Note

Dephosphorylation of Specific Proteins during Induction of Senescence in Immortal human Fibroblasts Expressing Thermolabile SV40 T Antigen

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Received December 15, 1998; Accepted February 2, 1999

Particular protein kinase inhibitors block a senescence-like phenomenon in SVts8 cells induced by a shift up in temperature. We characterized cellular proteins with affinity chromatography using one such inhibitor as a ligand. Two proteins of 56 and 100 kDa were found to be dephosphorylated specifically, probably due to induction of a protein phosphatase activity(s).

Key words: protein kinase inhibitor; protein phosphatase; H-9; senescence; human cell

An immortal cell line, SVts8, has been established from human embryonic lung fibroblasts (TIG3) after transfection with a plasmid encoding a thermolabile SV40 T antigen.1,2 When SVts8 cells are cultured at the non-permissive temperature (40°C), they undergo an irreversible arrest in cell division due to the inactivation of the T antigen.2,4 They also show morphological alterations, enlarged and flat cell shape, similar to normally senesced human fibroblasts,2,4,5 and show several senescence markers.4,5 As this phenomenon is very similar to cellular senescence,3-5 it is often used as a model of cellular senescence.

In our previous study,4 we have shown that the inhibitors defined in vitro as of those of cGMP-dependent protein kinase specifically block the induction of the senescence-like phenomenon in SVts8 cells. Among the inhibitors, the indolocarbazole type KT5823 and the isoquinoline sulfonamide derivatives H-9, H-88, and H-89 were found to be effective, although the latters were less effective than KT5823.2 In this phenomenon, p53 has been shown to play a major role,7 suggesting that a senescence signal is mediated by both p53 and a protein kinase(s) sensitive to the inhibitors. However, an in vivo target of the inhibitors remains to be identified.

To identify such a target, we used affinity chromatography using one of the inhibitors as a ligand, and searched for a protein that may change during the induction of the senescence-like phenomenon. We coupled H-9 to agarose beads, and packed them in a column as described.6 Using an H-9 affinity column like this, several kinds of protein kinases have been purified very efficiently from crude cell extracts.

We prepared cell extracts from SVts8 cells cultured at 35°C (permissive conditions) and at 40°C. As a control, we also used SVts8 (+T) cells that were obtained by transfection with a plasmid encoding the wild-type SV40 large T antigen and can grow normally at 40°C.5 We put the cell extracts on the affinity column, washed the column with a buffer, and eluted proteins with ATP, a competitor of protein kinases for binding to H-9.

The proteins eluted were resolved by electrophoresis on a SDS-polyacrylamide gel and detected by silver staining (Fig 1A). As H-9 inhibits various protein kinases,4,6 many protein bands were detected. However, there was no apparent difference in the band patterns between SVts8 cells cultured at 35°C and 40°C. Two-dimensional resolution of the samples gave no apparent difference either (not shown). These results show that quantities of individual proteins capable of binding to the ligand do not alter upon a shift up in temperature.

It is known that the activity of protein kinases is regulated post-translationally, that is, through phosphorylation and/or dephosphorylation of their serine, threonine, and/or tyrosine residues. Therefore, we examined whether some proteins are phosphorylated or dephosphorylated during the induction of the senescence-like phenomenon. For this purpose, we did Western blot analysis using antibodies against phosphoserine, phosphothreonine, or phosphotyrosine as probes. Probing the blot (Fig 1A) with the anti-phosphoserine antibody showed the disappearance of two phosphorylated protein bands corresponding to 56 and 100 kDa in the cell extracts of SVts8 cells cultured at 40°C (Fig 1B). The other differences were not reproducible. These changes were observed as early as one day after temperature shift, but were not in the cell extract of SVts8 (+T) cells cultured at 40°C, indicating that heat shock stress is not involved in this observation. In this respect, it is shown that upon a shift up in temperature p53 is released from SV40 large T antigen within a couple of hours in SVts8 cells, but it takes 2–3 days to commit the cells to the senescent-like phenomenon.4 Thus, the dephosphorylation of these proteins is an early event during the induction of this phenomenon. The other phosphorylated protein bands seemed to be unaltered. Also, we were not able to detect any significant difference with anti-phosphothreonine or -phosphotyrosine antibody (not shown).

Next we assessed a change in the phosphorylation sta-

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Abbreviation: SV40, simian virus 40
Fig. 1. Profiles of Proteins Bound to H-9 Affinity Column with Silver Staining (A) or Immunoblotting (B).

(A) Cell extracts were prepared from cells cultured under the conditions indicated as described. H-9 (Seikagaku Kogyo, Tokyo) was coupled to CNBr-activated Sepharose 4B beads (Pharmacia) as described, and the beads were packed in a column. Proteins were bound to the column, extensively washed with a buffer, and eluted with 80 mM ATP at 4°C. The eluted proteins were electrophoresed on SDS-polyacrylamide gel (7.5%), and the gel was silver stained.

(B) The gel was analyzed by Western blots using a mouse anti-phosphoserine antibody (Sigma) as a probe. The mouse antibody was detected with horseradish peroxidase-conjugated sheep anti-mouse antibody (Amersham) and a chemiluminescence detection kit (ECL, Amersham).

+T indicates a stable transformant of SVts8 isolated by transfection with pSV3neo and expressing the wild-type SV40 T antigen.

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Fig. 2. Profiles of Proteins Specifically Bound to H-9 Affinity Column after Phosphorylation in Vitro in the Absence (A) and Presence (B) of a Protein Phosphatase Inhibitor.

Protein samples prepared from cells cultured under the conditions indicated were incubated at 37°C in 10 μl of a reaction mixture containing 10 mM Hepes buffer (pH 7.3), 50 mM magnesium acetate, and 1 μl of [γ-32P]ATP (3000 Ci/mmole, ICN) for 5 minutes in the absence (A) or presence (B) of a protein phosphatase inhibitor. The samples were processed as described in Fig. 1, and autoradiograms were prepared.

Van, vanadate; OA, okadaic acid.

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tus of the 56 and 100-kDa protein in vitro. Cell extracts were prepared and incubated in a reaction mixture containing [γ-32P]ATP to label phosphoproteins. The labeled proteins were purified with the H-9 affinity column, resolved by SDS-polyacrylamide gel, and used to expose an X-ray film (Fig. 2A). In the samples of
SVts8 cells cultured at 40°C for 1–3 days, the 56-kDa protein was underphosphorylated specifically, while the samples of SVts8(+T) cells cultured at 40°C showed a normal level of phosphorylation of the protein. These results are in good agreement with the in vivo data shown in Fig. 1B.

As these data suggest activation of a protein phosphatase(s) in the samples of SVts8 cells cultured at 40°C, we investigated the effects of several inhibitors of protein phosphatases (Fig. 2B). Addition of 2 μM okadaic acid, an inhibitor of serine/threonine phosphatases,7 to the reaction mixtures significantly blocked dephosphorylation of the 56 kDa protein. The same results were obtained with the protein samples prepared from SVts8 cells cultured at 40°C for 1 or 2 days (not shown). Vanadate, another type of phosphatase inhibitor,9 did not show such an effect. The effect of okadaic acid was dose-dependent and negative with concentrations less than 0.2 μM (not shown). Two μM okadaic acid is much higher than its effective concentrations to inhibit protein phosphatase I and IIA (IC50=10–20 nM and 0.1 nM, respectively),7 and addition of a peptide inhibitor of protein phosphatase 19 or FK506, an inhibitor of IIB,10 had no effect (not shown). Taken together, these phosphatases does not seem to be involved in this dephosphorylation. As a specific inhibitor of protein phosphatase IIC is not known at present, we could not investigate the role of IIC in detail. Recently, a novel type of protein phosphatase that is activated by various stresses and mediated by p53 has been cloned.11,12 This phosphatase, resembling protein phosphatase IIC, is inhibited by a high concentration of okadaic acid (IC50=about 1–2 μM), suggesting the involvement of this kind of protein phosphatase(s) in the above observations.

Finally, we could not detect dephosphorylation of the 100 kDa protein under the various conditions we used. Although our data presented here are preliminary and further investigation is required, they suggest that a protein phosphatase is involved in the induction of the senescence-like phenomenon in SVts8 cells.

Acknowledgment

This work was supported by Public Trust Haraguchi Memorial Cancer Research Fund.

References