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EVIDENCE THAT THE VIRAL Ki-RAS PROTEIN, BUT NOT THE pp60$^{v\text{-}src}$ PROTEIN OF ASV, STIMULATES PROLIFERATION THROUGH THE PDGF RECEPTOR

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SUMMARY: Protamine sulfate (PS), a specific blocker of PDGF action, inhibited the proliferative response of tsKSV-NRK cells to a reactivated, temperature-sensitive viral Ki-RAS protein, but it did not affect the proliferative response of tsASV-NRK cells to a reactivated pp60$^{v\text{-}src}$ protein kinase. The inhibition by PS of the proliferation response of tsKSV-NRK cells to reactivated Ki-RAS protein was overcome by serum growth factors, notably EGF, and concentrated serum-free conditioned medium from cultured NRK cells infected with wild-type KSV, but not by a combination of PDGF and insulin. These observations suggest that the viral Ki-RAS protein, but not pp60$^{v\text{-}src}$, stimulates proliferation exclusively by inducing the host cells to produce PDGF or PDGF-like mitogenic factors.

The mechanisms by which viral oncogene products initiate and maintain the neoplastic state are unknown. However, evidence is mounting that some of these oncogene products are derivatives of normal growth factors or parts of growth factor receptors that normally generate the signals that drive cells through their cycles (1,2). Thus, the product of the simian sarcoma virus's v-sis oncogene is related to PDGF (platelet-derived growth factor), the product of the avian erythroblastosis virus's v-erb B oncogene is the tyrosine-protein kinase effector domain of the EGF receptor, and the p21 products of the Harvey and Kirsten sarcoma virus' Ha-ras and Ki-ras oncogenes are altered or "activated" versions of the cellular RAS proteins which appear to transduce membrane signals from growth factors that initiate G_1 transit and chromosome replication (3-10).

Altered forms of the cellular RAS proteins have been found in many human tumors (reviewed in 11), and they may have contributed to the failure of the normal growth controls that produced these tumors. Indeed, cells transformed by the Kirsten sarcoma virus (KSV) lose the need for external growth factors

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and can proliferate indefinitely in serum-free medium which does not support the proliferation of uninfected cells (12). Moreover, reactivating a temperature-sensitive viral Ki-RAS protein in quiescent NRK cells is a powerful mitogenic signal that activates the cells and drives them through their cycle without help from exogenous growth factors (13,14). One way by which the viral Ki-RAS protein might do this is to stimulate the cells to make and secrete their own growth factors. But this has yet to be shown.

The viral Ki-RAS protein might stimulate the proliferation of the host cells, at least in part, by inducing the production of PDGF, the principal mitogenic protein in serum for cells of mesenchymal origin. In fact it is thought that most transformed mesenchymal cells produce and secrete PDGF-like factors that activate the cells' own PDGF receptors (15). A way of testing this possibility for KSV-infected cells was suggested by the observation that the binding of PDGF to its cellular receptor is blocked specifically by protamine sulfate (PS) (16,17). PS blocks activities triggered by PDGF-occupied receptors such as chemotaxis (18) and, most significantly, the proliferation of 3T3 mouse cells by the PDGF-like product of the v-sis oncogene (19). By contrast, the binding of another major growth factor, EGF, to its receptor is not prevented by PS (17), and therefore it should not affect EGF-induced proliferation. Thus, if the viral Ki-RAS protein stimulates host cell proliferation through a PDGF-like autocrine factor(s), it should be prevented from doing so by PS. Provided the cells have functional EGF receptors this blockage by PS should be overcome either by EGF or fluids such as serum or conditioned medium that contain EGF or EGF-like factors. In this communication we show that this is indeed the case for NRK cells infected with KSV but not for the same cells transformed by another viral oncogene, the v-src of avian sarcoma virus.

MATERIALS AND METHODS

NRK cells infected with either the wild-type Kirsten sarcoma virus (KSV) or the temperature-sensitive ts371 mutant of KSV were generous gifts of M. Scolnick (Merck, Sharpe and Dhome, West Point PA). NRK cells, infected with the wild-type avian sarcoma virus (ASV) or the temperature-sensitive ts LA23 mutant of ASV were gifts of J. Vogt (University of California School of Medicine, Los Angeles CA). Stock cultures of tsKSV-NRK and tsASV-NRK cells were grown at the permissive 36°C in Dulbecco's modified Eagles medium (DMEM) containing 10% bovine calf serum (BCS). For experiments, cells were replated at a density of 2000/cm^2 in the same medium and incubated at 40°C for 48 hours. They were then rendered quiescent by incubation in DMEM-Ham's F12 (1:1) containing 10 mM HEPES and 0.2% BCS for 48 hours at 41°C. At that time more than 90 percent of the cells were arrested in a quiescent G_0 state.

Cell stimulation. Quiescent, serum-deprived tsKSV-NRK and tsASV-NRK cultures at 41°C were stimulated to start proliferating at "0 hours" as (1) nontransformed cells by adding either serum to 10% or defined growth factors at the nonpermissive 41°C, or as (2) transformed cells by shifting the cultures to the permissive 36°C in order to activate rapidly the p21 K-ras and pp60$^{v\text{-}src}$ viral oncogene products, respectively.

The entry of cells into the S-phase was determined autoradiographically by the procedure of Whitfield et al. (20) in which DNA-synthetic activity was expressed as the proportion of cells whose nuclei were labeled during an exposure to [^{3}H] thymidine (5 μCi/ml, specific activity 20 Ci/mmole: New England Nuclear).

Conditioned medium. wt KSV-NRK cells, cultured to high density in DMEM-10% BCS at 37°C were washed 3 times with DMEM, incubated for 8 hr in DMEM-Ham's F12 (1:1) medium, and then incubated in fresh DMEM:F12 (1:1) for 48 hours at 37°C. This medium was collected, centrifuged to remove cell material, concentrated 10-fold on an Amicon YM10 and stored at -80°C. This material stimulated quiescent NRK cells mitogenically at a 10-fold dilution.

RESULTS

tsKSV-NRK cells were phenotypically untransformed when incubated at 41°C because of the inactivation of the mutant tsKSV's abnormally thermolabile p21 Ki-RAS protein product (21). Consequently, the cells needed exogenous growth factors and could not proliferate in serum-deficient medium at 41°C. Reactivating the viral p21 Ki-RAS protein by lowering the temperature to a permissive 36°C stimulated the quiescent cells to transit the G_1 phase and start replicating DNA, despite the lack of exogenous growth factors (Fig. 1). The viral p21-triggered transit of cells from G_0 into the S phase after the 41°C to 36°C temperature shift was similar to that triggered by adding serum to the quiescent cultures at 41°C (Fig. 1).

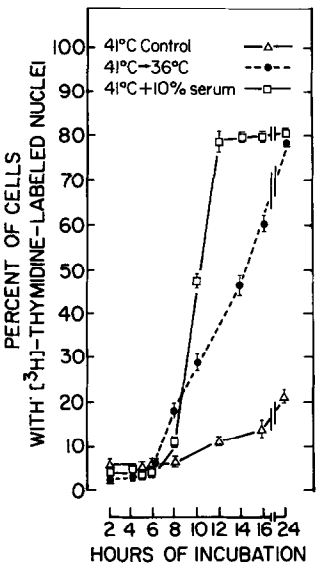
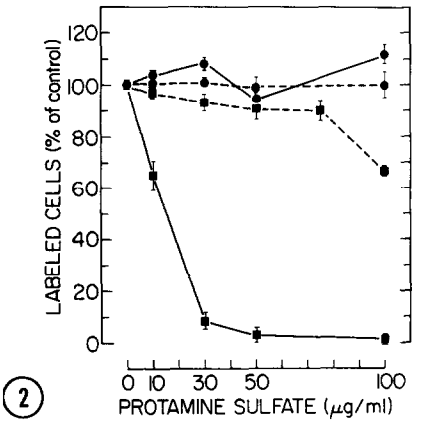


Fig. 1. The ability of reactivated viral Ki-RAS protein to stimulate quiescent serum-deprived tsKSV-NRK to transit G_1 and initiate DNA synthesis after a 41°C to 36°C shift. Cultures that were rendered quiescent by incubation in 0.2% BCS-DMEM for the previous 48 hours at 41°C were left unstimulated at 41°C (△), stimulated to transit G_1 phase by adding serum to 10% at 41°C (□), or shifted to the permissive 36°C to reactivate the Ki-RAS protein without any additions (●). Cultures were labeled continuously with [^{3}H]-thymidine from "0" hours and at the indicated times their DNA-synthetic activities were determined as described in Materials and Methods. Each point is the mean ± SEM of 4 determinations.



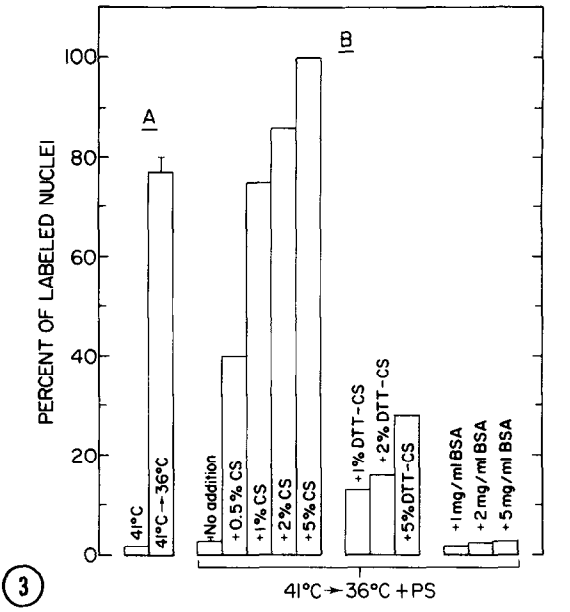


Fig. 2. The inhibition of $p21^{vKi}$-induced, but not $pp60^{v\text{-}src}$-induced, G_1-transit by protamine sulfate (PS). Cultures of tsKSV-NRK (——) and tsASV-NRK (---) cells were rendered quiescent at G_0 by serum deprivation at the nonpermissive 41°C. The cultures were then treated with the indicated concentrations of PS and the cells stimulated to transit G_1 phase by adding serum to 10% at 41°C (●), or by a $p21^{vKi}$- or $pp60^{v\text{-}src}$-activating shift to 36°C without added growth factors (■). The data is presented as the total proporation of cells that entered S-phase during the first 24 hours after stimulation, relative to the proportion of cells which entered S phase during the same period in the absence of PS. The points are the mean ± SEM of 4 separate determinations.

Fig. 3. The inhibition by PS of the proliferation triggered by the viral Ki-RAS protein was overcome by growth factors present in serum. Quiescent tsKSV-NRK cells in serum-deficient medium at 41°C were stimulated to transit G_1 phase by a p21-activating shift to 36°C in the (A) absence or (B) presence of 50 μg/ml PS. To some cultures either untreated bovine calf serum (BCS), calf serum whose growth factor activity had been inactivated by DTT treatment (DTT-CS; as described in text), or BSA was adding to the indicated concentrations at the time of stimulation. The total proportion of cells that entered S-phase during the first 24 hours after stimulation was determined autoradiographically as described in Materials and Methods. The bar heights are the mean ± SEM of 4 determinations.

PS concentrations of 10 μg/ml or more prevented serum-deprived tsKSV-NRK cells from initiating DNA replication when they were added to the culture medium at the time of the p21-activating 41°C to 36°C temperature shift (Fig. 2). By contrast, PS did not stop serum from stimulating the quiescent tsKSV-NRK cells at 41°C (Fig. 2). Indeed, PS concentrations as high as 300 μg/ml did not affect the stimulation by serum. Moreover, the blockage of the viral p21-induced G_1 transit by PS was overcome completely by serum (Fig. 3). Thus, adding serum to a final concentration of at least 2% to PS-treated cultures as late as 6 hours after dropping the temperature from

41°C to 36°C stimulated the cells to transit the G_1 phase and initiate DNA replication.

Serum did not act simply by adsorbing PS. To show this we used BSA and serum which had been treated with DTT and iodoacetamide to inactivate irreversibly its growth factors without affecting its non-specific adsorptive capacity (22). Unlike untreated serum, neither BSA nor DTT/iodoacetamide-treated serum could bypass the inhibitory action of PS (Fig. 3). Thus, it was likely that active growth factors present in serum overcame PS blockage of the mitogenic response to reactivation of the viral Ki-RAS protein.

PS is a highly basic protein (pI 12.3) and, as such, it could have non-specifically damaged the cells. However, another basic protein, histone H1, did not affect the ability of serum or reactivated p21 to stimulate quiescent tsKSV-NRK cells when added to the medium at a concentration as high as 0.1 mg/ml. On the other hand, poly-L-lysine, another highly basic polypeptide, which severely disturbs biomembranes (23), prevented both serum and reactivated p21 from stimulating quiescent tsKSV-NRK cells at 5 µg/ml. Therefore, the differential effect of PS on the abilities of serum and p21 to stimulate tsKSV-NRK cell proliferation was not simply due to it being a polycation.

The tyrosine-phosphorylating $pp60^{v\text{-}src}$ protein kinase product of the avian sarcoma virus's v-src oncogene is also a potent intracellular mitogen for NRK cells that can operate independently from exogenous growth factors (24). Thus, tsASV-NRK cells, like tsKSV-NRK cells, were rendered quiescent by incubation in serum-deficient medium at 41°C and were stimulated to transit the G_1 phase and start replicating DNA when $pp60^{v\text{-}src}$ was reactivated by dropping the temperature to 36°C (data not shown, see ref. 25). But the proliferative response triggered by $pp60^{v\text{-}src}$ reactivation was completely unaffected by the PS concentrations that strongly inhibited the response to p21 reactivation (Fig. 2). Very high PS concentrations (~100 µg/ml) did reduce [^{3}H]-thymidine-incorporation into tsASV-NRK cells stimulated by $pp60^{v\text{-}src}$ (Fig. 2), but this was due to non-specific toxicity. The differential effects of PS on proliferation driven by tsKSV and tsASV was also indicated by the ability of PS to inhibit the proliferation of wild-type KSV-infected cells, but not wild-type ASV-infected NRK cells, actively growing in serum-deficient medium at 36°C (data not shown). Thus, PS does not block the mitogenic response to all viral oncogene products.

NRK cells display both EGF and PDGF receptors on their surfaces, and quiescent tsKSV-NRK cells in serum-deficient medium at 41°C were stimulated to initiate DNA replication by either EGF or PDGF in the presence of insulin. As expected, neither PDGF (50 units/ml) nor a combination of PDGF (50 units/ml) and insulin (10 µg/ml) overcame the inhibitory action of PS on the

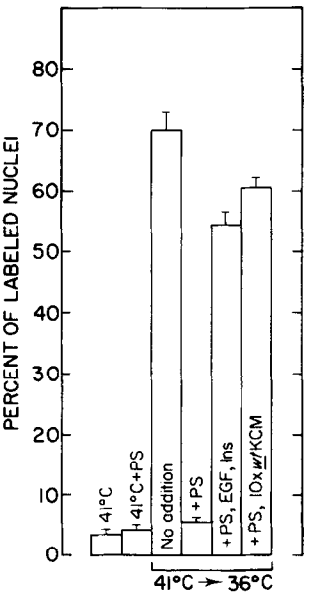


Fig. 4. The ability of EGF to overcome PS inhibition of proliferation induced by the viral Ki-RAS protein. Quiescent tsKSV-NRK cultures in serum-deficient medium at the nonpermissive 41°C were either left unstimulated at 41°C, or were stimulated to start proliferating by a p21- activating shift to 36°C. At the time of stimulation, PS(50 µg/ml) was added to the indicated cultures along with either EGF (20 ng/ml) plus insulin (10 µg/ml), or a 10-fold concentrate of serum-free conditioned medium from wtKSV-NRK cultures (10xwtKCM) prepared as described in Materials and Methods. The effect of these additives on the ability of $p21^{vKi}$ to induce DNA synthesis during the ensuing 24 hours was determined autoradiographically as described in Materials & Methods. The bar heights are means ± SEM of 4 cultures.

mitogenic response of tsKSV-NRK cells to reactivated p21. By contrast, a combination of EGF (20 ng/ml) and insulin (10 µg/ml) did overcome the inhibitory action of PS (Fig. 4). EGF (20 ng/ml) alone was able partially to overcome the inhibitory action of PS, but insulin was by itself totally ineffective (data not shown). Thus, the ameliorative effects of serum on PS inhibition may well be due to EGF or EGF-like factors present in serum. NRK cells transformed by wild-type KSV produce and secrete small amounts of the EGF-like TGF-α (25), which should also overcome the inhibitory action of PS if sufficiently concentrated. Indeed, a 10-fold concentrate of serum-free conditioned medium from wt KSV-NRK cultures also stimulated DNA synthesis in PS-inhibited tsKSV-NRK cells at 36°C (Fig. 4), presumably by supplying them with enough TGFα. Thus, PS-inhibited tsKSV-NRK cells are stimulated by growth factors which activate EGF receptors. These findings suggest that activation of the PDGF receptor, rather than the EGF receptor, triggers the G_0 to G_1 transition in p21-stimulated tsKSV-NRK cells. If this be true, then it is unlikely that TGFα, which acts at the EGF receptor, is the agent

normally responsible for stimulating these cells from the quiescent to the proliferative state.

DISCUSSION

NRK rat cells infected with the temperature-sensitive ts371 mutant of KSV have been used to demonstrate that the p21 product of the viral Ki-ras oncogene is a potent mitogen which relieves the cells' dependence on serum growth factors for proliferation (13). To proliferate, cells normally require a series of growth factors each of which operate at a specific point in the cell cycle. By contrast, the viral Ki-RAS protein is a complete, multifunctional mitogen which drives the host cell through all phases of its cycle without the intervention of external growth factors (12,13). This abnormally multifuntional activity of the viral Ki-RAS protein probably contributes to its oncogenicity.

The results of the present study have suggested a mechanism by which the viral Ki-RAS protein might trigger the first step in the proliferative response, the G_0 to G_1 transition. PDGF is the principal promotor of this transition in normal cells of mesenchymal origin (15). Following PDGF stimulation, the so-called serum "progression" factors complete the process by driving the cells through G_1 into S phase. The ability of the specific PDGF blocker PS to prevent the reactivated viral Ki-RAS protein from stimulating G_1 transit and the initiation of DNA replication in quiescent tsKSV-NRK cells indicates that viral $p21^{Ki}$ induces the host cell to make and secrete PDGF or a PDGF-like mitogenic factor (perhaps the c-sis gene product) which triggers the G_0 to G_1 transition by binding to and activating the PDGF surface receptor. Other, as yet unknown actions of the Ki-RAS protein are probably responsible for the subsequent progression of the stimulated cells through the rest of the cycle. By contrast, the $pp60^{v\text{-}src}$ protein kinase product of the v-src gene of ASV appears to induce the G_0 to G_1 transition by an entirely different route. The relative insensitivity of tsASV-NRK cells to the inhibitory effects of PS indicate that $pp60^{v\text{-}src}$ does not stimulate proliferation of NRK cells exclusively through PDGF or a PDGF-like mitogen.

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