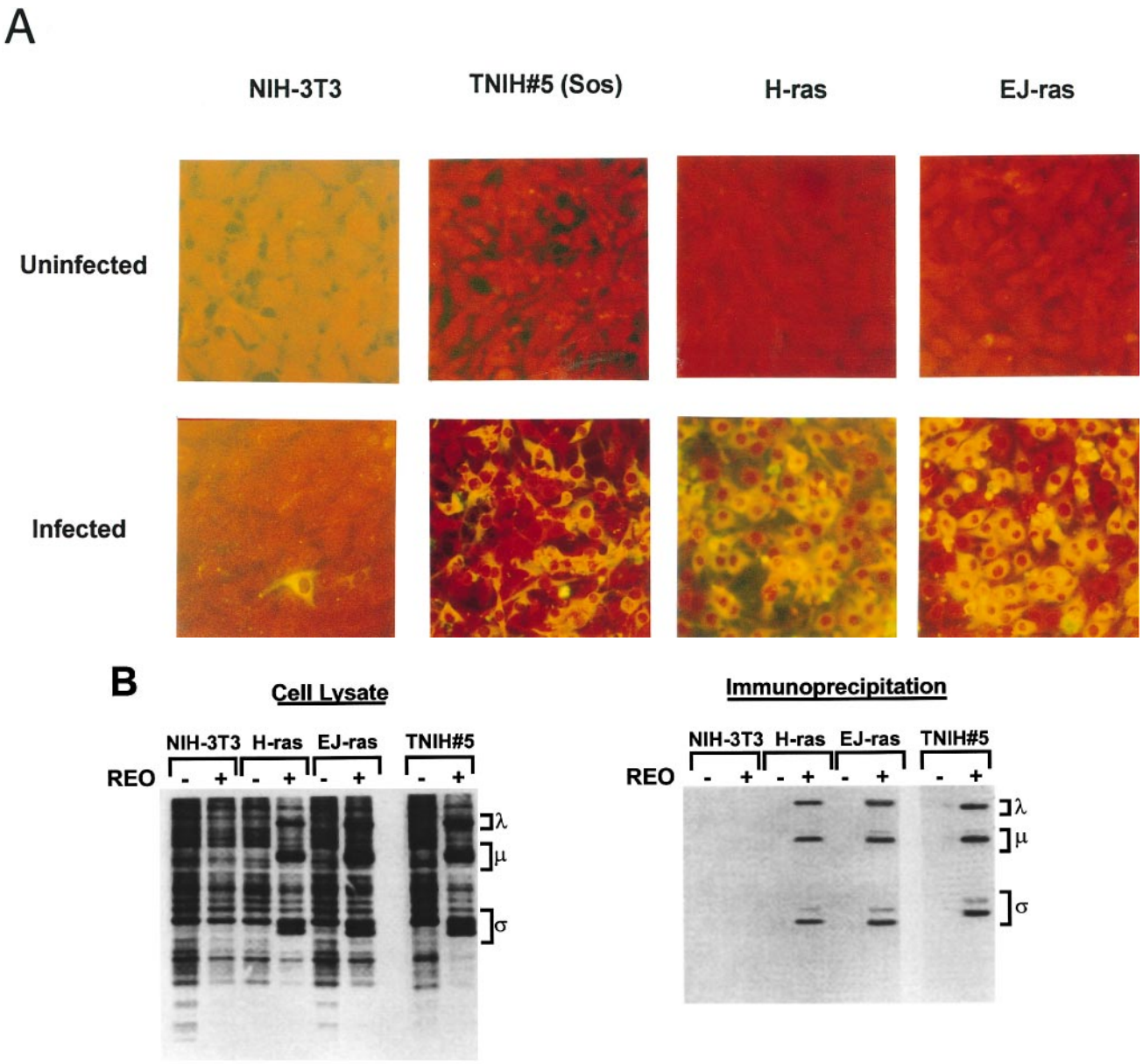

Fig. 1. Effect of activated Sos and activated Ras on host cell susceptibility to reovirus infection. (**A**) Immunofluorescence assay of viral proteins expressed in reovirus-infected NIH-3T3 cells, Sos-transformed cells (TNIH#5), H-Ras- and EJ-Ras-transformed cells. Cells were infected with reovirus at an estimated m.o.i. of 10 p.f.u. per cell. At 48 h post-infection, cells were fixed, processed and reacted with rabbit anti-reovirus type 3 antibody and then with FITC-conjugated goat anti-rabbit immunoglobulin G. The magnification for all panels is ×200. (**B**) Reovirus protein synthesis in mock-infected and reovirus-infected NIH-3T3 cells, Sos-transformed cells (TNIH#5), H-Ras- and EJ-Ras-transformed cells. Cells were labeled with [^{35}S]methionine from 12 to 48 h post-infection. Lysates were then prepared and either analyzed directly by SDS–PAGE (left panel) or immunoprecipitated with the polyclonal anti-reovirus type 3 serum and then analyzed by SDS–PAGE (right panel). The positions of reovirus proteins are indicated on the right.

protein synthesis in the Zn-induced 2H1 cells was confirmed further by metabolic labeling of the cells with [^{35}S]methionine followed by SDS–PAGE analysis of virus-specific proteins (Figure 2B). Based on these observations, we conclude that the augmentation of reovirus infection efficiency in the transformed cells is a direct result of the activated oncogene products, and not due to other factors that might have contributed to a stably transformed state of these established cell lines.

To show further that susceptibility to reovirus infection is not a result of transformation *per se* (i.e. the transformed state of the host cell), we examined NIH-3T3 cells containing a tetracycline-controlled human c-*myc* gene (tet-myc cells) (Helbing *et al*., 1997). These cells normally are maintained in tetracycline (2 μg/ml) which represses the expression of c-*myc*. Removal of tetracycline under normal growth conditions (10% fetal bovine serum) leads to accumulation of the c-Myc protein and the cells display a transformed phenotype. We found that these cells were unable to support virus growth in either the presence or absence of tetracycline (Figure 2C), which again suggests that susceptibility to reovirus infection is not due to the general transformed state of the host cell, but rather requires specific transformation by elements of the Ras signaling pathway.

A good indicator of activation of the Ras signaling

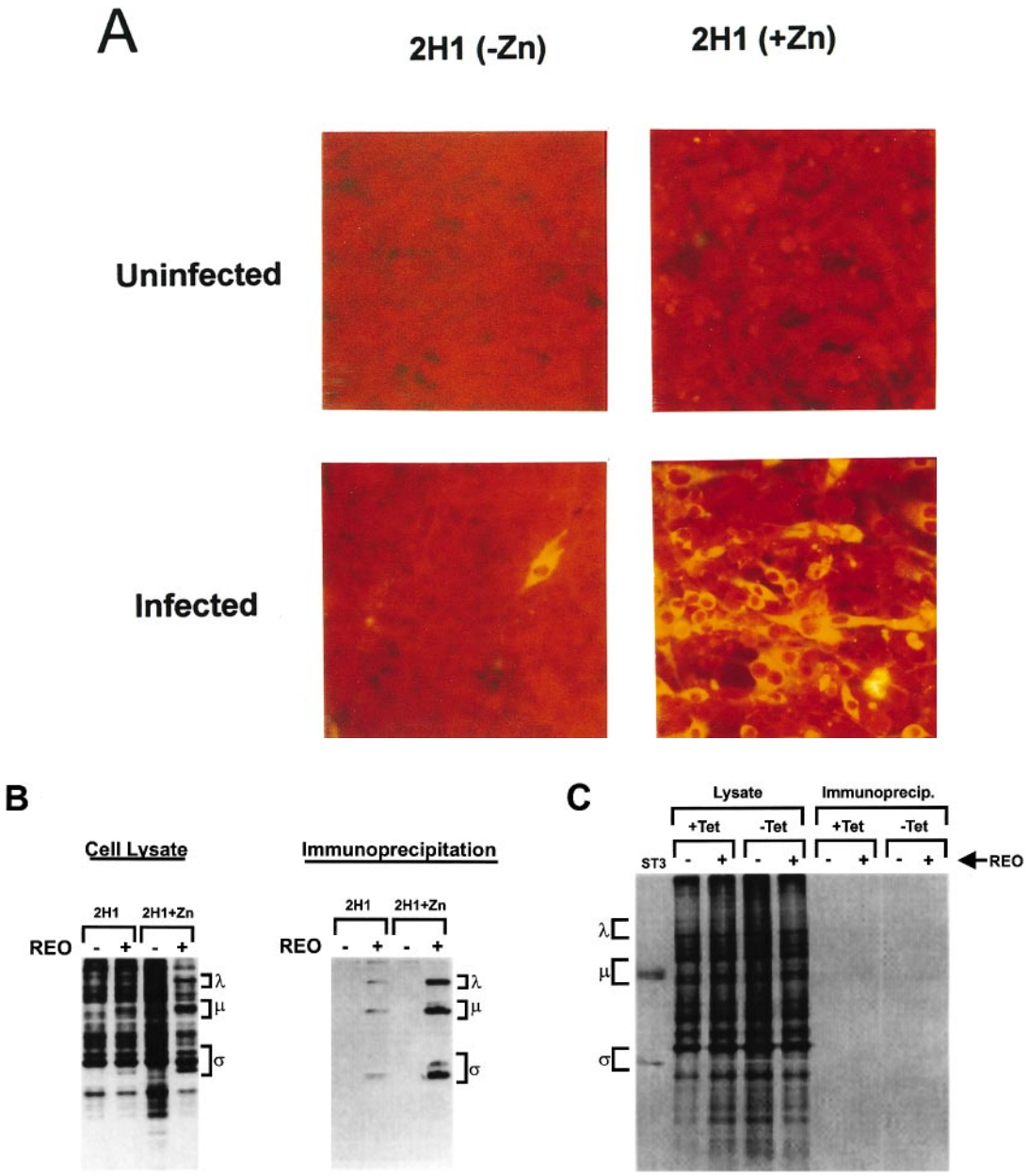

Fig. 2. Effect of transient induction of activated Ras or c-Myc on host cell susceptibility to reovirus infection. (**A**) Immunofluorescence assay of viral proteins expressed in reovirus-infected 2H1 cells (containing a Zn-inducible *ras* gene) in the presence or absence of zinc. 2H1 cells were pre-treated with 50 μM $ZnSO_4$ (+Zn) or mock treated (–Zn) for 18 h prior to infection. Cells were then infected with reovirus at an estimated m.o.i. of 10 p.f.u. per cell or mock infected. At 48 h post-infection, cells were fixed, processed and reacted with rabbit anti-reovirus type 3 antibody, followed by FITC-conjugated goat anti-rabbit IgG. The magnification for all panels is ×200. (**B**) Reovirus protein synthesis in mock-infected and reovirus-infected 2H1 cells in the presence or absence of $ZnSO_4$. Cells were labeled with [^{35}S]methionine from 12 to 48 h post-infection. Lysates were then prepared and either analyzed directly by SDS–PAGE (left panel) or immunoprecipitated with the polyclonal anti-reovirus type 3 serum and then analyzed by SDS–PAGE (right panel). The positions of reovirus proteins are indicated on the right. (**C**) NIH-3T3 tet-myc cells (containing the human c-*myc* gene whose expression is repressed in the presence of 2 μg/ml tetracycline) grown in the presence (+) or absence (–) of tetracycline were infected with reovirus and labeled with [^{35}S]methionine from 12 to 48 h post-infection. Lysates were then prepared and either analyzed directly by SDS–PAGE or immunoprecipitated with the polyclonal anti-reovirus serum and then analyzed by SDS–PAGE.

pathway is the activation of the MAP kinases ERK1 and ERK2 (for a review, see Robinson and Cobb, 1997). In this regard, we have found that compared with untransformed cells, Ras-transformed cells have a significantly higher ERK1/2 activity (unpublished observation). Furthermore, an examination of a number of human cancer cell lines has revealed an excellent correlation between the level of ERK1/2 activity and susceptibility to reovirus infection (unpublished observation), although ERK1/2 itself does not appear to play any role in it (see below). Not surprisingly, mouse L cells and human HeLa cells, in which reovirus grows very well, both manifest high ERK1/2 activity (data not shown).

Viral transcripts are generated, but are not translated, in reovirus-resistant NIH-3T3 cells

To elucidate the role(s) of these oncogenes in reovirus infection, it was important first to identify the step at which reovirus infection is blocked in the non-susceptible NIH-3T3 cells. We have demonstrated previously that

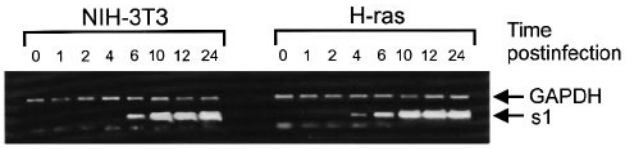

Fig. 3. Reovirus s1 mRNA levels in infected NIH-3T3 cells and H-Ras cells. Cells were infected with reovirus at an estimated m.o.i.of 10 p.f.u. per cell. At various times post-infection, cells were harvested and RNA was extracted from them. Equal amounts of RNA from each sample were then subjected to RT–PCR, followed by selective amplification of reovirus s1 cDNA and the constitutively expressed GAPDH, which served as a PCR and gel loading control. The PCR products were separated on a 2% agarose gel and visualized with ethidium bromide under UV light.

virus binding and virus internalization into non-susceptible cells are comparable with those observed for susceptible cells (Strong *et al*., 1993). It would therefore be of interest to determine whether early transcription of viral genes, a translation-independent process, proceeds normally in the non-susceptible cells. Accordingly, the relative amounts of reovirus s1 transcripts generated in NIH-3T3 cells and in the H-Ras-transformed cells during the first 12 h of infection were compared after amplification of these transcripts by quantitative PCR. The results, shown in Figure 3, demonstrate that the rates of accumulation of s1 transcripts in the two cell lines were similar, at least up to 12 h post-infection. Similar data (not shown) were obtained when other reovirus transcripts were compared. These experiments therefore demonstrate that infection block in the non-susceptible cells is not at the level of transcription of viral genes, but rather at the level of translation of these transcripts. At later times, the level of viral transcripts present in untransformed NIH-3T3 cells decreased significantly whereas transcripts in transformed cells continued to accumulate (data not shown). The inability of these transcripts to be translated in NIH-3T3 cells probably contributed to their degradation. As expected, the level of viral transcripts in infected L cells was at least comparable with that in infected Ras-transformed cells (data not shown).

The finding that viral transcripts were generated in untransformed NIH-3T3 cells led to the question as to whether these cells should still be identified as ‘resistant’, ‘non-susceptible’ or ‘not infectible’. We have opted not to change these long-held designations since there is little or no infectious outcome in these cells (i.e. the infection is abortive). Thus, cells that are ‘resistant’ or ‘non-susceptible’ to reovirus infection could still harbor viral transcripts but these transcripts are not translated.

A 65 kDa protein is phosphorylated in reovirus-treated NIH-3T3 cells, but not in reovirus-infected transformed cells

The above observation that viral transcripts were generated, but were not translated in NIH-3T3 cells led us to entertain the possibility that PKR could be activated (phosphorylated) in these cells [e.g. by s1 mRNA transcripts which have been shown to be potent activators of PKR (Bischoff and Samuel, 1989)], which in turn leads to inhibition of translation of viral genes. The corollary of such a scenario would be that in the case of the transformed cells, this activation is prevented, allowing viral protein synthesis to ensue.

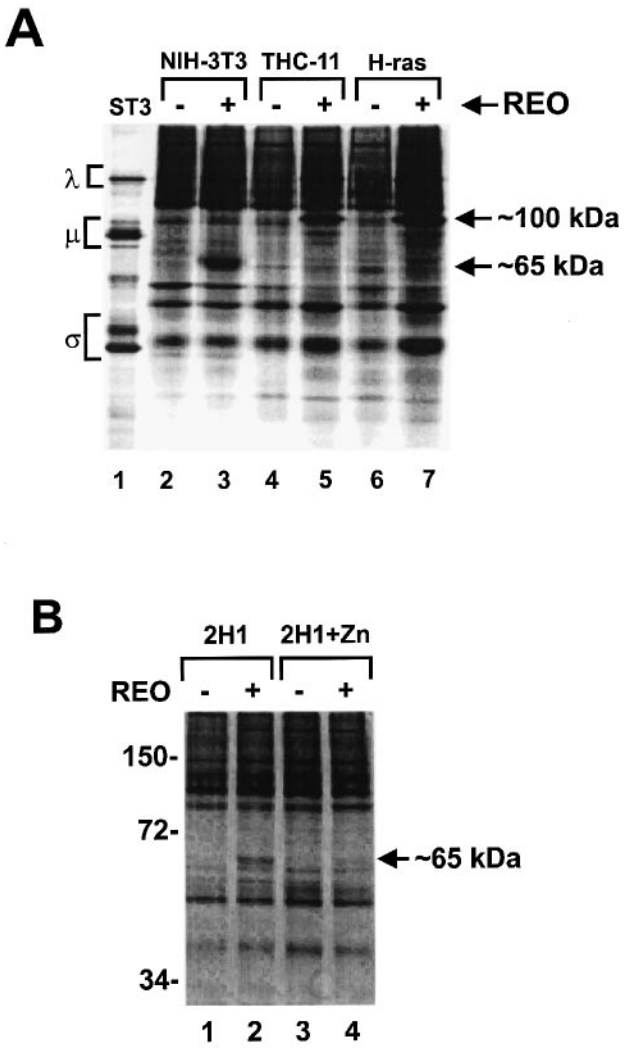

Fig. 4. *In vitro* kinase reactions of lysates from uninfected and reovirus-infected cells. (**A**) *In vitro* kinase reactions of lysates from uninfected and reovirus-infected NIH-3T3, THC-11 and H-Ras cells. Cells were harvested at 48 h post-infection and lysates were prepared. After normalizing for total protein concentration, the lysates were incubated with [γ-^{32}P]ATP for 30 min at 37°C, followed by SDS–PAGE analysis. Lane 1 represents the marker lane which shows the positions of reovirus type 3 structural proteins. (**B**) *In vitro* kinase reactions of lysates from uninfected and reovirus-infected 2H1 cells and Zn-induced 2H1 cells. The positions of molecular weight markers are indicated on the left.

To test the above hypothesis, NIH-3T3 cells and v-erbB- or Ras-transformed cells (designated THC-11 and H-ras, respectively) were treated with reovirus (or mock-treated), and at 48 h post-infection were subjected to *in vitro* kinase reactions, followed by SDS–PAGE for autoradiographic analysis. The results (Figure 4A) clearly show that there was a distinct phosphoprotein migrating at ~65 kDa (the expected size of PKR) only in the NIH-3T3 cells and only after exposure to reovirus (lane 3). This protein was not labeled in the lysates of either the uninfected or infected transformed cell lines (lanes 4–7), although it was present in comparable amounts in all the cell lines tested (data not shown). Instead, a protein migrating at ~100 kDa was found to be labeled in the transformed cell lines after viral infection (Figure 4A, lanes 5 and 7). This protein was absent in both the pre-

infection and post-infection lysates of the NIH-3T3 cell line (lanes 2 and 3), and was not a reovirus protein since it did not react with an anti-reovirus serum that precipitated all reovirus proteins (data not shown). A similar 100 kDa protein was also found to be ^{32}P-labeled in *in vitro* kinase reactions of post-infection lysates of the Sos- or Raf-1-transformed cell lines (data not shown). The identity of this 100 kDa protein presently is unknown.

That intermediates in the Ras signaling pathway are responsible for the lack of phosphorylation of the 65 kDa protein was confirmed further by the use of the 2H1 cells which contain a Zn-inducible *ras* oncogene. As shown in Figure 4B, uninduced 2H1 cells [shown above (Figure 2A and B) to be relatively resistant to reovirus infection] were capable of activating the 65 kDa protein only after exposure to reovirus (compare lanes 1 and 2). However, 2H1 cells subjected to Zn induction of the H-*ras* oncogene showed significant impairment of the activation of this protein (compare lanes 2 and 4). This impairment coincided with the enhancement of viral synthesis in the presence of zinc as shown in Figure 2A and B. Our results therefore eliminated the possibility that phosphorylation of the 65 kDa protein is strictly an NIH-3T3-specific event, and clearly established the role of Ras in preventing (or reversing) this phosphorylation. It is interesting that the Zn-induced 2H1 cells did not produce the 100 kDa phosphoprotein seen in the infected, stably transformed H-Ras cells. The reason for this is unclear at present.

Identification of the 65 kDa phosphoprotein as PKR

To determine whether the 65 kDa protein was indeed PKR, a dsRNA-binding experiment was carried out in which poly(I)–poly(C)–agarose beads were added to the ^{32}P-labeled lysates. After incubation for 30 min at 4°C, the beads were washed, and bound proteins were released and analyzed by SDS–PAGE. The results (Figure 5A) show that the 65 kDa phosphoprotein present in the post-infection NIH-3T3 cell lysates was capable of binding to dsRNA, a well-recognized characteristic of PKR. In contrast, the 100 kDa phosphoprotein detected in the infected H-Ras-transformed cell line did not bind to the poly(I)–poly(C)–agarose. That the 65 kDa phosphoprotein was indeed PKR was confirmed further by the demonstration that it was immunoprecipitable with a PKR-specific antibody (Figure 5B).

Induction of PKR phosphorylation requires active viral transcription

Since phosphorylation of PKR occurred only in non-susceptible cells, and only after the cells had been exposed to reovirus, it was of interest to determine whether active viral transcription is required for this event. To this end, reovirus was UV treated to inactivate its genome prior to addition to NIH-3T3 cells. Such treatment efficiently abolished viral gene transcription (as analyzed by PCR), and hence viral infectivity (data not shown). The cells were then incubated for 48 h and lysates were prepared and subjected to *in vitro* ^{32}P labeling as before. The results are shown in Figure 6. Again, NIH-3T3 cells infected with untreated reovirus produced a prominent 65 kDa ^{32}P-labeled band (PKR) not found in uninfected cells (compare lanes 2 and 3). However, cells exposed to

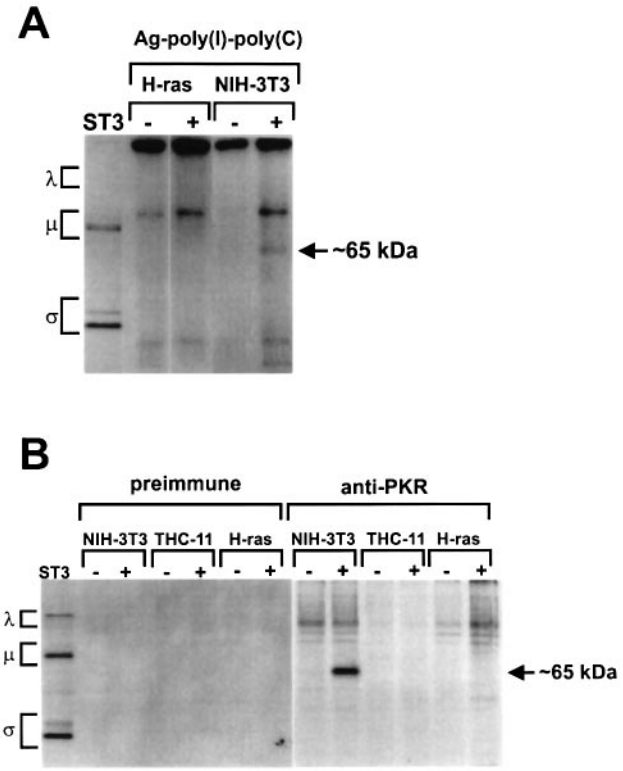

Fig. 5. Identification of the 65 kDa phosphoprotein as PKR. (**A**) Interaction of the 65 kDa phosphoprotein with poly(I)–poly(C). Cytoplasmic extracts were made from uninfected or reovirus-infected NIH-3T3 or H-Ras cells and subjected to *in vitro* kinase reaction in the presence of [γ-^{32}P]ATP. The labeled extracts were then mixed with a 50% agarose–poly(I)–poly(C) slurry and incubated at 4°C for 1 h. The beads were then washed extensively and the adsorbed proteins were released and analyzed by SDS–PAGE. The leftmost lane represents the marker lane which shows the reovirus serotype 3 (ST3) structural proteins. (**B**) Immunoprecipitation of the 65 kDa phosphoprotein with anti-PKR antibody. *In vitro* kinase reactions of cytoplasmic extracts from uninfected or reovirus-infected NIH-3T3, THC-11 or H-Ras cells were subjected to immunoprecipitation with either pre-immune or anti-PKR serum, followed by SDS–PAGE analysis.

the UV-inactivated reovirus behaved similarly to the uninfected controls, manifesting little PKR phosphorylation (lane 4). These results demonstrate that the induction of PKR phosphorylation requires the transcription of viral genes, and is not due to the presence of dsRNA in the input virus. This observation is compatible with the notion that PKR is phosphorylated upon direct binding with the viral transcripts.

PKR inactivation or deletion results in enhanced infectibility of untransformed cells

If PKR phosphorylation is responsible for the shut-off of viral gene translation in NIH-3T3 cells, and one of the functions of the activated oncogene products in the transformed cells is the prevention (or reversal) of this phosphorylation event, then inhibition of PKR phosphorylation in NIH-3T3 cells by other means (e.g. drugs) should result in the enhancement of viral protein synthesis, and hence infection, in these cells. To test this idea, we used the drug 2-aminopurine which has been shown to possess relatively specific inhibitory activity towards PKR autophosphorylation (Samuel and Brody, 1990; Hu and

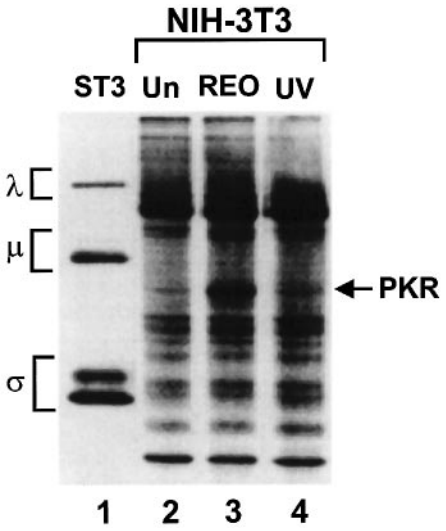

Fig. 6. Comparison of reovirus and UV-inactivated reovirus in their ability to induce phosphorylation of the 65 kDa protein in NIH-3T3 cells. Lysates from uninfected NIH-3T3 cells (Un), and from NIH-3T3 cells exposed to reovirus (REO) or to UV-inactivated reovirus (UV) for 48 h were prepared and subjected to *in vitro* kinase reaction in the presence of [γ-^{32}P]ATP, followed by SDS–PAGE analysis. Lane 1 represents the marker lane which shows the positions of reovirus serotype 3 (ST3) structural proteins.

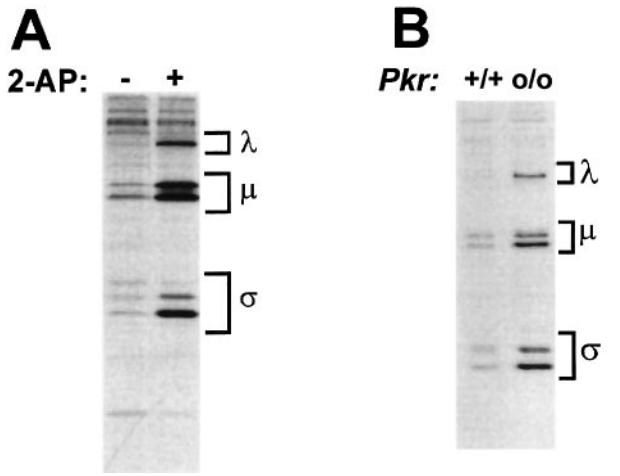

Fig. 7. Effect of PKR inactivation (by 2-aminopurine) or deletion of the *Pkr* gene on the susceptibility of untransformed cells to reovirus infection. (**A**) Effect of PKR inactivation by 2-aminopurine (2-AP). NIH-3T3 cells were treated with 2-aminopurine (+) or mock-treated (–) at the time of infection, and labeled from 12 to 48 h post-infection. Lysates were then immunoprecipitated with the polyclonal anti-reovirus type 3 serum and then analyzed by SDS–PAGE. (**B**) Effect of deletion of the *Pkr* gene. $Pkr^{+/+}$ and $Pkr^{o/o}$ mouse embryo fibroblasts were infected with reovirus and labeled from 12 to 48 h post-infection. Lysates were prepared, immunoprecipitated and analyzed by SDS–PAGE.

Conway, 1993). Accordingly, NIH-3T3 cells were exposed to 5 mM 2-aminopurine at the same time as the addition of reovirus. The cells were labeled with [^{35}S]methionine from 12 to 48 h post-infection and lysates were prepared, immunoprecipitated with the anti-reovirus serum, and analyzed by SDS–PAGE. Figure 7A shows that exposure to 2-aminopurine resulted in a significantly higher level of viral protein synthesis in untransformed NIH-3T3 cells. These results are compatible with the view that PKR phosphorylation leads to the inhibition of viral gene translation, and that inhibition of this phosphorylation event releases the translation block.

A more direct approach to defining the role of PKR in reovirus infection would be through the use of cells that are devoid of PKR. Accordingly, we compared primary embryo fibroblasts from wild-type $PKR^{+/+}$ and $PKR^{o/o}$ mice (Yang *et al*., 1995) in terms of susceptibility to reovirus infection. The results (Figure 7B) clearly show that reovirus proteins were synthesized at a significantly higher level in the $PKR^{o/o}$ cells than in the $PKR^{+/+}$ cells.

The above experiments demonstrate that PKR inactivation or deletion enhances host cell susceptibility to reovirus infection in the same way as does transformation by Ras or elements of the Ras signaling pathway. Together with the PKR phosphorylation data, these results provide strong support for the notion that elements of the Ras signaling pathway negatively regulate PKR, leading to enhanced infectibility of cells transformed by them.

Inactivation of PKR in transformed cells does not involve MEK

Receptor tyrosine kinases such as EGFRs are known to stimulate the mitogen-activated or extracellular signal-regulated kinases (ERK1/2) via Ras (for a review, see Robinson and Cobb, 1997). This stimulation requires the phosphorylation of ERK1/2 by the mitogen-activated extracellular signal-regulated kinase kinase MEK which itself is activated (phosphorylated) by Raf, a serine-threonine kinase downstream of Ras. To determine whether MEK activity is required for PKR inactivation in the transformed cells, we studied the effect of the recently identified MEK inhibitor PD98059 (Dudley *et al*., 1995; Waters *et al*., 1995) on infected Ras-transformed cells. The results (Figure 8A) show that PD98059, at a concentration that effectively inhibited ERK1/2 phosphorylation, did not inhibit reovirus protein synthesis in the transformed cells. On the contrary, PD98059 treatment consistently caused a slight enhancement of viral protein synthesis in these cells; the reason for this is unknown and is under investigation. Consistent with the lack of inhibition of viral protein synthesis in the presence of PD98059, the PKR in these cells remained unphosphorylated (Figure 8B, lanes 6–9). As expected, PD98059 had no effect on reovirus-induced PKR phosphorylation in untransformed NIH-3T3 cells (Figure 8B, lanes 2–5). These results suggest that MEK and ERK1/2 are probably not involved in PKR inactivation. Although presently it is not clear whether Raf plays a role in reovirus infection, elimination of the involvement of the ERK pathway would lead us logically to focus on the stress-activated JNK/SAPK pathway which is also regulated by Ras.

Discussion

Our previous demonstration that EGFRs or v-erbB enhance host cell susceptibility to reovirus infection suggests that the cell growth signaling pathway plays an important role in this event (Strong *et al*., 1993; Strong and Lee, 1996). The present study shows that it is the Ras pathway that is usurped by reovirus. How does Ras (or a downstream element) promote reovirus infection? A major clue was obtained by following the fate of the virus in reovirus-resistant NIH-3T3 cells. We previously demonstrated that

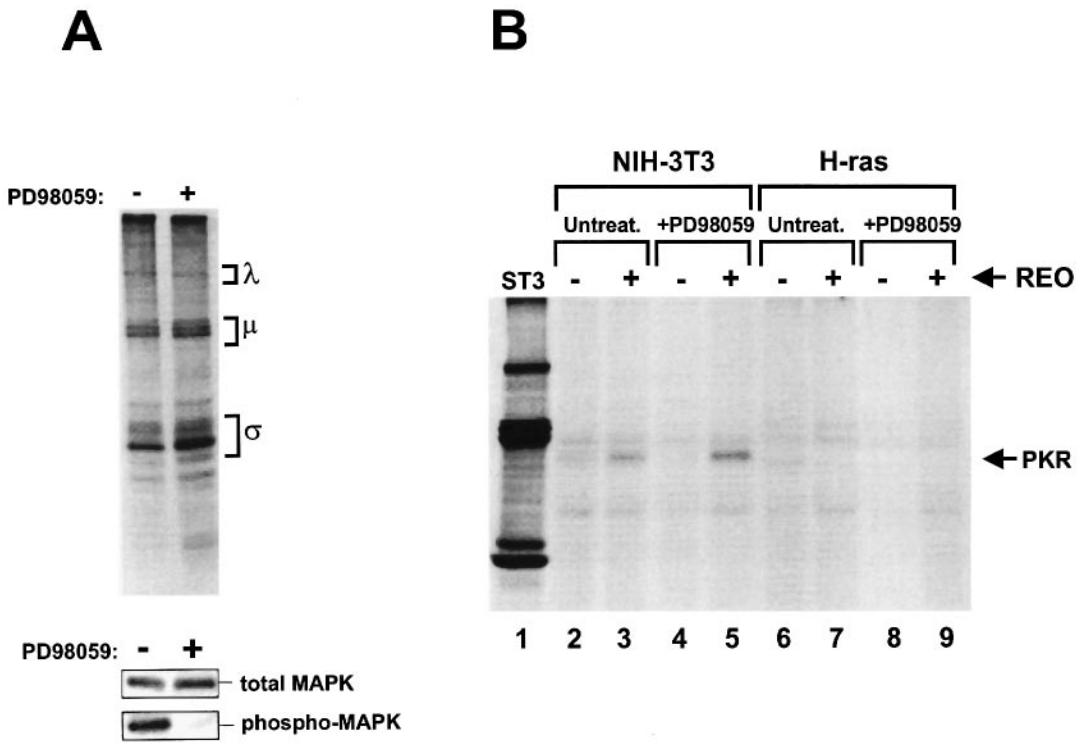

Fig. 8. Effect of the MEK inhibitor PD98059 on reovirus protein synthesis in Ras-transformed cells and on PKR phosphorylation. (**A**) Effect of PD98059 on reovirus protein synthesis in Ras-transformed cells. H-Ras-transformed cells were grown to 80% confluency and infected with reovirus at an m.o.i. of ~10 p.f.u./cell. PD98059 (Calbiochem) [dissolved in dimethylsulfoxide (DMSO)] was applied to the cells at the same time as the virus (final concentration of PD98059 was 50 μM). The control cells received an equivalent volume of DMSO. The cells were labeled with [^{35}S]methionine from 12 to 48 h post-infection. Lysates were then prepared, immunoprecipitated with the polyclonal anti-reovirus serotype 3 serum and then analyzed by SDS–PAGE (top panel). The bottom panel shows the inhibition of MAPK activivity in uninfected H-Ras cells after exposure to 50 μM PD98059 for 48 h. (**B**) Effect of PD98059 on PKR activity of uninfected and reovirus-infected NIH-3T3 and H-Ras transformed cells. NIH-3T3 and H-Ras cells were infected with reovirus in the presence or absence of PD98059 (50 μM final concentration). Cells were harvested at 48 h post-infection and lysates were prepared and subjected to *in vitro* kinase reaction in the presence of [γ-^{32}P]ATP. They were then immunoprecipitated with the anti-PKR antibody and analyzed by SDS–PAGE. Lane 1 represents the marker lane which shows the positions of reovirus serotype 3 (ST3) structural proteins.

neither binding nor internalization is hindered in the non-susceptible cells. In the present study, it is revealed that viral transcription (at least early transcription) also proceeds normally in these cells, leading to the conclusion that inhibition of viral gene translation is the cause of abortive infection. The function of Ras in the reovirus-susceptible transformed cells is therefore the release of this block. A major cause of inhibition of viral protein synthesis is PKR activation. Indeed, PKR was found to be activated in the non-susceptible NIH-3T3 cell, but only after exposure to reovirus. UV-inactivated reovirus does not produce viral transcripts and is unable to activate PKR, an observation consistent with the previous demonstration that the reovirus s1 transcript is a potent activator of PKR, presumably due to its extensive secondary structure (Bischoff and Samuel, 1989). This experiment also rules out the possibility of genomic dsRNA of the input virus being responsible for PKR activation. The end result of reovirus invasion in the non-susceptible NIH-3T3 cells is therefore a suicidal loop in which the viral transcripts are the cause of inhibition of their own translation (Figure 9). Alternatively, one can view this inhibition as a defense mechanism of the host cell against reovirus infection. Interestingly, PKR is not activated in v-erbB-, Sos- or Ras-transformed NIH-3T3 cells exposed to reovirus, and this is consistent with the ability of these cells to support viral gene translation and the subsequent virus replication. The demonstration that inhibition of PKR activation by 2-aminopurine, or deletion of the *Pkr* gene, leads to viral protein synthesis in otherwise non-susceptible cells is also consistent with the view that oncogenes support reovirus protein synthesis by preventing PKR activation. The usurpation of the Ras signaling pathway is, therefore, the molecular basis of reovirus oncolysis.

Compared with other viruses, reovirus is certainly unique in its strategy to down-regulate PKR. It is believed that PKR activation is an inevitable event during the course of a viral infection in which dsRNAs or RNAs with extensive secondary structure are often synthesized. Viruses, therefore, have evolved different mechanisms to prevent PKR activation (reviewed by Mathews, 1993; Katze, 1995). In the case of adenovirus, a viral gene product VAI RNA is synthesized in large amounts and, with its extensive secondary structure, inactivates PKR by acting as a competitive inhibitor of activator dsRNA (Katze *et al.*, 1987). Vaccinia virus encodes two gene products (K3L and E3L) to down-regulate PKR with different mechanisms. The K3L gene product has limited homology with the N-terminal region of eIF-2α and therefore may act as a pseudosubstrate for PKR (Beattie *et al.*, 1991; Davies *et al.*, 1991), whereas the E3L gene product is a dsRNA-binding protein and may well function

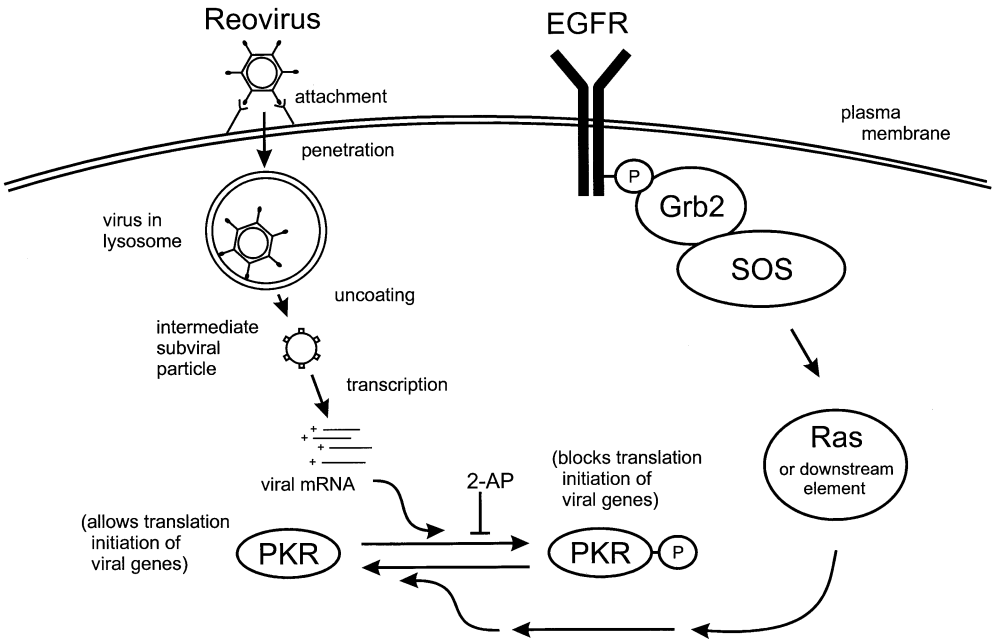

Fig. 9. The molecular basis of reovirus oncolysis: usurpation of the host cell Ras signaling pathway. For both untransformed (reovirus-resistant) and EGFR-, Sos- or Ras-transformed (reovirus-susceptible) cells, virus binding, internalization, uncoating and early transcription of viral genes all proceed normally. In the case of untransformed cells, secondary structures on the early viral transcripts inevitably trigger the phosphorylation of PKR, thereby activating it, leading to the phosphorylation of the translation initiation factor eIF-2α, hence the inhibition of viral gene translation. In the case of EGFR-, Sos- or Ras-transformed cells, the PKR phosphorylation step is prevented or reversed by Ras or one of its downstream elements, thereby allowing viral gene translation to ensue. The action of Ras (or a downstream element) in promoting viral gene translation (and hence reovirus infection) in the untransformed cells can be mimicked by deletion of the *Pkr* gene or by blocking PKR phosphorylation with 2-aminopurine (2-AP).

by squelching activator dsRNAs (Chang and Jacobs, 1993; Davies *et al*., 1993). Poliovirus has been shown to recruit a cellular protease that apparently works in conjunction with poliovirus-specific dsRNA to degrade PKR (Black *et al*., 1993). Influenza virus also triggers a host cell protein (p58) that is a member of the tetratricopeptide repeat (TPR) family of proteins to down-regulate PKR (Lee *et al*., 1994). Thus, the exploitation of host cell factors to cope with the PKR problem is by no means unique to reovirus; the usurpation of a major cell signaling pathway for this purpose certainly is.

It deserves mention that a Ras–PKR connection (not in the context of viral infection) has been noted previously by Mundschau and Faller (1992, 1994). Based on the previous observation that introduction of a transforming Ras gene into BALB/c-3T3 fibroblasts blocks induction of responsive genes by platelet-derived growth factor and interferon (Zullo and Faller, 1988; Offermann and Faller, 1989), these investigators examined the effect of transforming Ras genes on PKR activity in these cells. Their results indicate that dsRNA-mediated activation of PKR is blocked in these transformed cells and that the block is at the level of activation of autophosphorylation, and not PKR synthesis (Mundschau and Faller, 1992). Our results extend this connection to include upstream events of the Ras signaling pathway (EGFR, v-erbB and Sos) as being capable of inhibiting the PKR activity and, more importantly, demonstrate for the first time that a virus is capable of utilizing this connection surreptitiously for infecting a host cell. Exactly how activated Ras down-regulates PKR remains to be seen, but it does not appear to involve MEK or ERK1/2. Elimination of the ERK pathway would infer a possible involvement of another Ras-regulated pathway, the stress-activated JNK/SAPK pathway (for a review, see Robinson and Cobb, 1997), in PKR inactivation, which we currently are investigating.

Our data show that only viral transcripts, but not host cell transcripts, are the targets of virus-induced activated PKR, since host cell protein synthesis proceeds normally in NIH-3T3 cells exposed to reovirus. Although the reason for this bias presently is unknown, several possibilities can be entertained. The first possibility is that viral transcripts are synthesized in a compartment separate from the bulk cytoplasm that harbors cellular transcripts which would essentially be physically shielded from the viral transcript-activated PKR. The second possibility is that intrinsic differences between viral and cellular transcripts (particularly at the 5′ end) necessitate distinct translation initiation events in the two systems. As a result, translation initiations that occur on viral transcripts could be more sensitive to PKR activation (and hence eIF-2α phosphorylation) than those on cellular transcripts. The third possibility is that there may well be a physical association between newly synthesized viral transcripts and the PKR which they inadvertently (and inevitably) activate. This would lead to localized eIF-2α activation (and hence localized inhibition of translation initiation) which, accordingly, would be a strictly viral transcript-specific event. These three alternative scenarios for NIH-3T3 cells are all applicable to the transformed cells, with the exception that PKR activation would now be blocked by elements of the host cell Ras signaling pathway.

It is necessary to reconcile our present findings with the currently held notion that the reovirus major outer capsid protein σ3 is responsible for blocking the activation of PKR (Imani and Jacobs, 1988; Lloyd and Shatkin,

1992). This protein can bind dsRNA [e.g. poly(I)(C)] and inhibit PKR activity *in vitro* (Imani and Jacobs, 1988). Expression of σ3 has been shown to stimulate translation of reporter gene mRNA in transfected COS cells (Giantini and Shatkin, 1989), and to rescue a VAI-defective adenovirus (Lloyd and Shatkin, 1992) or an E3L-defective mutant of vaccinia virus (Beattie *et al*., 1995). These observations are compatible with the presumptive anti-PKR role of σ3. However, there are several unsettling issues. First, there has been no direct evidence that σ3 inactivates PKR in reovirus-infected cells. On the contrary, σ3 has been implicated in serotype-specific inhibition of host mRNA translation in reovirus-infected cells (Sharpe and Fields, 1982). Second, protein σ3 is known to bind dsRNA, and not single-stranded (ss) RNA (including reovirus transcripts). However, accumulated evidence (including our present finding) suggests that it is the ssRNA transcripts, and not the genomic dsRNA segments, that trigger PKR activation (Bischoff and Samuel, 1989). Third, although the various reovirus mRNA species vary greatly with respect to the efficiency with which they are translated, there is no evidence that protein σ3 synthesis precedes that of the other viral proteins (Gaillard and Joklik, 1985). Fourth, the very notion that σ3 is responsible for promoting the synthesis of the other viral proteins by inactivating PKR begs the question as to what is responsible for promoting the synthesis of σ3 itself. The above considerations have made it unlikely, in our opinion, that σ3 is used by reovirus as an anti-PKR agent, at least, not as a primary one. It seems easier to envisage σ3 as playing a subsidiary role in PKR down-regulation, one that supplements the initial assault on PKR by elements of the host cell Ras pathway. Such a view would be compatible with the propensity of reovirus to target transformed cells, and may well compromise the puzzle surrounding the σ3/PKR dilemma.

Materials and methods

Cells and virus

Parental NIH-3T3 cell lines along with NIH-3T3 cells transformed with a number of oncogenes were obtained from a variety of sources. Parental NIH-3T3 and NIH-3T3 cells transfected with the Harvey-*ras* (H-Ras) and EJ-*ras* oncogenes were a generous gift of Dr Douglas Faller (Boston University School of Medicine). NIH-3T3 cells along with their Sos-transformed counterparts (designated TNIH#5) were a generous gift of Dr Michael Karin (University of California, San Diego). Dr H.-J.Kung (Case Western Reserve University) kindly donated parental NIH-3T3 cells along with NIH-3T3 cells transfected with the v-*erbB* oncogene (designated THC-11). 2H1 cells, a derivative of the C3H 10T1/2 murine fibroblast line, containing the Harvey-*ras* gene under the transcriptional control of the mouse metallothionein-I promoter, were obtained from Dr Nobumichi Hozumi (Mount Sinai Hospital Research Institute). These 2H1 cells are conditional Ras transformants that express the H-Ras oncogene in the presence of 50 μM $ZnSO_4$. All the above cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS).

The NIH-3T3 tet-myc cells were obtained from Dr R.N.Johnston (University of Calgary) and were grown in DMEM containing 10% heat-inactivated FBS and antibiotics in the presence or absence of 2 μg/ml tetracycline (Helbing *et al*., 1997). In the presence of tetracycline, expression of the human c-*myc* gene is repressed. Removal of tetracycline results in the elevation of expression of c-*myc* by up to 100-fold in these cells, which also display a transformed phenotype.

The $Pkr^{+/+}$ and $Pkr^{o/o}$ mouse embryo fibroblasts (MEFs) were obtained from Dr B.R.G.Williams (The Cleveland Clinic Foundation) and were grown in α-MEM containing 10% fetal bovine serum and antibiotics as previously described (Yang *et al*., 1995; Der *et al*., 1997).

The Dearing strain of reovirus serotype 3 used in these studies was propagated in suspension cultures of L cells and purified according to Smith *et al*. (1969) with the exception that β-mercaptoethanol was omitted from the extraction buffer. Reovirus labeled with [^{35}S]methionine was grown and purified as described by McCrae and Joklik (1978). The particle/p.f.u. ratio for purified reovirus was typically 100/1.

Immunofluorescent analysis of reovirus infection

For the immunofluorescent studies, the NIH-3T3, TNIH#5, H-Ras, EJ-Ras, 2H1 (±$ZnSO_4$) and THC-11 cells were grown on coverslips, and infected with reovirus at a multiplicity of infection (m.o.i.) of ~10 p.f.u./cell, or mock-infected. At 48 h post-infection, cells were fixed in an ethanol/acetic acid (20/1) mixture for 5 min, then rehydrated by sequential washes in 75, 50 and 25% ethanol, followed by four washes with phosphate-buffered saline (PBS). The fixed and rehydrated cells were then exposed to the primary antibody (rabbit polyclonal anti-reovirus type 3 serum diluted 1/100 in PBS) for 2 h at room temperature. Following three washes with PBS, the cells were exposed to the secondary antibody [goat anti-rabbit IgG (whole molecule)–fluorescein isothiocyanate (FITC) conjugate diluted 1/100 in PBS containing 10% goat serum and 0.005% Evan's blue counterstain] for 1 h at room temperature. Finally, the fixed and treated cells were washed three more times with PBS and then once with double-distilled water, dried and mounted on slides in 90% glycerol containing 0.1% phenylenediamine, and viewed with a Zeiss Axiophot microscope on which a Carl Zeiss camera was mounted (the magnification for all panels was ×200).

Detection of MAP kinase (ERK) activity

The 'PhosphoPlus' p44/42 MAP kinase (Thr202/Tyr204) Antibody kit (New England Biolabs) was used for the detection of MAP kinase in cell lysates according to the manufacturer's instructions. Briefly, subconfluent monolayer cultures were lysed with the recommended SDS-containing sample buffer, and subjected to SDS–PAGE, followed by electroblotting onto nitrocellulose paper. The membrane was then probed with the primary antibody (anti-total MAPK or anti-phospho-MAPK), followed by the horseradish peroxidase (HRP)-conjugated secondary antibody as described in the manufacturer's instruction manual.

Radiolabelling of reovirus-infected cells and preparation of lysates

Confluent monolayers of NIH-3T3, TNIH#5, H-Ras, EJ-Ras, 2H1 (±$ZnSO_4$), THC-11 cells, tet-myc cells and the $Pkr^{+/+}$ and $Pkr^{o/o}$ MEFs were infected with reovirus (m.o.i. ~10 p.f.u./cell). At 12 h post-infection, the medium was replaced with methionine-free DMEM containing 10% dialyzed FBS and 0.1 mCi/ml of [^{35}S]methionine. After further incubation for 36 h at 37°C, the cells were washed in PBS and lysed in the same buffer containing 1% Triton X-100, 0.5% sodium deoxycholate and 1 mM EDTA. The nuclei were then removed by low-speed centrifugation and the supernatants were stored at –70°C until use.

Preparation of cytoplasmic extracts for in vitro kinase assays

Confluent monolayers of the various cell lines were grown on 96-well cell culture plates. At the appropriate time post-infection, the medium was aspirated off and the cells were lysed with a buffer containing 20 mM HEPES (pH 7.4), 120 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5% NP-40, 2 μg/ml leupeptin and 50 μg/ml aprotinin. The nuclei were then removed by low-speed centrifugation and the supernatants were stored at –70°C until use.

Cytoplasmic extracts were normalized for protein concentrations before use by the Bio-Rad protein microassay method. Each *in vitro* kinase reaction contained 20 μl of cell extract, 7.5 μl of reaction buffer [20 mM HEPES (pH 7.4), 120 mM KCl, 5 mM $MgCl_2$, 1 mM DTT and 10% glycerol) and 7.0 μl of ATP mixture (1.0 μCi of [γ-^{32}P]ATP in 7 μl of reaction buffer), and was incubated for 30 min at 37°C (Mundschau and Faller, 1992). Immediately after incubation, the labeled extracts were either boiled in Laemmli SDS sample buffer or were precipitated with agarose–poly(I)–poly(C) beads or immunoprecipitated with an anti-PKR antibody.

Agarose–poly(I)–poly(C) precipitation

To each *in vitro* kinase reaction mixture, 30 μl of a 50% Ag poly(I)–poly(C) Type 6 slurry (Pharmacia LKB Biotechnology) was added, and the mixture was incubated at 4°C for 1 h. The Ag poly(I)–poly(C) beads with the absorbed, labeled proteins were then washed four times with wash buffer [20 mM HEPES (pH 7.5), 90 mM KCl, 0.1 mM EDTA, 2 mM DTT, 10% glycerol] at room temperature and mixed with 2×

Laemmli SDS sample buffer. The beads were then boiled for 5 min, and the released proteins were analyzed by SDS–PAGE.

Polymerase chain reaction

Cells at various times post-infection were harvested and resuspended in ice-cold TNE [10 mM Tris (pH 7.8), 150 mM NaCl, 1 mM EDTA] to which NP-40 was then added to a final concentration of 1%. After 5 min, the nuclei were pelleted and RNA was extracted from the supernatant using the phenol:chloroform procedure. Equal amounts of total cellular RNA from each sample were then subjected to RT–PCR (Wong *et al*., 1994) using random hexanucleotide primers (Pharmacia) and reverse transcriptase (Gibco-BRL) according to the manufacturers' protocol. The cDNAs from the RT–PCR step was then subjected to selective amplification of reovirus s1 cDNA using the primers 5′-AATTCGATTTAGGTGACACTATAGCTATTGGTCGGATG-3′ and 5′-CCCTTTTGACAGTGATGCTCCGTTATCACTCG-3′ that amplify a predicted 116 bp fragment. These primer sequences were derived from the S1 sequence determined previously (Nagata *et al*., 1984). The GAPDH primers (Wong *et al*., 1994) 5′-CGGAGTCAACGGATTTGGT-CGTAT-3′ and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ were used to amplify a predicted 306 bp GAPDH fragment which served as a PCR and gel loading control. Selective amplification of the s1 and GAPDH cDNAs was performed using *Taq* DNA polymerase (Gibco-BRL) according to the manufacturer's protocol using a Perkin Elmer Gene Amp PCR system 9600. PCR was carried out for 28 cycles, with each cycle consisting of a denaturing step for 30 s at 97°C, an annealing step for 45 s at 55°C and a polymerization step for 60 s at 72°C. PCR products were analyzed by electrophoresis through an ethidium bromide-impregnated TAE–2% agarose gel and photographed under UV illumination with Polaroid 57 film.

Immunoprecipitation and SDS–PAGE analysis

Immunoprecipitation of ^{35}S-labeled reovirus-infected cell lysates with anti-reovirus serotype 3 serum was carried out as previously described (Lee *et al*., 1981). Immunoprecipitation of ^{32}P-labeled cell lysates with an anti-PKR antibody (from Dr Michael Mathews, Cold Spring Harbor) similarly was carried out. Immunoprecipitates were analyzed by discontinuous SDS–PAGE according to the protocol of Laemmli (1970).

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