Features of replicative senescence induced by direct addition of antennapedia-p16^{INK4A} fusion protein to human diploid fibroblasts

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Received 23 March 1998

Abstract The p16^{INK4A} cyclin-dependent kinase (Cdk) inhibitor is now recognized as a major tumor suppressor that is inactivated by a variety of mechanisms in a wide range of human cancers. It is also implicated in the mechanisms underlying replicative senescence since p16^{INK4A} RNA and protein accumulate as cells approach their proscribed limit of population doublings in tissue culture. To obtain further evidence of its role in senescence, we have sought ways of overexpressing p16^{INK4A} in primary human diploid fibroblasts (HDF). To circumvent the low transfection efficiency of primary cells we have exploited a recombinant form of the full-length p16^{INK4A} protein fused to a 16 amino acid peptide from the *Drosophila* antennapedia protein. This peptide has the capacity to cross both cytoplasmic and nuclear membranes allowing the direct introduction of the active protein to primary cells. Here, we show that antennapedia-tagged wild-type p16^{INK4A} protein, but not a functionally compromised tumor-specific variant, causes G1 arrest in early passage HDFs by inhibiting the phosphorylation of the retinoblastoma protein. Significantly, the arrested cells display several phenotypic features that are considered characteristic of senescent cells. These data support a role for p16^{INK4A} in replicative senescence and raise the possibility of using the antennapedia-tagged protein therapeutically.

Key words: p16^{INK4A}; Antennapedia; Cellular senescence; Cell cycle

1. Introduction

p16^{INK4A} (also known as the tumor suppressor gene, MTS1) is a G1 specific cell cycle inhibitor which negatively regulates the cyclin-dependent kinases (Cdk) Cdk4 and Cdk6 by binding in competition with D-type cyclins [1–3]. Cdk4 and Cdk6 kinases are important for the phosphorylation and inactivation of the retinoblastoma susceptibility gene product, pRB [4,5]. Therefore, p16^{INK4A} plays a critical role in blocking the G1/S transition by preventing the inactivation of pRB [6,7]. There are at present two different classes of Cdk inhibitors (CdkI): the KIP/CIP family (p21, p27 and p57) and the INK4 family (p15^{INK4B}, p16^{INK4A}, p18^{INK4C} and p19^{INK4D}) [8]. In contrast to the KIP/CIP family, which inhibits a broad range of Cdks, the INK4 family is specific for Cdk4 and Cdk6. The INK4 proteins, which contains four or more ankyrin repeats, are expressed in distinct tissue-specific patterns, suggesting that, although they have essentially indistinguishable biochemical properties [9], they are not strictly redundant [10]. Among these CdkI genes, p16^{INK4A} is the only gene which is frequently mutated and/or deleted in human cancer cell lines and, to lesser extent, in primary tumors [2,3,11].

Cellular senescence is considered to be genetically programmed and induced by the expression of dominant-acting growth suppressor(s) [12,13]. There are also evidence that tumorigenesis entails, at least in part, mechanisms that permit cells to escape senescence [13]. Therefore, cellular senescence could be a mechanism for tumor suppression. Several lines of evidence suggest that two well-recognized tumor suppressors, the p53 and Rb genes, appear to be critical for the senescence of human diploid fibroblasts (HDF) [14,15]. In the case of Rb, the growth suppressive (unphosphorylated) form of the protein accumulates in senescent cells, due to the loss of phosphorylation by Cdks [16]. The observation that p16^{INK4A} levels rise as cells senesce suggest that p16^{INK4A} may be an inducer of cellular senescence, whose expression may record the number of cell divisions completed and subsequently promote cell cycle exit [17,18]. Moreover, mice carrying a targeted deletion of the p16^{INK4A} locus develop spontaneous tumors at an early age and mouse primary embryo fibroblasts (MEF) grow for multiple passages apparently without undergoing senescence or passing through a characteristic crisis [19]. These data suggest the possibility that p16^{INK4A} acts as a tumor suppressor by the induction of cellular senescence. This may explain why the p16^{INK4A} gene, but not other cdkI genes, is frequently mutated in immortalized cancer cell lines. However, a recent report by Kamijo and coworkers argues for this possibility [20]. They report that much of the phenotype ascribed to p16^{INK4A}-null mice may in fact be attributed to disruption of p19^{ARF}, an alternative spliced transcript whose product is completely distinct from p16^{INK4A} in its sequence and functional properties [20].

To resolve this controversy, we have introduced a large amount of p16^{INK4A} protein into early passage HDF and determined whether cells acquire a senescent phenotype. Because of the low transfection efficiency typically achieved in primary cells, we employed a technique to deliver the p16^{INK4A} protein into the cells. A 16 amino acid peptide derived from the third antennapedia homeodomain (ant tag) has been shown to allow the intracellular delivery of antisense oligonucleotides or biologically active peptides to the nucleus [21]. Interestingly, this ant-tagged peptide is efficiently translocated through the plasma membrane and into the nucleus in the absence of

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Abbreviations: Cdk, cyclin-dependent kinase; HDF, human diploid fibroblasts; Rb, retinoblastoma susceptibility gene; MEF, mouse embryo fibroblasts; β-gal, β-galactosidase; HTLV-1, human T-cell leukemia virus type 1; GST, glutathione *S*-transferase

PII S0014-5793(98)00426-8

exogenously provided energy [21]. This kind of carrier peptide has recently become a popular tool for introducing synthetic peptides into cells [22–24]. However, synthetic peptides retain only a part of the function of an intact protein. In order to retain the full range of $p16^{INK4A}$ biological activities, we engineered an intact $p16^{INK4A}$ protein with an ant tag at its N-terminus. The modified $p16^{INK4A}$ gene was cloned into a bacterial expression vector. A histidine tag (his tag) was added to the N-terminus of the ant-tagged $p16^{INK4A}$ protein, which allows the one step purification of the bacterially expressed recombinant protein [25].

In the present study, we introduced the bacterially expressed ant-tagged full-sized $p16^{INK4A}$ protein into early passage HDF by simple addition of the recombinant protein to the tissue culture medium. The ant-tagged $p16^{INK4A}$ protein efficiently inhibited cell growth. This growth inhibition was accompanied by a change in phenotype resembling that of senescent cells. These data support a role for $p16^{INK4A}$ in cellular senescence, and raise the possibility that recombinant penetrative full-sized proteins prepared in bacteria offer a novel therapeutic approach towards tumor suppression.

2. Materials and methods

2.1. Plasmid construction and preparation of recombinant protein

The DNA fragment encoding the 16 amino acids (aa 43–58) of the antennapedia homeodomain (ant tag) was cloned into the multi-cloning site of the his tag expression plasmid, pRSETa (Invitrogen), then the DNA fragment encoding wild-type or R87P mutant of the $p16^{INK4A}$ coding region was subcloned under the ant tag. The recombinant proteins were expressed in 4 l of *Escherichia coli* BL21 (DE3) pLysE culture and recovered from the soluble fraction of bacterial lysate using non-denaturing condition for his-tagged protein purification [25]. The purified proteins were dialyzed to remove imidazole and concentrated by the Selective absorbent (ATTO #AB-1100 Japan).

2.2. In vitro binding assay

[^{35}S]Methionine-labeled Cdk2 and Cdk4 were synthesized by coupled transcription and translation of plasmid DNAs using TNT expression system (Promega). Samples (5 μl) of reaction products were mixed with 50 ng of bacterially expressed his-tagged $p16^{INK4A}$ proteins and incubated for 30 min at 30°C. After the incubation, the mixtures were analyzed as described previously [26].

2.3. Cell culture and [^{3}H]thymidine labeling

Normal human diploid fibroblasts, TIG-3 (obtained from the Japanese Cancer Research Resources Bank, Tokyo, Japan) were cultured as described previously [17]. Sparse cells ($1–5\times10^3$ per cm^2) were given 10 μCi of [^{3}H]thymidine (Amersham TRK686) per ml for 2 h, washed in phosphate-buffered saline (PBS), rinsed twice in methanol, and processed for autoradiography, as described [27].

2.4. β-Galactosidase (β-gal) staining

Cells were washed in PBS, fixed for 3–5 min in 2% formaldehyde/0.2% glutaraldehyde, washed, and incubated at 37°C (no CO_2) with freshly prepared senescence-associated β-Gal (SA-β-Gal) staining solution [27]: 1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) per ml/40 mM citric acid/40 mM sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM $MgCl_2$. Staining was evident in 2–6 h and maximal in 16 h.

3. Results

3.1. Construction of antennapedia-tagged $p16^{INK4A}$ proteins

Published results have established that the chemical conjugation of an antennapedia homeodomain-derived peptide extending from amino acids 43–58 facilitated the cellular internalization of synthetic peptides [21], such as 20 amino acids derived from $p16^{INK4A}$ [22], p53 [23] and DP-1 [24]. However, 20 amino acid residues rarely if ever exhibit all the activities of an intact protein. This is especially true in the case of proteins that have multi-functional domains, or whose functional domain is unknown. Therefore, it would be of interest to develop a system to produce penetrative full-sized proteins. For this purpose, we constructed a recombinant full-size $p16^{INK4A}$ protein with a his tag and ant tag at its N-terminus (H-ant-p16wt). The recombinant protein was expressed and purified from bacteria using non-denaturing conditions and Ni-affinity chromatography, as described by Hoffmann and Roeder [25]. To more critically examine the internalization and biological activities of the ant-tagged $p16^{INK4A}$ protein, we also constructed two other recombinant his-tagged $p16^{INK4A}$ proteins: one lacks the ant tag (H-p16wt), and the other (H-ant-p16mut) has a loss-of-function mutation (R87P) in the $p16^{INK4A}$ coding region associated with familial melanoma [26]. Because the $p16^{INK4A}$ protein tends to aggregate, we used only freshly prepared proteins for all experiments. The quality of the freshly prepared $p16^{INK4A}$ proteins (H-p16wt, H-ant-p16wt and H-ant-p16mut) was confirmed by SDS-PAGE stained by Coomassie brilliant blue, and also by Western blotting using an anti-his-tag antibody (Fig. 1A) and anti-$p16^{INK4A}$ antibody (data not shown). To confirm the activity of these recombinant proteins, an in vitro binding assay was performed using in vitro translated Cdk2 and Cdk4 proteins. Equal amounts of $p16^{INK4A}$ proteins were mixed with ^{35}S-labeled Cdk2 or Cdk4 proteins, immunoprecipitated with the anti-his-tag antibody, and analyzed by SDS-PAGE. Although the amount of input Cdk2 protein is slightly less than that of Cdk4 (Fig. 1B), both wild-type $p16^{INK4A}$ proteins (H-p16wt and H-ant-p16wt) specifically bound to Cdk4 and

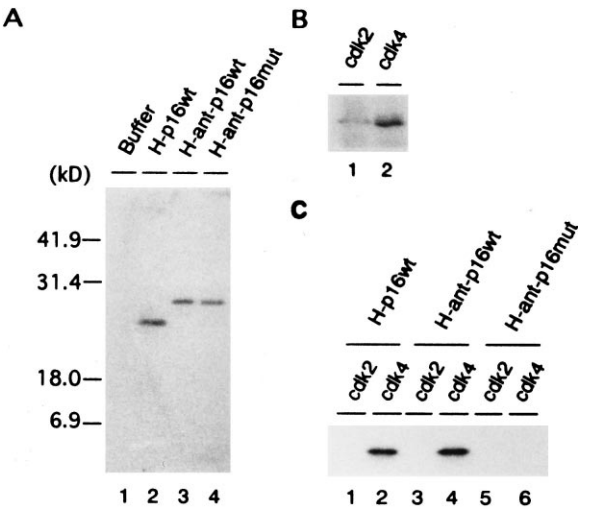

Fig. 1. The activity of recombinant $p16^{INK4A}$ protein. Histidine-tagged $p16^{INK4A}$ proteins prepared from bacteria were fractionated by SDS-PAGE in a 12% gel and immunoblotted with a monoclonal antibody against the his tag. Immune complexes were detected with enhanced chemiluminescence (ECL) (Amersham) (A). Human Cdk2 and Cdk4 were radiolabeled by in vitro translation (B) and mixed with 50 ng of bacterially expressed his-tagged $p16^{INK4A}$ proteins, H-p16wt (C, lanes 1, 2), H-ant-p16wt (C, lanes 3, 4), H-ant-p16mut (lanes 5, 6). The samples were analyzed either directly (B) or after immunoprecipitation with anti-$p16^{INK4A}$ antibody (C). The proteins were then fractionated by SDS-PAGE in a 12% gel and the labeled Cdks were detected by autoradiography.

did not bind to Cdk2 (Fig. 1C, lanes 1–4). The R87P mutant (H-ant-p16mut) did not bind either Cdk2 or Cdk4, as expected (Fig. 1C, lanes 5, 6) and previously reported [26]. These data demonstrated that bacterially expressed wild-type $p16^{INK4A}$ proteins have the expected binding activity in vitro.

3.2. Internalization of recombinant proteins

To confirm intracellular penetration, three his-tagged $p16^{INK4A}$ proteins were added to the tissue culture medium of early passage (35PDL) human diploid fibroblasts, TIG-3 (20 μM final concentration). The presence of roughly equal amounts of his-tagged proteins in the tissue culture medium was confirmed by Western blotting (Fig. 2A). Twelve hours after addition, the cells were harvested, nuclear extracts were prepared to avoid the contamination of the $p16^{INK4A}$ protein stuck to the surface of the cell membrane and subjected to Western blotting using anti-his-tag antibody (Fig. 2B). Both ant-tagged proteins (H-ant-p16wt and H-ant-p16mut) were detected in the nuclear extracts (Fig. 2B, lanes 3, 4). However, the his-tagged p16 protein lacking the ant tag (H-p16wt) was not observed in the nuclear extracts (Fig. 2B, lane 2). We observed the same results using the anti-$p16^{INK4A}$ antibody to detect the proteins in the nuclear extracts (data not shown). These data demonstrated that the ant tag efficiently delivered

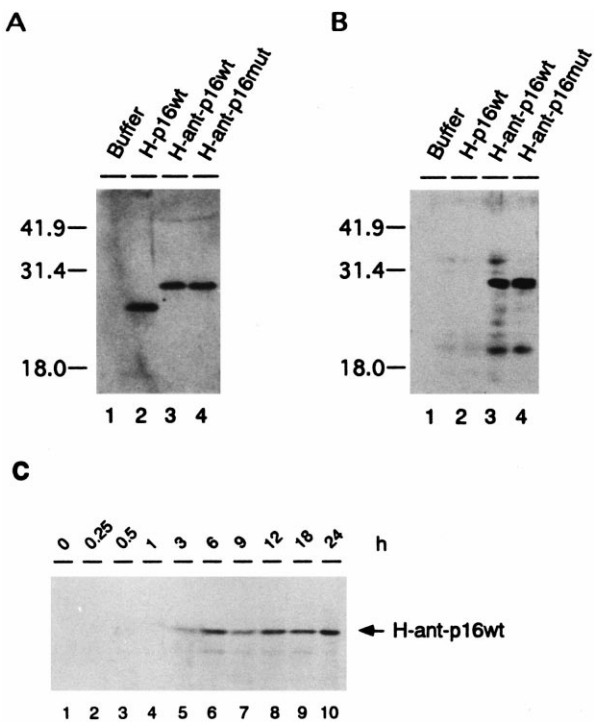

Fig. 2. Internalization of recombinant $p16^{INK4A}$ proteins. Recombinant $p16^{INK4A}$ proteins (20 μM concentration) were added to the tissue culture medium of early passage (35PDL) TIG-3 cells (lane 1: mock, buffer alone; lane 2: H-p16wt; lane 3: H-ant-p16wt; lane 4: H-antp16mut). 5 μl of each medium was subjected to SDS-PAGE in a 12% gel and immunoblotted with monoclonal antibody against the his tag. Immune complexes were detected with ECL (A). Nuclear extracts prepared from the treated cells were fractionated by SDS-PAGE in a 12% gel and immunoblotted with monoclonal antibody against the his tag. Immune complexes were detected with ECL (B). Nuclear extracts were prepared from the cells treated with H-ant-p16wt for 0, 15, and 30 min, 1, 3, 6, 9, 12, 18 and 24 h. Each sample was subjected to Western blotting using the monoclonal antibody against $p16^{INK4A}$ (C).

full-length $p16^{INK4A}$ protein to the nucleus, whereas the his-tagged $p16^{INK4A}$ protein by itself did not translocate to the nucleus. These results were confirmed by immunofluorescence (data not shown). Therefore, the ant tag is essential for the internalization of $p16^{INK4A}$ protein.

We next determined the time required for internalization of the ant-tagged $p16^{INK4A}$ protein. Early passage (35PDL) TIG-3 cells were incubated with 20 μM H-ant-p16wt protein for 15 and 30 min, 1, 3, 6, 9, 12, 18 and 24 h. Nuclear extracts were then prepared and subjected to Western blotting using the anti-$p16^{INK4A}$ antibody. As shown in Fig. 2C, H-ant-p16wt protein was detectable in the nucleus within 3 h, reaching a maximal level in 6 h. These results suggest that it takes about 3 h for the internalization of ant-tagged $p16^{INK4A}$ protein in TIG-3 cells.

3.3. Cell growth inhibition by antennapedia-tagged $p16^{INK4A}$ protein

$p16^{INK4A}$ is thought to be a strong inhibitor of the G1/S transition. Therefore, we have examined the activity of ant-tagged $p16^{INK4A}$ proteins by [^{3}H]thymidine incorporation, which measures DNA synthesis. Asynchronously growing early passage (35PDL) TIG-3 cells were incubated with recombinant $p16^{INK4A}$ proteins (20 μM) for 48 h, and [^{3}H]thymidine incorporation during 2 h was then measured as described in Section 2. As shown in Fig. 3A,B, an equal percentage (approximately 40%) of cells incorporated [^{3}H]thymidine in mock (Fig. 3A) and H-p16wt (Fig. 3B) treated cells. This result is consistent with our earlier results showing that H-p16wt does not internalize (Fig. 2B, lane 2) and thus the his-tagged $p16^{INK4A}$ protein has little or no effect on S phase entry in TIG-3 cells. The same experiment was done using penetrative $p16^{INK4A}$ proteins (H-ant-p16wt, H-ant-p16mut). Although the H-ant-p16mut protein slightly inhibited [^{3}H]thymidine incorporation (Fig. 3D), these effects were negligible. However, Fig. 3C clearly shows that the both number of cells and [^{3}H]thymidine incorporation are significantly decreased in H-ant-p16wt treated cells. Similar results were obtained using synchronized TIG-3 cells (Fig. 3E–H). Serum-starved TIG-3 cells reached mid-S-phase around 16 h after the addition of serum. Therefore, serum-starved early passage TIG-3 cells were treated with recombinant proteins for 2 days, then the cells were stimulated with serum for 16 h and examined for [^{3}H]thymidine incorporation. [^{3}H]Thymidine incorporation was drastically reduced in H-ant-p16wt treated cells. In synchronized cells, a lower concentration (10 μM) of H-ant-p16wt protein was sufficient to inhibit cell growth (Fig. 3G). These data clearly suggest that we can deliver a functional full length $p16^{INK4A}$ protein into cells and inhibit S-phase entry by using the antennapedia tag.

3.4. Inhibition of pRB phosphorylation and induction of a phenotype resembling that of cellular senescence by antennapedia-tagged $p16^{INK4A}$ protein

To confirm that the $p16^{INK4A}$-induced growth arrest (Fig. 3C,G) is caused by inhibition of Cdk activity, we examined the phosphorylation status of pRB. Serum-starved early passage TIG-3 cells were treated with either H-ant-p16wt or H-ant-p16mut for 2 days, then the cells were stimulated with serum. At the indicated times thereafter, whole cell extracts were prepared and subjected to Western blotting using an anti-RB antibody. As shown in Fig. 4, pRB is phosphoryl-

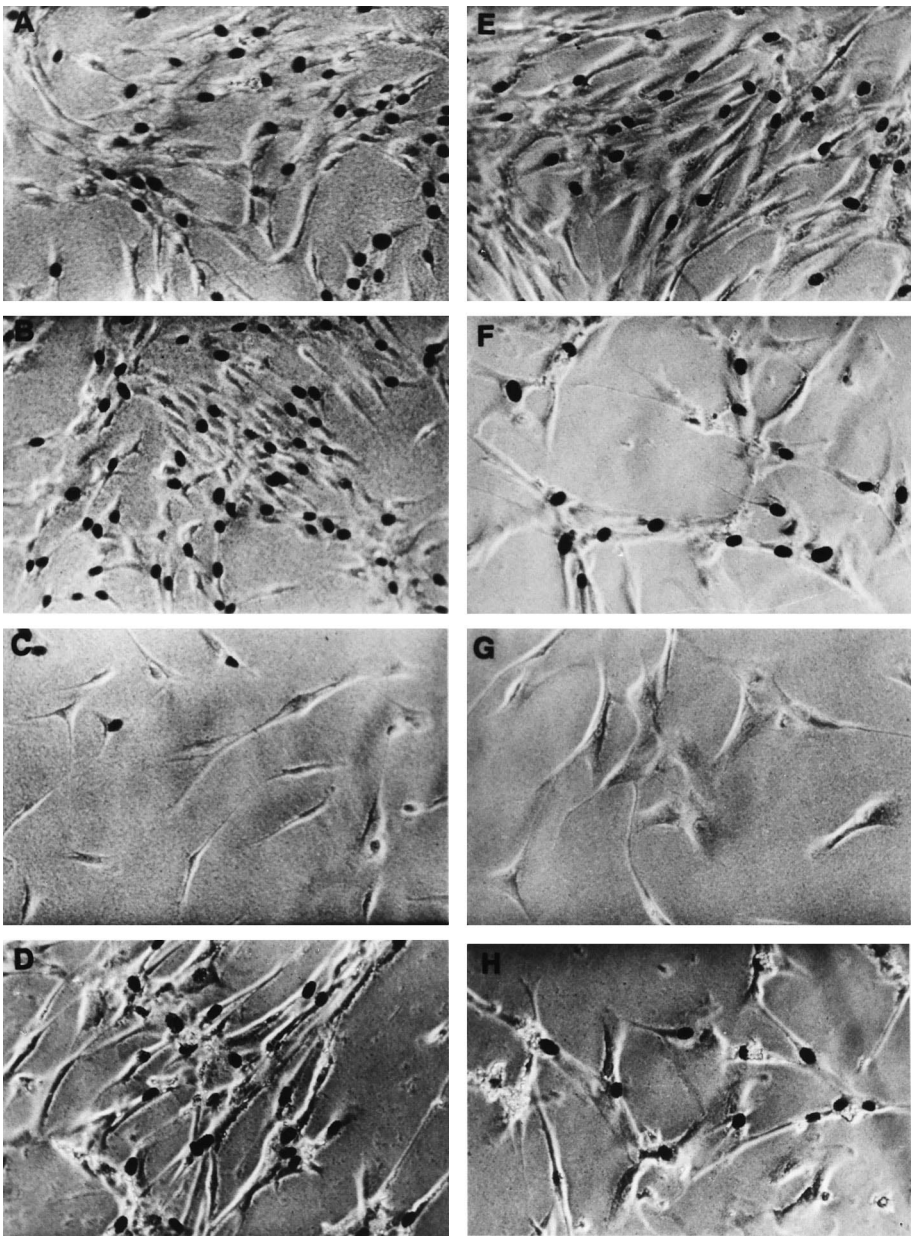

Fig. 3. [^{3}H]Thymidine incorporation. Asynchronously growing early passage TIG-3 cells were incubated with a buffer (A), H-p16wt (B), H-ant-p16wt (C), H-ant-p16mut (D) for 48 h and labeled with [^{3}H]thymidine. Serum-starved TIG-3 cells were incubated with just a buffer (E), H-p16wt (F), H-ant-p16wt (G), H-ant-p16mut (H) for 48 h and labeled with [^{3}H]thymidine. Cells were fixed and visualized by autoradiogaphy.

ated following serum stimulation in cells treated (Fig. 4, lanes 5, 6) or untreated (data not shown) with the H-ant-p16mut protein. However, pRB phosphorylation was not observed in cells treated with the H-ant-p16wt protein (Fig. 4, lanes 2, 3). These data confirm that the penetrative p16^{INK4A} protein inhibits cell growth by inhibiting pRB phosphorylation.

We next asked whether cells arrested by the H-ant-p16wt protein displayed any characteristics of senescent cells. Asynchronously growing early passage TIG-3 cells were incubated with H-ant-p16wt protein (20 μM) for 7 days. The cells were then fixed and stained for SA-β-gal, described to be expressed by senescent cells [27]. The enlarged growth-arrested cells gave a positive staining reaction (Fig. 5B). As described elsewhere, untreated early passage TIG-3 cells were negative for SA-β-gal staining (Fig. 5A), but late passage TIG-3 cells, which had lost growth potential, showed significant SA-β-gal activity (Fig. 5C) [27–29]. Moreover, the treated cells do not return to the cell cycle, but rather remain growth arrested and retain the SA-β-gal activity even 7 days after removal of ant-tagged p16^{INK4A} protein (Fig. 5D). We confirmed that the H-ant-p16wt protein level decreased to the background level by 7 days after removal of the protein from tissue culture medium (data not shown). This result is consistent with several other reports suggesting that cellular senescence is an essentially irreversible phenotype [13,28]. These results demonstrated that ectopic expression of p16^{INK4A} protein in early passage human primary fibroblasts caused the rapid appearance of three phenotypes associated with senescence: growth arrest in G1, accumulation of unphosphorylated pRB, and expression of SA-β-gal.

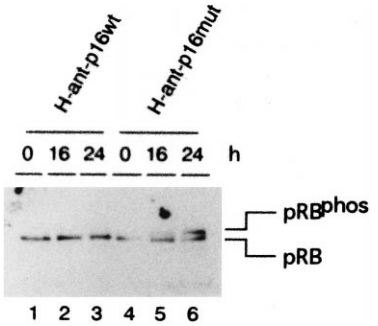

Fig. 4. Inhibition of phosphorylation of pRB by penetrative $p16^{INK4A}$ protein. Serum-starved early passage TIG-3 cells were incubated with H-ant-p16wt (lanes 1–3) or H-ant-p16mut (lanes 4–6) for 48 h, then cells were treated with a medium containing 20% fetal bovine serum with ant-tagged p16 proteins. Cell lysates were fractionated by SDS-PAGE in a 8% gel and immunoblotted with monoclonal antibody against pRB. Immune complexes were detected with ECL. pRB: hypophosphorylated form of RB protein. pRB^{phos}: hyperphosphorylated form of RB protein.

4. Discussion

In this study, we delivered the full-length $p16^{INK4A}$ protein into primary human cells using an antennapedia tag. We developed a system to produce a penetrative $p16^{INK4A}$ protein by a one step purification from bacteria. Because bacterially expressed his-tagged $p16^{INK4A}$ proteins aggregate in a relatively short time, all experiments were done using freshly prepared recombinant $p16^{INK4A}$ proteins. Antennapedia-tagged $p16^{INK4A}$ proteins, both wild-type and mutant $p16^{INK4A}$, were efficiently taken up by cells from the tissue culture medium (Fig. 2). In general, the transfection efficiency of plasmid DNA into primary cells is extremely low. Therefore, antennapedia-tagged proteins provide many advantages for biological studies and clinical applications.

Addition of the antennapedia-tagged wild-type $p16^{INK4A}$ protein into the tissue culture medium efficiently inhibited the phosphorylation of pRB (Fig. 4) and caused growth arrest (Fig. 3) of early passage human diploid fibroblasts. This was not the case when we added the antennapedia-tagged mutant $p16^{INK4A}$ protein, or the simply histidine-tagged $p16^{INK4A}$ protein. Moreover, the morphological change that occurred when $p16^{INK4A}$ protein was introduced into the early passage cells led us to investigate the possibility that these early passage cells might have been induced to enter senescence. Introduction of the $p16^{INK4A}$ protein induced cell enlargement and the inability to proliferate at subconfluent cell densities despite the presence of serum. Using SA-β-gal staining, which has been shown to be expressed by senescent cells [27], we subsequently showed a high degree of SA-β-gal in the early passage TIG-3 cells treated with antennapedia-tagged $p16^{INK4A}$ protein (Fig. 5B). These results are consistent with recent reports suggesting that $p16^{INK4A}$ might be a key factor in the block to phosphorylation of the RB protein in senescent cells. The level of $p16^{INK4A}$ protein is very low in presenescent growing cells [30], but is elevated at the end of the replicative life span [17,18]. In addition, targeted deletion of the $p16^{INK4A}$ gene causes many type of cancer in mice, and primary fibroblasts from these mice do not senesce in culture [19]. Although another cdk inhibitor, $p21^{CIP1/WAF1/SDI1}$, is also known as a gene whose expression is increased as cells senesce [31], it is rarely mutated in cancer cell lines. Moreover, $p21^{CIP1/WAF1/SDI1}$ knock out mice develop normally and primary fibroblasts from these mice senesce as expected [32,33]. Therefore, expression of $p21^{CIP1/WAF1/SDI1}$ may not be required for the senescence of fibroblasts [34]. Although $p19^{ARF}$, an alternative reading frame protein encoded by the INK4A locus, seems also to be important for cell senescence [20], our data presented here, and the evidence that overexpression of $p16^{INK4A}$ induces cell senescence in human glioma cells [35], strongly suggest that $p16^{INK4A}$ plays an important role in cel-

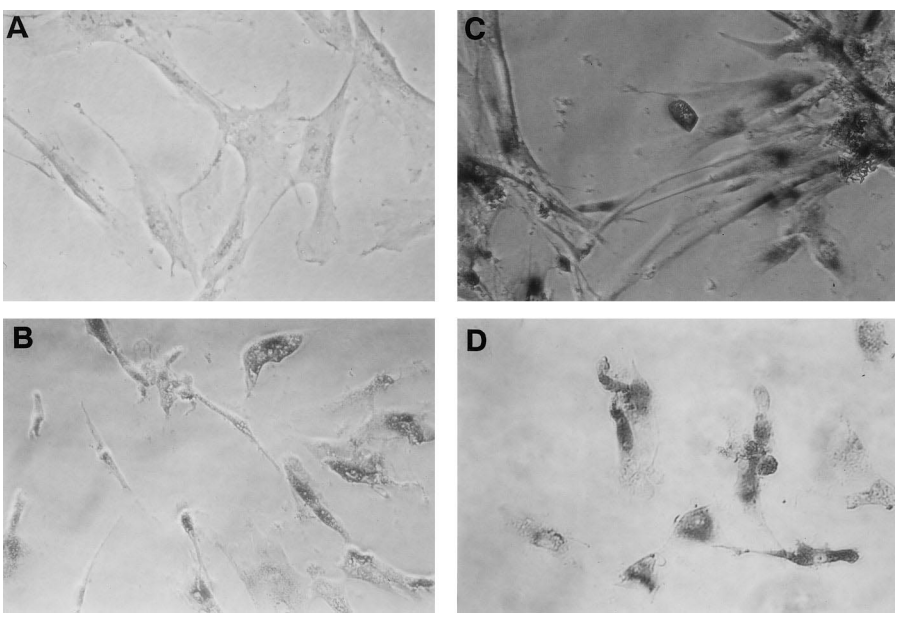

Fig. 5. Senescence-associated β-galactosidase (SA-β-gal) staining of TIG-3 cells. Early passage TIG-3 cells were treated with buffer (mock) (A) or H-ant-p16wt (B) for 7 days and stained for SA-β-gal activity. As positive control, late passage TIG-3 cells (C) were stained for SA-β-gal activity. H-ant-p16wt treated cells were washed with PBS and incubated with the tissue culture medium without H-ant-p16wt protein for a further 7 days. Then cells were stained for SA-β-gal activity (D).

lular senescence. Furthermore, recent findings suggest that the functional inactivation of $p16^{INK4A}$ by Tax oncoprotein of human T-cell leukemia virus type 1 (HTLV-1) through protein-protein interaction contributes to cellular immortalization induced by HTLV-1 infection [36]. Taking these evidences together, $p16^{INK4A}$ acts as a tumor suppressor which prevent cellular immortalization.

Our work presented here also raises the possibility that bacterially produced penetrative tumor suppressor proteins offer a novel therapeutic approach towards controlling aberrant cellular proliferation. In our hands, histidine-tagged $p16^{INK4A}$ protein prepared from bacteria aggregate rapidly, specially at high concentrations. Therefore, we are currently trying further application to increase the solubility of antennapedia-tagged $p16^{INK4A}$ protein. Our recent observation suggests that glutathione *S*-transferase (GST)-tagged $p16^{INK4A}$ protein is much more soluble than his-tagged $p16^{INK4A}$ protein and is efficiently internalized by the addition of the ant tag (Kato and Hara, unpublished results).

Acknowledgements: We are grateful to our many colleagues, particularly Naoya Tanida, Naoko Ohtani-Fujita, Takeshi Nakamura and Shinichiro Niwa for their technical support and valuable suggestions. We are also indebted to Michel Volovitch for his useful information about the antennapedia tag. Judith Campisi provided helpful comments on the manuscript. This work was supported by Sumitomo Electric Industries and the Imperial Cancer Research Fund.

References

[1] Serrano, M., Hannon, G.J. and Beach, D. (1993) Nature 366, 704–707.
[2] Kamb, A., Gruis, N.A., Weaver-Feldhaus, J., Liu, Q., Harshman, K., Tavtigian, S.V., Stockert, E., Day, R.S., Johnson, B.E. and Skolnick, M.H. (1994) Science 264, 436–440.
[3] Nobori, T., Miura, K., Wu, D.J., Lois, A., Takabayashi, K. and Carson, D.A. (1994) Nature 368, 753–756.
[4] Sherr, C.J. (1994) Cell 79, 551–555.
[5] Hunter, T. and Pines, J. (1994) Cell 79, 573–582.
[6] Lukas, J., Parry, D., Aagaard, L., Mann, D.J., Bartkova, J., Strauss, M., Peters, G. and Bartek, J. (1995) Nature 375, 503–506.
[7] Koh, J., Enders, G.H., Dynlacht, B.D. and Harlow, E. (1995) Nature 351, 506–510.
[8] Sherr, C.J. and Roberts, J.M. (1995) Genes Dev. 9, 1149–1163.
[9] Hirai, H., Roussel, M.F., Kato, J.Y., Ashmun, R.A. and Sherr, C.J. (1995) Mol. Cell. Biol. 15, 2672–2681.
[10] Sherr, C.J. (1996) Science 274, 1672–1677.
[11] Okamoto, A., Demetrick, D.J., Spillare, E.A., Hagiwara, K., Hussain, S.P., Bennett, W.P., Forrester, K., Gerwin, B., Serrano, M., Beach, D.H. and Harris, C.C. (1994) Proc. Natl. Acad. Sci. USA 91, 11045–11049.
[12] Lumpkin Jr., C.K., McClung, J.K., Pereira-Smith, O.M. and Smith, J.R. (1986) Science 232, 393–395.
[13] Campisi, J., Dimiri, G. and Hara, E. (1996) Handbook of the Biology of Aging, 4th edn., pp. 121–149, Academic Press, New York.
[14] Shay, J.W., Pereira-Smith, O.M. and Wright, W.E. (1991) Exp. Cell Res. 196, 33–39.
[15] Hara, E., Tsurui, H., Shinozaki, A., Nakada, S. and Oda, K. (1991) Biochem. Biophys. Res. Commun. 179, 528–534.
[16] Stein, G.H., Beeson, M. and Gordon, L. (1990) Science 249, 666–669.
[17] Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S. and Peters, G. (1996) Mol. Cell. Biol. 16, 859–867.
[18] Alcorta, D.A., Xiong, Y., Phelps, D., Hannon, G., Beach, D. and Barrett, J.C. (1996) Proc. Natl. Acad. Sci. USA 93, 13742–13747.
[19] Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D. and DePinho, R.A. (1996) Cell 85, 27–37.
[20] Kamijo, T., Zindy, F., Roussel, M.F., Quell, D.E., Downing, J.R., Ashmun, R.A., Grosveld, G. and Sherr, C.J. (1997) Cell 91, 649–654.
[21] Derossi, D., Chassaing, G. and Prochiantz, A. (1998) Trends Cell Biol. 8, 84–87.
[22] Fahraeus, R., Paramio, J.M., Ball, K.L., Lain, S. and Lane, D.P. (1996) Curr. Biol. 6, 84–91.
[23] Selivanova, G., Iotsova, V., Okan, I., Fritsche, M., Strom, M., Groner, B., Grafstrom, R.C. and Wiman, K.G. (1997) Nature Med. 3, 632–638.
[24] Bandara, L.R., Girling, R. and La Thangue, N.B. (1997) Nature Biotechnol. 15, 896–901.
[25] Hoffmann, A. and Roeder, R.G. (1991) Nucleic Acids Res. 19, 6337–6338.
[26] Parry, D. and Peters, G. (1996) Mol. Cell. Biol. 16, 3844–3852.
[27] Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M. and Campisi, J. (1995) Proc. Natl. Acad. Sci. USA 92, 9363–9367.
[28] Ogryzko, V.V., Hirai, T.H., Russanova, V.R., Barbie, D.A. and Howard, B.H. (1996) Mol. Cell. Biol. 16, 5210–5218.
[29] Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D. and Lowe, S.W. (1997) Cell 88, 593–602.
[30] Quelle, D.E., Ashmun, R.A., Hannon, G.J., Rehberger, P.A., Trono, D., Richter, K.H., Walker, C., Beach, D., Sherr, C.J. and Serrano, M. (1995) Oncogene 11, 635–645.
[31] Noda, A., Ning, Y., Venable, S.F., Pereira-Smith, O.M. and Smith, J.R. (1994) Exp. Cell Res. 211, 90–98.
[32] Deng, C., Zhang, P., Harper, J.W., Elledge, S.J. and Leder, P. (1995) Cell 82, 675–684.
[33] Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks, T. and Hannon, G.J. (1995) Nature 377, 552–557.
[34] Medcalf, A.S.C., Klein-Szanto, A.J.P. and Cristofalo, V.J. (1996) Cancer Res. 56, 4582–4585.
[35] Uhrbom, L., Nister, M. and Westermark, B. (1997) Oncogene 15, 505–514.
[36] Suzuki, T., Kitao, S., Matsushime, H. and Yoshida, M. (1996) EMBO J. 15, 1607–1614.