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Role of the Retinoblastoma Protein in Cell Cycle Arrest Mediated by a Novel Cell Surface Proliferation Inhibitor

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Abstract A novel cell regulatory sialoglycopeptide (CeReS-18), purified from the cell surface of bovine cerebral cortex cells has been shown to be a potent and reversible inhibitor of proliferation of a wide array of fibroblasts as well as epithelial-like cells and nontransformed and transformed cells. To investigate the possible mechanisms by which CeReS-18 exerts its inhibitory action, the effect of the inhibitor on the posttranslational regulation of the retinoblastoma susceptibility gene product (RB), a tumor suppressor gene, has been examined. It is shown that CeReS-18 mediated cell cycle arrest of both human diploid fibroblasts (HSBP) and mouse fibroblasts (Swiss 3T3) results in the maintenance of the RB protein in the hypophosphorylated state, consistent with a late G1 arrest site. Although their normal nontransformed counterparts are sensitive to cell cycle arrest mediated by CeReS-18, cell lines lacking a functional RB protein, through either genetic mutation or DNA tumor virus oncoprotein interaction, are less sensitive. The refractory nature of these cells is shown to be independent of specific surface receptors for the inhibitor, and another tumor suppressor gene (p53) does not appear to be involved in the CeReS-18 inhibition of cell proliferation. The requirement for a functional RB protein product, in order for CeReS-18 to mediate cell cycle arrest, is discussed in light of regulatory events associated with density-dependent growth inhibition.

Key words: cell regulatory sialoglycopeptide, retinoblastoma protein product, cell cycling, phosphorylation, signal transduction

The regulation of cell cycling is most likely a result of a fine balance between the interactions of mitogens (growth factors and tumor promoters) and growth inhibitors (negative signals). While a great deal is known about the structure and mechanism of action of numerous growth factors, relatively little is known about the nature and biological activity of proliferation inhibitors, and very few have been purified and extensively characterized. CeReS-18, released from intact bovine cerebral cortex cells by mild proteolytic hydrolysis, has been characterized as a biologically active 18 kDa sialoglycopeptide fragment, obtained from a parental 66 kDa cell surface glycoprotein [Fattaey et al., 1993; Sharifi et al., 1986c]. CeReS-18 has been shown to bind to a specific 150 kDa cell surface receptor [Bascom et al., 1986; Sharifi et al., 1986a; Sharifi and Johnson, 1989] and to reversibly arrest and synchronize a wide variety of exponentially growing cells in late G1 [Fattaey et al., 1989, 1991]. In addition, the inhibitor is a potent antagonist of the mitogenic activity of EGF, PDGF, bombesin, and the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) [Bascom et al., 1987; Chou et al., 1987; Johnson and Sharifi, 1987]. The expression of several cell proliferation-associated genes, however, (i.e., JE, KC, c-myc, c-fos, c-ras, ornithine decarboxylase, and thymidine kinase), in response to serum stimulation, is not reduced by CeReS-18 [Fattaey et al., 1991]. The biological properties of CeReS-18 have been shown to be consistent with those expected for a membrane-associated component that serves as a negative regulator of cell cycling and responsible for events affiliated with density-dependent growth arrest [Fattaey et al., 1989; Johnson et al., 1992].

Studies on the RB product has shown it to be an important cell cycle regulatory element [DeCaprio et al., 1989; Mihara et al., 1989; Alberts et al., 1993; Knudson, 1993]. The nuclear RB protein has been shown to exist in two states: a 110 kDa hypophosphorylated ($RB^{hypophos}$) form, found at G0/G1, that may be important in the

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maintenance of cells at a reversible late G1 cell cycle arrest point; and 112–116 kDa hyperphosphorylated (RB^{phos}) forms that are found in the S, G2, and M phases of the cell cycle [Buchkovich et al., 1989; DeCaprio et al., 1989; Ludlow et al., 1990]. The phosphorylation of $RB^{hypophos}$ appears to be necessary for cell progression through the cell cycle [Mihara et al., 1989; Laiho et al., 1990]. The RB protein has been extensively studied with respect to tumorigenesis of retinoblastoma and osteosarcoma cell lines and the transforming properties of certain DNA tumor viruses (i.e., SV40, adenoviruses, and papillomaviruses) [Knudson, 1971; Whyte et al., 1988; Ludlow et al., 1989; Romanczuk et al., 1991]. The ability of DNA virus transforming antigens to interact with the RB product and the variety of cancerous cells that have been shown to have RB mutations have led to its recognition as an important cell regulatory protein and tumor suppressor. Although the precise mechanism by which the RB product plays a central role in cell cycling is not yet complete, it has recently been demonstrated that the RB product binds several transcription factors and that RB has a cell cycle–specific affinity for the nuclear matrix [Alberts et al., 1993]. It has been proposed that the RB gene product may be a necessary element of c-myc expression in keratinocytes that are growth-inhibited by transforming growth factor-β (TGF-β) [Laiho et al., 1990; Pietenpol et al., 1990a; Murphy et al., 1991]. The exact nature of this complex pathway, however, presently is not clear.

In light of the late G1 cell cycle arrest that is mediated by CeReS-18 and the reversible nature of the inhibition, we were led to explore the possibility that the inhibitor may exert an effect on the phosphorylation state of the RB protein. Preliminary studies have illustrated that serum-deprived and quiescent human diploid fibroblasts did not phosphorylate the RB protein in the presence of CeReS-18 upon serum stimulation, while control cells did phosphorylate the RB protein and resume cell cycling [Johnson et al., 1992].

METHODS

CeReS-18 Purification

The sialoglycopeptide CeReS-18 inhibitor was released from intact bovine cerebral cortex cells by mild proteolysis and purified to apparent homogeneity as previously described [Sharifi et al., 1986c]. Briefly, bovine cerebral cortex cells were treated with dilute protease, and the released molecules precipitated with ethanol; the precipitates were extracted with chloroform/methanol (2:1), and CeReS-18 was purified by DEAE ion-exchange chromatography, lectin affinity chromatography, and HPLC with a TSK-3000 size exclusion column. The samples were then dialyzed against distilled water, lyophilized, and resuspended in phosphate buffered saline (PBS) (145 mM NaCl, 5 mM potassium phosphate, pH 7.2). Protein determinations were carried out by the method of Bradford [1976] using bovine serum albumin as a protein standard, and the purified CeReS-18 preparations were stored at −70°C.

Cell Culture

Cultures were grown as monolayers in a humidified incubator with a 5% CO_2/95% air atmosphere [Fattaey et al., 1989]. Mouse fibroblast Swiss 3T3 cells, from the American Type Culture Collection, and the SV40 transformed 3T3 cell lines (SVT2 and F5B), provided by Dr. S.K. Chapes of the Division of Biology, Kansas State University, were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO/BRL, Grand Island, NY) with 10% calf serum. Human diploid foreskin fibroblasts (HSBP), human osteosarcoma cells (U2OS and SAOS-2), human bladder carcinoma cells (J82), human prostate carcinoma cells (DU145), and adenovirus transformed human epithelial cells (293) were kindly provided by Dr. Ali Fattaey (Massachusetts General Hospital, Charlestown, MA) and grown in DMEM with 10% fetal calf serum. Human fibroblast keratinocytes (HFK), and HFK cells transformed with papillomaviruses (18-NCO and 1321) [Pietenpol et al., 1990b; Romanczuk et al., 1991] were provided by Dr. Richard Schlegel (Georgetown University Medical School, Washington, DC) and grown in KGM media with growth factors (Clonetics, San Diego, CA).

Protein Synthesis Inhibition Assay

Protein synthesis inhibition was tested essentially as described by Sharifi et al. [1986c]. Various concentrations of the purified CeReS-18 were added to 5×10^5 cells in 100 μl of methionine-free minimal Eagle's medium (MEM/HEPES). The cells were preincubated with the inhibitor for 30 min at 37°C to allow CeReS-18 to bind to the cell surface receptor, and then 2.0 μCi of [^{35}S]methionine in 10 μl of methionine-free MEM/HEPES were added, and the cells were

incubated for an additional 15 min. The cell proteins were precipitated with trichloroacetic acid (TCA), the precipitates were resolubilized in alkaline water and reprecipitated by the addition of an equal volume of 20% TCA, and the amount of radioactivity incorporated into acid-insoluble protein was measured in a liquid scintillation system [Sharifi et al., 1986c].

Cell Proliferation Assay

Cells were plated in 48 well culture plates (Costar, Cambridge, MA) and allowed to attach for at least 4 h. Then 9×10^{-8} M CeReS-18, diluted in the appropriate culture medium or medium alone, was added and the cell number determined at various times with a Coulter counter, model ZM [Fattaey et al., 1989; Edson et al., 1991].

RB Protein Immunoprecipitation and SDS-PAGE

Cell cultures, incubated with and without CeReS-18 for 24 h, were radiolabelled for 3.5 h with 300 μCi/ml of [^{35}S]methionine (TRANS^{35}SLABEL; ICN, Irvine, CA) in methionine-free DMEM and immunoprecipitated from cell lysates for 12 h as described by Harlow and Lane [1988], using monoclonal anti-human RB IgG_1 (PMG3-245; Pharminigen, San Diego, CA). Due to the lower reactivity between the mouse RB product and the PMG3-245 antibody, Swiss 3T3 lysates were incubated with the antibody for 24 h. The immunoprecipitates were boiled for 5 min in sample buffer [Laemmli, 1970] and separated on a 7.5% SDS-PAGE at 15 mA for ~3 h (samples were equalized with regard to the amount of radiolabelled protein loaded). After electrophoresis the proteins were electroblotted to a PVDF membrane (Millipore, Bedford, MA), prepared for fluorography (EN^{3}HANCE spray; NEN/DuPont, Wilmington, DE), and exposed to X-ray film for 24 h at −70°C.

CeReS-18 Binding Assay

CeReS-18 was radioiodinated, and the binding studies were carried out as described by Bascom et al. [1986]. Briefly, radioiodination was by the chloramine T method (Sigma Chemical Co., St. Louis, MO) that resulted in a biologically active CeReS-18 with a specific radioactivity of $\sim 1 \times 10^4$ cpm/ng protein. Cultures were grown in 24 well plates, and various concentrations of the ^{125}I-labelled CeReS-18 (in 300 μl of culture medium), with or without a thirtyfold excess of nonradioactive CeReS-18 to measure nonspecific binding, were added to duplicate subconfluent cell cultures ($\sim 1.5 \times 10^5$ cells/well). The cells were incubated with the radiolabelled inhibitor preparations at 37°C for 30 min and then quickly washed three times with PBS. The cells were then lysed by the addition of 300 μl of distilled water containing 100 μl of 1 M NaOH. The samples were collected, the bound radiolabelled CeReS-18 was determined with a gamma counter, and the number of receptors per cell and their Kd were determined as described by Bascom et al. [1986].

RESULTS

Exponentially growing human diploid fibroblasts (HSBP) and Swiss 3T3 cells were used to study the potential effect of CeReS-18 mediated cell cycle arrest on the phosphorylation states of the RB gene product. The cultures were incubated with or without CeReS-18 for 24 h and radiolabelled for 3.5 h with [^{35}S]methionine, and the RB protein was immunoprecipitated with the monoclonal anti-human RB IgG as described in Methods. Both exponentially growing cell cultures exhibited newly synthesized RB protein in both the hypo- and hyperphosphorylated states (Fig. 1, lanes 1, 3), while cells arrested by the CeReS-18 inhibitor contained only RB protein in the hypophosphorylated state (Fig. 1, lanes 2, 4). These observations were consistent with the proposed G1 regulatory state of the RB protein [DeCaprio et al., 1989; Mihara et al., 1989] and the site of cell cycle arrest mediated by the CeReS-18 inhibitor [Fattaey et al., 1989; Edson et al., 1991].

To further examine the potential role of post-translational modification of the RB product in the biological inhibitory action of CeReS-18, both HSBP and Swiss 3T3 cells were plated and allowed to grow to confluence. When the cultures reached confluence they were incubated for an additional 24 h to ensure density-dependent arrest of the majority of the cells. A second set of cultures were plated at the same time at approximately one-third the density, and at the time of immunoprecipitation these cultures remained subconfluent. The results clearly showed that both HSBP and 3T3 density-dependent growth arrested cultures solely displayed the $RB^{hypophos}$ protein (Fig. 2, lanes, 2,4). Exponentially dividing HSBP and 3T3 cells, however, again expressed the expected hyper- and hypophosphorylated forms of the RB product (Fig. 2, lanes 1,3).

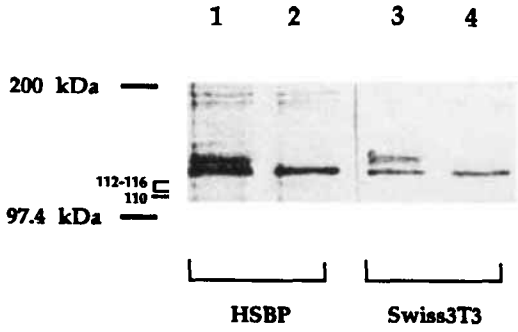

Fig. 1. RB protein immunoprecipitation of SGP arrested HSBP and Swiss 3T3 fibroblasts. Sparse HSBP and Swiss 3T3 cultures were treated for 24 h with either 9×10^{-8} M CeReS-18 in DMEM with 10% newborn calf serum or DMEM alone as a control. The cells were then radiolabelled for 3.5 h with [^{35}S]methionine, immunoprecipitated with monoclonal mouse anti-human RB IgG_1, and the proteins separated by SDS-PAGE as described in Methods. **Lane 1:** Control logarithmically growing HSBP cells. **Lane 2:** CeReS-18 arrested HSBP cells. **Lane 3:** Control logarithmically growing Swiss 3T3 cells. **Lane 4:** CeReS-18 arrested Swiss 3T3 cells.

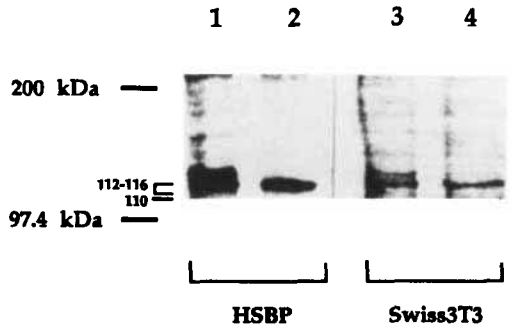

Fig. 2. RB protein immunoprecipitation of density-dependent quiescent HSBP and Swiss 3T3 fibroblasts. HSBP and Swiss 3T3 cells were plated and allowed to grow to confluence as described in Methods. After reaching confluency the cultures were incubated an additional 24 h, and the cells were then radiolabelled and immunoprecipitated as described. Another set of HSBP and Swiss 3T3 cultures were plated on the same day at approximately one-third the density. These cells were treated in the same fashion as the first set but at the time of immunoprecipitation were subconfluent. **Lane 1:** Subconfluent HSBP cultures. **Lane 2:** Confluent HSBP cultures. **Lane 3:** Subconfluent Swiss 3T3 cultures. **Lane 4:** Confluent Swiss 3T3 cultures.

The results from these experiments indicated that cell cycle arrest, mediated by CeReS-18, was consistent with a block at or near the G1 arrest site since the state of phosphorylation of the RB protein, under CeReS-18 mediated cell cycle arrest and density-dependent arrest, was indistinguishable.

These observations, however, did not necessarily establish a direct relationship between the RB protein and signal transduction events associated with the CeReS-18 inhibitor. If the maintenance of the RB protein in the $RB^{hypophos}$ state is necessary for CeReS-18 mediated inhibition, it would be expected that cell lines either lacking a functional RB product or having the RB protein sequestered by a viral oncoprotein would lead to an insensitivity to the cell cycle regulatory activity of the sialoglycopeptide.

To explore this possible relationship we compared the sensitivity of two human osteosarcoma cell lines, U2OS (RB^+) and SAOS-2 (RB^-) [Shew et al., 1990], to CeReS-18. When 9×10^{-8} M CeReS-18 was added to the culture medium, the U2OS cells were efficiently inhibited within 20 h, while the SAOS-2 cells were refractory to inhibition throughout the incubation period (Fig. 3). Consistent with the possibility that a functional RB product was necessary for CeReS-18 cell cycle arrest, the human bladder carcinoma cell line J82 (RB^-) [Horowitz et al., 1989] and prostate carcinoma cell line DU145 (RB^-) [Bookstein et al., 1990] also were resistant to the inhibitory action of CeReS-18 (Fig. 4). Higher concentrations (1.3×10^{-7} M) of the inhibitor were also ineffective in blocking cell cycling in RB^- cell lines (data not shown).

Since cells that express a normal RB product can phenotypically act like RB^- cell lines when

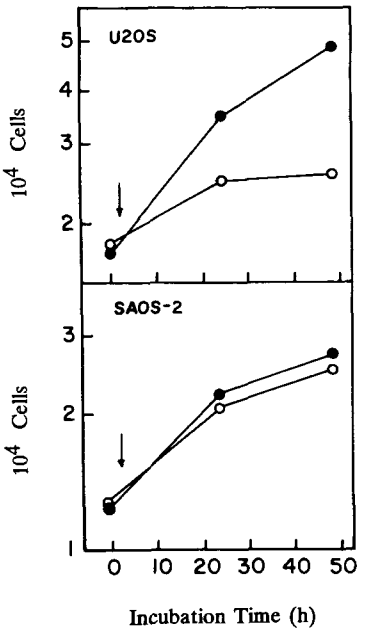

Fig. 3. CeReS-18 cell proliferation inhibition assays carried out on the human osteosarcoma U2OS (RB^+) and SAOS-2 (RB^-) cell lines. Osteosarcoma cells grown in DMEM and 10% fetal calf serum, and either 9×10^{-8} M CeReS-18 (○) or an equal volume of PBS (●) was added at the time indicated by the arrows. Data are plotted as the average of duplicate wells.

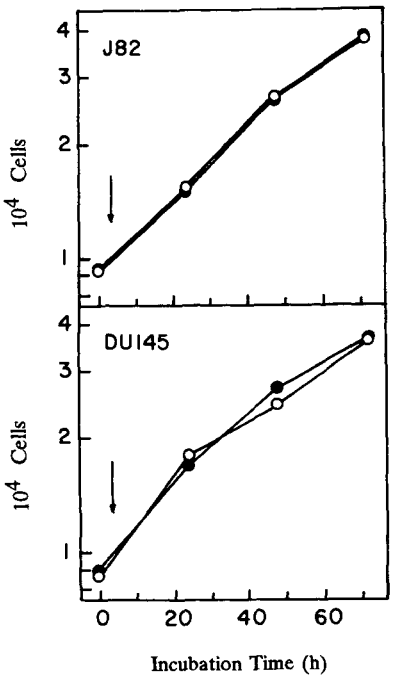

Fig. 4. CeReS-18 cell proliferation inhibition assays carried out on the human bladder carcinoma J82 (RB^-) and human prostate carcinoma DU145 (RB^-) cell lines. Carcinoma cells grown in DMEM and 10% fetal calf serum, and either 9×10^{-8} M CeReS-18 (○) or an equal volume of PBS (●) was added at the time indicated by the arrows. Data are plotted as the average of duplicate wells.

transformed with certain DNA tumor antigens, we explored the possibility that these cell lines might also be resistant to the inhibitory influence of CeReS-18. While normal human keratinocytes were readily arrested by CeReS-18, growth was not inhibited with the 1321 and NCO papillomavirus E6/E7 protein transformed cell lines (Fig. 5). The adenovirus E1A protein transformed human epithelial cell line 293 also was resistant to the cell cycle arrest mediated by the CeReS-18 inhibitor (Fig. 6).

Swiss 3T3 cells were found to be sensitive to the inhibitory action of CeReS-18, while the proliferation of both the SV40 large T antigen transformed cell lines SVT2 and F5B were not inhibited by the sialoglycopeptide (Fig. 7). These results were consistent with the observations of papillomavirus and adenovirus transformed human cell lines. Clearly, the transformation of both human and mouse cells by the transforming antigens of several DNA oncogenic viruses, that are known to sequester the nuclear RB product, resulted in a refractory phenotype with regard to CeReS-18 action. This observation alone, however, is insufficient to establish a role for the RB protein in CeReS-18 action since

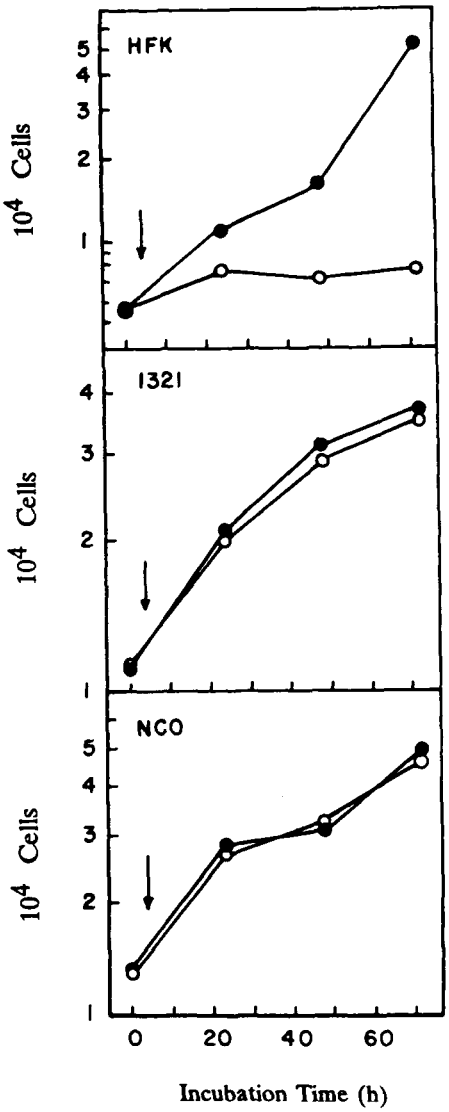

Fig. 5. CeReS-18 cell proliferation inhibition assays carried out on the human keratinocyte HFK (normal) and human papillomavirus transformed (1321 and NCO) cell lines. Cells were grown in KGM medium with appropriate growth factors (Clonetics, San Diego, CA), and either 9×10^{-8} M CeReS-18 (○) or an equal volume of PBS (●) was added at the time indicated by the arrows. Data are plotted as the average of duplicate wells.

several cellular nuclear proteins, including the p53 protein, can be tightly associated to viral gene products [Whyte et al., 1988].

Since it has been shown that cells resistant to the inhibitory action of TGF-β can be a reflection of a decrease in the surface receptor population for the ligand [Kimchi et al., 1988], the number of receptors and the K_d of the inhibitor-receptor interaction was measured with the human SAOS-2 and mouse SVT2 cell lines and compared to the sensitive human U2OS cells.

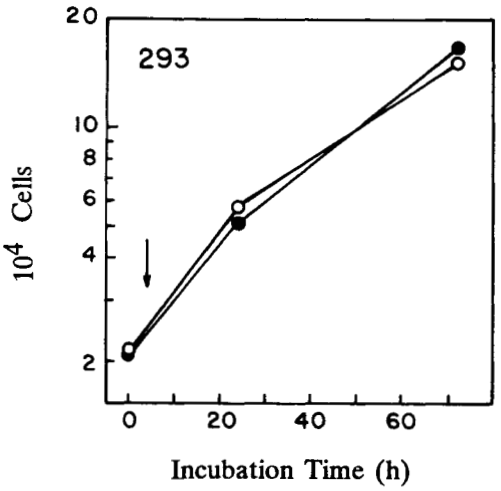

Fig. 6. CeReS-18 cell proliferation inhibition assays carried out on the adenovirus transformed human kidney epithelial cell line 293. Cells were grown in DMEM and 10% fetal calf serum, and either 9×10^{-8} M CeReS-18 (○) or an equal volume of PBS (●) was added at the time indicated by the arrows. Data are plotted as the average of duplicate wells.

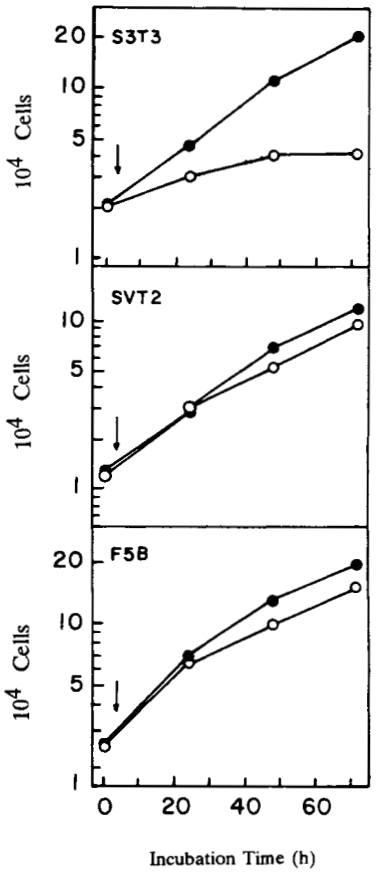

Fig. 7. CeReS-18 cell proliferation inhibition assays carried out on the murine fibroblast Swiss 3T3 (normal) and SV40 transformed (SVT-2 and F5B) cell lines. Fibroblasts were grown in DMEM and 10% calf serum, and either 9×10^{-8} M CeReS-18 (○) or an equal volume of PBS (●) were added at the time indicated by the arrows. Data are plotted as the average of duplicate wells.

The number of CeReS-18 receptors per cell and their K_d were remarkably similar (Table I). The reason for the refractory nature of RB^- and viral transformed cell lines to CeReS-18 cell cycle arrest clearly could not be attributed to a change in cell surface receptors. Consistent with this observation, all cell lines were sensitive to the transient CeReS-18 inhibition of protein synthesis, which requires occupancy of the CeReS-18 receptor to inhibit translational events [Bascom et al., 1986], whether or not the more enduring cell cycle arrest was affected (data not shown).

The cell lines used in this study also provided an examination of the potential role of a second tumor suppressor gene product, p53, with regard to the inhibitory action of CeReS-18. The human carcinoma cell lines J82 and DU145 are $p53^+$ while being resistant to the cell proliferation inhibitory action of CeReS-18 (Table II). HL-60 cells, however, are RB^+ and $p53^-$ [Wolf and Rotter, 1985] and are sensitive target cells to the inhibitor [Edson et al., 1991]. These observations delineate that the RB protein, and not p53 product, appears to play a central role in the ability of CeReS-18 to mediate arrest in the G1 phase of the cell cycle.

DISCUSSION

Previous experiments have shown that serum stimulation of RB phosphorylation associated with cell cycling of confluent and quiescent human fibroblast cultures readily can be abrogated by the presence of CeReS-18 [Johnson et al., 1992]. These data suggested that the site of CeReS-18 mediated cell cycle arrest was at the time of RB phosphorylation or somewhat upstream of this posttranslational modification. The present experiments confirm and extend these observations in that cell cycle arrest of exponentially dividing human and mouse fibroblasts by the sialoglycopeptide inhibitor results in cells primarily having the tumor suppressor protein in the $RB^{hypophos}$ state (Fig. 1).

It is interesting that CeReS-18, derived from a parental cell surface component of bovine cerebral cortex cells, has such an unusually broad target cell range. Its ability to mediate cell cycle arrest of cells obtained from mouse, human, rat, avian, and insect species [Fattaey et al., 1989], all of which necessarily have specific cell surface receptors for the inhibitor, suggests that

TABLE I. CeReS-18 Receptors on Sensitive and Insensitive Cell Lines*

Cell line	Cell type	Receptors per cell	K_d (nM)	CeReS-18 mediated arrest
SVT2	Mouse fibroblast	2.6×10^4	8.5	Insensitive
U2OS	Human osteosarcoma	2.9×10^4	9.7	Sensitive
SAOS-2	Human osteosarcoma	2.3×10^4	6.1	Insensitive

*Specific cell receptors for CeReS-18, and their K_d, were determined with ^{125}I-radiolabelled inhibitor as described in Methods.

TABLE II. CeReS-18 Inhibition of Cell Proliferation

Cell line	Cell type	Functional tumor suppressor product (RB)	Functional tumor suppressor product (p53)	CeReS-18 mediated inhibition[a]
Swiss 3T3	Mouse fibroblast	+	+	+
HSBP	Human fibroblast	+	+	+
HFK	Human keratinocyte	+	+	+
U2OS	Human osteosarcoma	+	+	+
SAOS-2	Human osteosarcoma	−	−	−
DU145	Human prostate carcinoma	−	+	−
J82	Human bladder carcinoma	−	+	−
1321	Human keratinocyte	v[b]	v	−
NCO	Human keratinocyte	v	v	−
293	Human kidney epithelial	v	v	−
F5B	Mouse fibroblast	v	v	−
SVT2	Mouse fibroblast	v	v	−

[a]The cell lines were examined for sensitivity of cell proliferation with 9×10^{-8} M of the CeReS-18 inhibitor.
[b]v denotes the presence of viral oncoproteins capable of sequestering RB and p53.

CeReS-18 may represent a class of evolutionarily conserved cell cycle regulators. In addition, many tumorigenic cell lines, derived by mutation or retroviruses, have been shown to be highly sensitive to the proliferation inhibitor [Fattaey et al., 1989]. For the most part, reversal experiments also have shown that this broad array of cells primarily is arrested in the G1 phase of the cell cycle [Fattaey et al., 1989; Edson et al., 1991]. The present studies with mouse and human cells confirm a G1 phase block by the presence of solely the underphosphorylated form of the RB in the inhibited cells. Although the exact site of arrest is not completely mapped, the kinetics of reversal of DNA synthesis, cell doubling, and the state of the RB protein are all consistent with the restriction (R) point, near the G1/S interphase, described by Pardee [1989]. The one exception at the present time to this generality is the CeReS-18 mediated arrest of HL-60 cells. Unlike most other cell types that appear to be synchronously released from cell cycle arrest when the inhibitor is removed, HL-60 cells are irreversibly arrested by the sialoglycopeptide, and even after CeReS-18 is removed the cells progress through differentiation [Edson et al., 1991]. This is of particular relevance to the present study since the HL-60 cells are $p53^-$ and RB^+ [Wolf and Rotter, 1985]. The human osteosarcoma SAOS-2 cell line is $p53^-$ and RB^- [Kuerbitz et al., 1992], but it appears that the RB protein is most likely a relevant gene product with regard to CeReS-18 inhibition of cell cycling. The central role of the RB product in CeReS-18 action was confirmed by the insensitivity of the human bladder J82 (RB^- and $p53^+$) and human prostate DU145 (RB^- and $p53^+$) carcinoma cell lines to the inhibitor (Fig. 4).

The insensitivity of RB^- mutants and DNA tumor virus transformed cell lines was not associated with either a reduced level of receptors or the measured binding affinities of CeReS-18 to U2OS, SAOS-2, and SVT2 cells (Table I). In fact, the number of CeReS-18 receptors per cell was quite comparable whether or not the cells were growth arrested by the sialoglycopeptide inhibitor and consistent with earlier measurements of 2×10^4 receptors per Swiss 3T3 cell that serve as the standard cell line for many of the CeReS-18 studies [Bascom et al., 1986].

How the RB protein product is involved in the signal transduction pathway elicited by CeReS-18 presently is unknown. It is clear, however, that the RB product is more than a casual player in the series of metabolic events that mediate cell cycle arrest by the inhibitor. On one hand, it may not be surprising that CeReS-18 arrests cells at a site where the $RB^{hypophos}$ state is the dominant form of the tumor suppressor

protein. It was not intuitively obvious, however, that RB^- cell lines would be refractory to cell cycle inhibition by the sialoglycopeptide. Either its absence as a functional protein by mutation or its being sequestered by transforming antigens of certain DNA oncoviruses led to an insensitivity of cell cycle arrest by the sialoglycopeptide inhibitor (Table II). Consistent with the information that the RB protein regulates progression through the cell cycle [Goodrich et al., 1991], there appears to be an absolute requirement for a functional RB protein in order for the cell surface inhibitor to mediate cell cycle arrest.

CeReS-18 is one of the few naturally occurring potential growth regulators that has been shown to abrogate the phosphorylation of the RB protein [Laiho et al., 1990; Chao et al., 1992; Resnitzky et al., 1992]. As a cell surface component [Lakshmanarao et al., 1991; Fattaey et al., 1993] that influences cell cycling of a wide variety of cell types, the similarity at a molecular level between the CeReS-18 arrested cells and those that naturally reach confluency and quiescence, and the reversibility of its inhibitory action, CeReS-18 likely represents a wider class of cell growth regulators that play a fundamental role in density-dependent growth inhibition. In this regard, the inhibitor could be a valuable agent for studies of cell cycling and provide a controlled and synchronous population of cells in their progression through the cell cycle, as well as delineate the genetic and molecular events associated with the posttranslational modifications of the RB product that regulate cell proliferation.

Previous studies have shown that the mobilization of Ca^{2+}, either by influx or release from intracellular stores, also plays a fundamental role in CeReS-18 mediated inhibition. Early signal transduction events induced by CeReS-18 appear to be involved with intracellular Ca^{2+} activity since its inhibitory activity could be antagonized by the calcium ionophore A23187 but not the sodium ionophore Monensin [Sharifi et al., 1986b], and the inhibitor has been shown to block Ca^{2+} mobilization and cytosol alkalinization induced by the mitogenic action of TPA [Toole-Simms et al., 1991]. These events, however, occur as very early signal transduction events. The time between the addition of CeReS-18 and the ionophore or tumor promoter has been shown to be crucial, and the interval between their introduction to the target cells is a very narrow window (i.e., within minutes) [Sharifi et al., 1986b; Toole-Simms et al., 1991]. This observation is consistent with the report of Wahl and Gruenstein [1993] that a transient and abrupt increase in intracellular Ca^{2+}, occurring within 2–3 min after serum stimulation of quiescent human fibroblasts, is necessary but not alone sufficient for cellular progression into S phase. Furthermore, a recent study by Takuwa et al. [1993] has shown that Ca^{2+}/calmodulin regulates the signaling cascade that leads to $p34^{cdc2}$ kinase activation, subsequent RB protein phosphorylation, and the initiation of DNA synthesis. These investigators also have shown that transformation by SV40 led to the abrogation of this Ca^{2+}-dependent regulation of the $p34^{cdc2}$ kinases. It is, therefore, conceivable that the early signal events that are intercepted by the sialoglycopeptide inhibitor involve the initial mobilization of intracellular Ca^{2+}, followed by the subsequent downstream posttranslational modification of an active RB protein that is associated with cell progression.

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