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S100C/A11 is a key mediator of Ca^{2+}-induced growth inhibition of human epidermal keratinocytes

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An increase in extracellular Ca^{2+} induces growth arrest and differentiation of human keratinocytes in culture. We examined possible involvement of S100C/A11 in this growth regulation. On exposure of the cells to high Ca^{2+}, S100C/A11 was specifically phosphorylated at ^{10}Thr and ^{94}Ser. Phosphorylation facilitated the binding of S100C/A11 to nucleolin, resulting in nuclear translocation of S100C/A11. In nuclei, S100C/A11 liberated Sp1/3 from nucleolin. The resulting free Sp1/3 transcriptionally activated $p21^{CIP1/WAF1}$, a representative negative regulator of cell growth. Introduction of anti-S100C/A11 antibody into the cells largely abolished the growth inhibition induced by Ca^{2+} and the induction of $p21^{CIP1/WAF1}$. In the human epidermis, S100C/A11 was detected in nuclei of differentiating cells in the suprabasal layers, but not in nuclei of proliferating cells in the basal layer. These results indicate that S100C/A11 is a key mediator of the Ca^{2+}-induced growth inhibition of human keratinocytes in culture, and that it may be possibly involved in the growth regulation in vivo as well.

Introduction

Exquisite spatial and temporal control of cell growth and differentiation is a prerequisite for embryonic development and maintenance of fine tissue architecture. The epidermis is a typical tissue in which compartments of growing cells and growth-arrested terminally differentiating cells are clearly demarcated. In normal epidermis, proliferating cells are only observed in the basal layer. On moving to the upper layers, the cells stop growing, progressively follow a terminal differentiation pathway, and finally shed off from the surface. The critical step determining whether the cells withdraw from the growing cell population is regulated by a complex network involving many genes, but the precise molecular mechanisms remain largely unknown.

A number of factors have been reported to trigger differentiation of human and mouse epidermal keratinocytes in culture, i.e., increased extracellular Ca^{2+} concentration (Hennings et al., 1980), TGFβ (Shipley et al., 1986), forced expression of PKC (Ohba et al., 1998), and detachment of cells from the substrate (Zhu and Watt, 1996). Normal human keratinocytes (NHKs) continuously proliferate only in a culture medium with Ca^{2+} of <0.1 mM. An increase in the Ca^{2+} concentration to 1.2–2.0 mM results in termination of cell growth and induction of terminal differentiation phenotypes (Hennings et al., 1980). An increase in the extracellular Ca^{2+} level resulted in a sustained higher intracellular Ca^{2+} concentration (Sharpe et al., 1989). Some other conditions inducing differentiation of keratinocytes also lead to increased intracellular Ca^{2+} levels (Sharpe et al., 1989; Missero et al., 1996). An increasing gradient of Ca^{2+} concentration is present from the basal to the cornified layers of the epidermis in vivo (Menon et al., 1992). Mice lacking the expression of full-length extracellular Ca^{2+}-sensing receptors showed deteriorated epidermal differentiation (Komuves et al., 2002). These results indicate that higher Ca^{2+} levels lead to induction of epidermal differentiation not only in culture, but also in vivo.

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Abbreviations used in this paper: NHK, normal human keratinocyte; PEI, polyethyleneimine; siRNA, small interfering RNA.

An elevation in the Ca^{2+} level triggers a number of intracellular signal transductional events, including production of inositol 1,4,5-triphosphate and 1,2-diacylglycerol; activation of calcineurin, PKCs, and Raf/MEK/ERK pathway; and tyrosine phosphorylation of p62 and fyn (Dotto, 1999; Schmidt et al., 2000). On the other hand, it has been shown that $p21^{CIP1/WAF1}$ was induced 4 or 8 h after an increase in the extracellular Ca^{2+} level, leading to inhibition of Cdk activity and blockage of cell cycle progression (Missero et al., 1996). $p21^{CIP1/WAF1}$ protein has been detected in cells of the suprabasal layers, but not in those cells of the basal layer of the human epidermis (Ponten et al., 1995). An important missing link is how the Ca^{2+}-induced initial events lead to the induction of $p21^{CIP1/WAF1}$.

In a previous work on density-dependent growth inhibition of normal human fibroblasts, we identified S100C/A11 (calgizzarin), a member of the Ca^{2+}-binding S100 protein family, as a key mediator of growth arrest (Sakaguchi et al., 2000). In a confluent state, S100C/A11 was phosphorylated at ^{10}Thr and translocated into nuclei, and it eventually inhibited DNA synthesis through the induction of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$. S100C/A11 is comprised in the epidermal differentiation complex (EDC) located on chromosome 1q21 in humans. EDC encodes nearly 30 genes. About half of them are specifically expressed during Ca^{2+}-dependent terminal differentiation of keratinocytes (e.g., profilaggrin and loricrin), and the other half are members of the S100 protein family. The S100 family proteins have been assumed to play signal transduction roles in the differentiation of epidermis and other tissues. Some of the S100 protein family members were differentially expressed in normal human skin and melanocytic lesions (Boni et al., 1997). S100C/A11 was reported to be up- or down-regulated in malignant tumors (Van Ginkel et al., 1998). These results prompted us to examine possible involvement of S100C/A11 in growth regulation of epidermal keratinocytes. Here, we show that S100C/A11 is a key mediator of the high Ca^{2+}-induced growth arrest in human keratinocytes.

Results

Increase in extracellular Ca^{2+} concentration inhibits the growth of NHK and HaCaT cells

At first, we confirmed that an increase in extracellular Ca^{2+} concentration from 0.03 to 1.5 mM results in inhibition of DNA synthesis of NHK cells in dose- and time-dependent manners (Fig. 1, A and B). HaCaT cells, an immortalized human keratinocyte line (Boukamp et al., 1988), were routinely cultivated in a medium with 1.5 mM Ca^{2+}. When the concentration of Ca^{2+} was increased to 5 or 10 mM, DNA synthesis of HaCaT cells was inhibited (Fig. 1, A and B). Under similar conditions, Sharpe et al. (1989) and Gonczi et al. (2002) reported that there was no remarkable difference in intracellular Ca^{2+} level (70–100 nM) of NHK and HaCaT cells, despite different extracellular Ca^{2+} concentrations. Intracellular Ca^{2+} concentrations in NHK and HaCaT cells increased by ~40% within 30 min after increasing the extracellular Ca^{2+} concentrations from 0.03 to 1.5 mM and from 1.5 to 10 mM, respectively (Fig. 1 C). Overall, the response of HaCaT cells to 10 mM Ca^{2+} was similar to that of NHK cells to 1.5 mM Ca^{2+}. No immediate cytotoxicity was noted in HaCaT cells in a medium with 10 mM Ca^{2+} during the observation period. Therefore, throughout the present work, 0.03 and 1.5 mM Ca^{2+} was used as low Ca^{2+} media and 1.5 and 10 mM Ca^{2+} was used as high Ca^{2+} media for NHK and HaCaT cells, respectively.

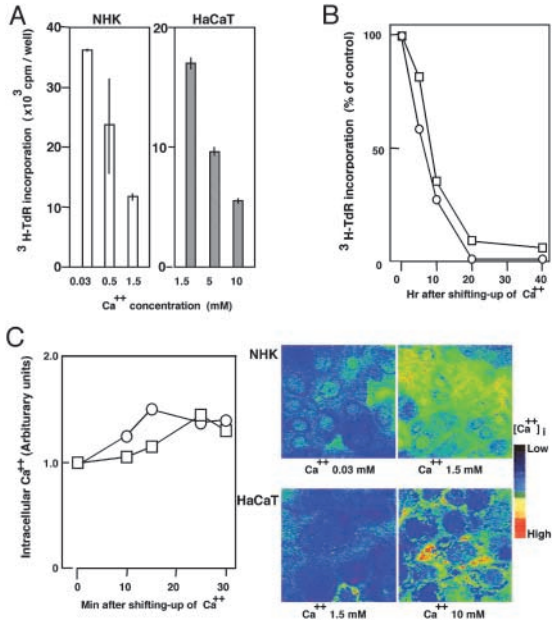

Figure 1. **Growth arrest and increased intracellular Ca^{2+} levels of NHK and HaCaT cells exposed to high Ca^{2+} media.** (A) Dose-dependent inhibition of [^{3}H]TdR incorporation into an insoluble fraction in NHK (left) and HaCaT (right) cells exposed to high Ca^{2+} for 6 h. (B) Time-dependent inhibition of 1 μCi/ml [^{3}H]TdR incorporation by NHK (circles) and HaCaT (squares) cells exposed to high Ca^{2+}. (C) Increases in intracellular Ca^{2+} levels of NHK (circles) and HaCaT (squares) cells. Relative intracellular Ca^{2+} level (left; the concentrations in cells cultured in low Ca^{2+} media are 1.0) and representative images (right).

S100C/A11 inhibits the growth of human keratinocytes

To examine the possible involvement of S100C/A11 in growth regulation of human keratinocytes, we transfected various constructs of S100C/A11 flanking SV40-derived NLS to exponentially growing HaCaT cells. As shown in Fig. 2 A, incorporation of BrdU was specifically inhibited in the cells transfected with wild-type S100C/A11 (indicated by arrows), whereas GFP alone did not show any effects. DNA synthesis was inhibited by ~25% by the wild-type S100C/A11 (Fig. 2 B). This reduction is substantial, considering that the transfection efficiency was at most 40%. Introduction of recombinant S100C/A11 protein into HaCaT cells by the aid of the protein transduction domain of TAT protein (YGRKKRRQRRR; Schwarze et al., 1999) efficiently inhibited DNA synthesis (Fig. 2 C). The TAT-flanked protein was transferred to cell nuclei with an efficiency of nearly 100%.

Increase in extracellular Ca^{2+} concentration results in phosphorylation and nuclear translocation of S100C/A11

When extracts of NHK and HaCaT cells labeled with [^{32}P]phosphate were analyzed by a two-dimensional gel electrophoresis, increased phosphorylation of S100C/A11 (as identified by sequencing) was noted in both cell types ex-

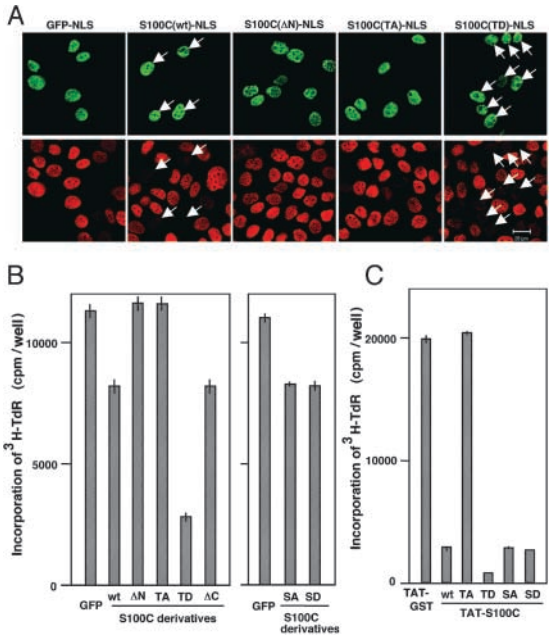

Figure 2. **Growth inhibition of human epidermal keratinocytes by S100C/A11.** (A) Various constructs of S100C/A11-NLS-Myc (top row) were transfected to HaCaT cells 48 h before the addition of 1 μM BrdU. The cells were further incubated for 12 h before fixation and staining for Myc (top row) and BrdU (bottom row). Arrows indicate Myc-positive and BrdU-negative cells. ΔN, NH_2-terminal 23 aa–deleted form; TA, Ala-substituted form of ^{10}Thr; TD, Asp-substituted form of ^{10}Thr (a ^{10}phosphothreonine-equivalent form). Bar, 20 μm. (B) Inhibition of 1 μCi/ml [^{3}H]TdR incorporation (12 h) by various S100C/A11-NLS-Myc constructs in HaCaT cells under conditions similar to A. SA, Ala-substituted form of ^{94}Ser; SD, Asp-substituted form of ^{94}Ser; ΔC, COOH-terminal 19 aa-deleted form. For the other abbreviations, see A. (C) Inhibition of [^{3}H]TdR incorporation by TAT-S100C/A11 protein variants in HaCaT cells. Recombinant His_6-TAT–fused S100C/A11 proteins were purified using a nickel-NTA chelating column and gel electrophoresis. The cells were incubated with each protein (10 μM) for 6 h. 1 μCi/ml [^{3}H]TdR was added 1 h before harvest. For abbreviations, see B.

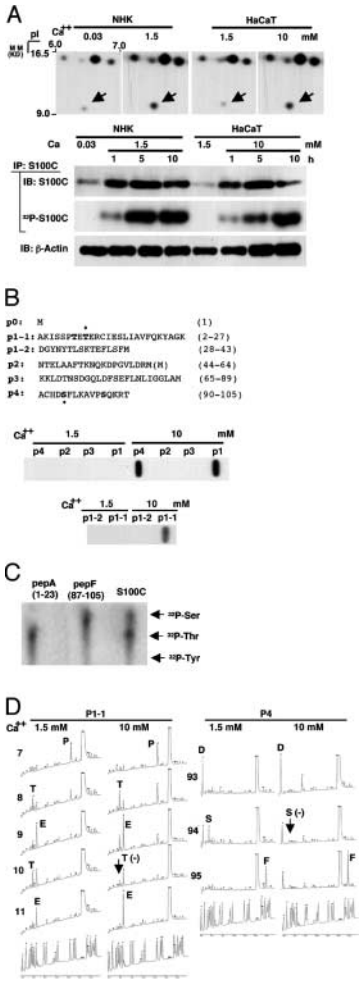

Figure 3. **Phosphorylation of S100C/A11 in NHK and HaCaT cells exposed to high Ca^{2+} concentrations.** (A) Top: two-dimensional electrophoresis of [^{32}P]phosphate-labeled cell extracts. Only a part covering pI (isoelectric point) 6.0–7.0, molecular marker of 9.0–16.5 kD, is shown. The arrows show phosphorylated S100C/A11 as identified by sequencing. Bottom: after increasing the Ca^{2+} concentrations, cell extracts were immunoprecipitated with anti-S100C/A11 antibody and analyzed by Western blotting or autoradiography. (B) Top: expected partial peptides of S100C/A11 cleaved by cyanogen bromide (for p0 and p2-4) or by cyanogen bromide followed by an asparatic endopeptidase (for p1-1 and p1-2). Asterisks indicate potential phosphorylation sites as determined in D. Bottom: autoradiography of the purified peptides prepared from [^{32}P]phosphate-labeled HaCaT cells exposed to high Ca^{2+}. (C) Phosphorylated amino acid residues of synthetic pepA (aa residues 1–23), pepF (87–105), and endogenous S100C/A11. pepA and pepF were phosphorylated in vitro by a cell extract prepared from HaCaT cells exposed to high Ca^{2+}. (D) Determination of phosphorylation sites of S100C/A11. The purified p1-1 and p4 (A) were analyzed by an automated amino acid sequencer. Missing peaks (arrows) indicate the phosphorylation sites. Numerals indicate the amino acid residues of S100C/A11.

posed to high Ca^{2+} (Fig. 3 A). Exposure to high Ca^{2+} increased the immunoprecipitable amount of S100C/A11 and more remarkably the level of phosphorylation in both cell types (Fig. 3 A, bottom), whereas the S100C/A11 protein levels only slightly increased by high Ca^{2+} (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200304017/DC1).

To determine the phosphorylation sites of S100C/A11, endogenous S100C/A11 protein was purified from ^{32}P-labeled HaCaT cells and degradated to peptides. As shown in Fig. 3 B (middle and bottom), only NH_2-terminal p1-1 and COOH-terminal p4 were phosphorylated in a totally high Ca^{2+}-depending manner. When the synthesized peptides of S100C/A11 were incubated with cell extracts in vitro, NH_2-terminal pepA (amino acid residues 1–23) and COOH-terminal pepF (87–105), but not four other peptides covering the middle part of S100C/A11, were significantly phosphorylated by the extracts prepared from NHK and HaCaT cells cultivated in the high Ca^{2+} media (unpublished data). Thr and Ser residues were phosphorylated in pepA and pepF, respectively (Fig. 3 C). Finally, phosphorylation sites in p1-1 and p4 were determined as ^{10}Thr and ^{94}Ser, respectively, by an amino acid sequencer (Fig. 3 D). In accordance with this, Ala-substituted forms at ^{10}Thr of pepA and at ^{94}Ser of pepF were not phosphorylated by the cell extracts prepared after cultivating in the high Ca^{2+} media (unpublished data).

Increases in Ca^{2+} in the media resulted in partial translocation of endogenous S100C/A11 to nuclei of NHK and HaCaT cells as demonstrated by immunostaining (Fig. 4 A) and by Western blotting and autoradiography (Fig. 4 B).

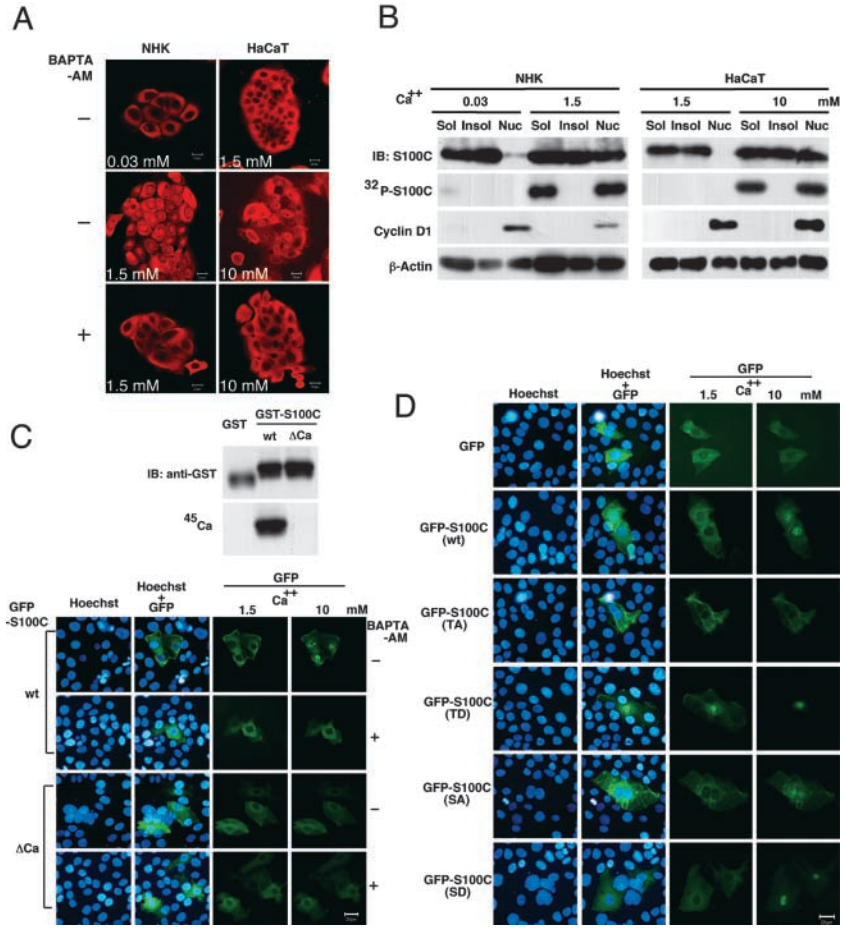

Figure 4. **Nuclear translocation of S100C/A11 in NHK and HaCaT cells by high Ca^{2+}.** (A) Translocation of S100C/A11 to nuclei by high Ca^{2+}. NHK and HaCaT cells were immunostained with anti-S100C/A11 antibody. A membrane-permeable Ca^{2+} chelator, BAPTA-AM, was added at 30 μM from 1 h before the increase of Ca^{2+} concentrations. (B) Cells labeled with [^{32}P]phosphate were fractionated into soluble (Sol) and insoluble (Insol) cytoplasmic fractions and a nuclear fraction (Nuc). Immunoblotting of cyclin D1 was used to show successful fractionation. (C) Loss of Ca^{2+}-binding capacity of S100C/A11 inhibits the nuclear translocation induced by high Ca^{2+} in HaCaT cells. Top: successful elimination of Ca^{2+}-binding capacity of S100C/A11 as demonstrated by native PAGE of the recombinant proteins premixed with ^{45}Ca. Bottom: necessity of Ca^{2+} binding to S100C/A11 for the nuclear translocation. Plasmid constructs of wild-type (wt) S100C/A11 and a variant of the protein lacking Ca^{2+}-binding (ΔCa) were transfected 36 h before the increase of Ca^{2+} concentration. BAPTA-AM, 30 μM. The cells were observed in living conditions before and 6 h after increasing the extracellular Ca^{2+} level. (D) Nuclear translocation of S100C/A11 is affected by the 10th residue. Various GFP-S100C constructs were transfected to HaCaT cells under conditions similar to C. TA, ^{10}Thr to A; TD, ^{10}Thr to D; SA, ^{94}Ser to A; SD, ^{94}Ser to D. Bars, 20 μm (A, C, and D).

Preincubation with a membrane-permeable Ca^{2+} chelator, BAPTA-AM, inhibited the nuclear translocation of endogenous S100C/A11 by high Ca^{2+} (Fig. 4 A). An S100C/A11 variant protein lacking Ca^{2+}-binding capacity was not translocated to nuclei by high Ca^{2+} (Fig. 4 C). Transfection of the wild-type S100C/A11-GFP construct before the shift to high Ca^{2+} resulted in nuclear translocation of the protein in HaCaT cells (Fig. 4 D). This nuclear translocation was not observed when ^{10}Thr was substituted with Ala (Fig. 4 D) or when the NH_2-terminal 23 amino acids were deleted (unpublished data). ^{10}Thr-to-Asp–substituted protein (TD) was observed in nuclei even in the low Ca^{2+} medium. On the contrary, ^{94}Ser-substituted forms behaved in a manner similar to the wild-type protein. A COOH-terminal truncated form of S100C/A11 lacking the residues from 87 to 105 was transferred to nuclei by the high Ca^{2+} (unpublished data). These results indicate that the phosphorylation of ^{10}Thr, but not of ^{94}Ser, is critical for the Ca^{2+}-induced nuclear localization of S100C/A11, and that binding of Ca^{2+} to S100C/A11 is necessary for the translocation.

Binding of S100C/A11 to nucleolin is essential for nuclear translocation

Because S100C/A11 lacks the canonical NLS sequence, we screened for possible interacting protein(s) using a pepA(TD)-immobilized column (pepA with Asp at the 10th residue, a phosphorylated pepA equivalent). An ~100-kD protein was identified in extracts of HaCaT cells as showing specific binding (Fig. 5 A, left). Sequencing of the eluted protein gave an amino acid sequence of VKLAKAGKANQGD, corresponding to the NH_2 terminus of human nucleolin. The binding of S100C/A11 and nucleolin was confirmed in vitro (Fig. 5 A, right; Fig. 5 B). The bound amounts of nucleolin depended on the amounts of S100C/A11 protein immobilized, and S100C/A11(TD), a ^{10}Thr phosphorylation equivalent form, showed higher affinity to nucleolin than did the wild type (Fig. 5 B). Synthesized pepA having phosphothreonine at the 10th residue showed a higher nucleolin-binding capacity similar to pepA(TD) (Fig. S6, available at http://www.jcb.org/cgi/content/full/jcb.200304017/DC1). Only pepA, and not the other five partial peptides of S100C/A11, binds to nucleolin. Furthermore, neither GST-S100A2 nor GST-S100A6, the other members of S100 family, bound to nucleolin as assayed in vitro (unpublished data).

In the Ca^{2+}-stimulated HaCaT cells, S100C/A11 protein that was phosphorylated and bound to nucleolin was predominantly found in the nuclei (Fig. 5 C). Among various GFP-conjugated ^{10}Thr- or ^{94}Ser-substituted forms of S100C/A11 transfected to HaCaT cells, only ^{10}Thr-to-Asp–substituted forms were coprecipitated with nucleolin in the low Ca^{2+} medium (Fig. 5 D). In the high Ca^{2+} medium, phosphorylation of S100C/A11 was observed when either ^{10}Thr or ^{94}Ser was intact. Nucleolin was coprecipitated with ^{10}Thr-intact forms in addition to ^{10}Thr-to-Asp–substituted forms. BAPTA-AM inhibited the phosphorylation and interaction of wild-type S100C/A11 with nucleolin by high

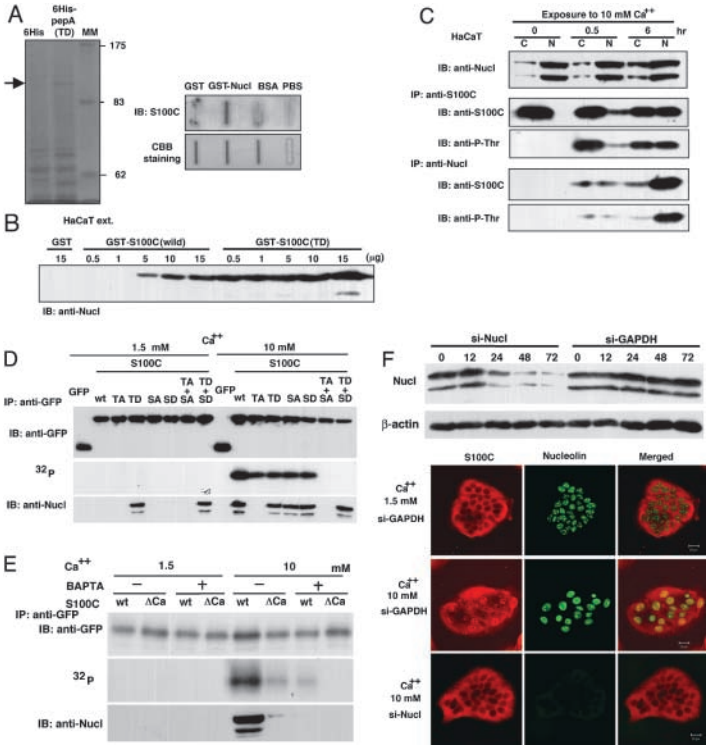

Figure 5. **Binding of phosphorylated S100C/A11 to nucleolin as a prerequisite for its nuclear translocation.** (A) Binding of S100C/A11 to nucleolin. Left: HaCaT cell extract was applied onto a pepA(TD) column (a phosphorylated pepA equivalent), and the bound fraction was electrophoresed and silver stained. MM, molecular marker. An ~100-kD protein band was identified (arrow). Right: S100C/A11 in the cell extract bound to recombinant nucleolin immobilized on a membrane. (B) Binding of nucleolin to S100C/A11-GST or S100C/A11(TD)-GST beads. Extracts from HaCaT cells were applied onto the beads, and the bound fractions were analyzed by Western blotting using anti-nucleolin antibody. (C) At various times after increasing the Ca^{2+} level, cytoplasmic (C) and nuclear (N) extracts were prepared and analyzed by immunoblotting (IB) with or without prior immunoprecipitation (IP) using indicated antibodies. On exposure to high Ca^{2+}, S100C/A11 was phosphorylated, bound to nucleolin, and transferred to nuclei. (D) Binding of S100C/A11 to nucleolin is affected by the 10th residue. Various GFP-S100C constructs were transfected to HaCaT cells 36 h before the increase of Ca^{2+} concentration. TA, ^{10}Thr to A; TD, ^{10}Thr to D; SA, ^{94}Ser to A; SD, ^{94}Ser to D. TA + SA and TD + SD were double mutants. Immunoprecipitates of ^{32}P-labeled cell extracts by anti-GFP antibody were immunoblotted with either anti-GFP (top) or anti-nucleolin antibody (bottom) or autoradiographed (middle). (E) Necessity of Ca^{2+} binding to S100C for the interaction with nucleolin in HaCaT cells. Plasmid constructs of wild-type (wt) S100C/A11 and a variant lacking Ca^{2+}-binding capacity (ΔCa) were transfected and analyzed under conditions similar to D. 30 μM BAPTA-AM was added 1 h before increasing Ca^{2+} concentration. (F) Top: time-dependent reduction of the nucleolin levels in HaCaT cells by 0.2 μM siRNA. Bottom: 48 h after the application of siRNA, extracellular Ca^{2+} concentration was shifted up and incubated for a further 6 h. The cells were then fixed and immunostained. Bar, 20 μm.

Ca^{2+} (Fig. 5 E). The S100C/A11 variant protein lacking Ca^{2+}-binding capacity was neither phosphorylated nor coprecipitated with nucleolin by high Ca^{2+} (Fig. 5 E).

When small interfering RNA (siRNA) against nucleolin was applied to HaCaT cells, the protein levels were substantially reduced (Fig. 5 F, top), whereas the control siRNA against GAPDH showed no effects. Increase in Ca^{2+} concentration translocated endogenous S100C/A11 into nuclei in the siRNA against GAPDH-treated cells, but not in the siRNA against nucleolin-treated cells (Fig. 5 F, bottom). The necessity of nucleolin for the Ca^{2+}-induced nuclear translocation of S100C/A11 was also confirmed in an in vitro translocation assay using permeabilized HaCaT cells by depleting nucleolin with a specific antibody (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200304017/DC1). These results indicate that (1) high Ca^{2+} treatment of HaCaT cells induces endogenous activity to phosphorylate S100C/A11 at ^{10}Thr and ^{94}Ser, and eventually enhances the binding of S100C/A11 to nucleolin; (2) S100C/A11 binds to nucleolin through its NH_2-terminal region in a ^{10}Thr phosphorylation–dependent manner; (3) the binding of S100C/A11 to nucleolin is essential for the translocation of S100C/A11 to nuclei; and (4) the binding of Ca^{2+} to S100C/A11 is necessary for the phosphorylation, binding to nucleolin, and nuclear translocation of S100C/A11.

The effector protein for growth inhibition by S100C/A11 is p21$^{CIP1/WAF1}$

The next question is how nuclear S100C/A11 exerts its inhibitory action on the growth of keratinocytes. We screened a number of possible candidates and found that p21$^{CIP1/WAF1}$ mRNA was remarkably induced in NHK cells either cultivated at 1.5 mM Ca^{2+} or exposed to TAT-S100C/A11 (Fig. 6 A). p16^{INK4a} was also induced, but to a moderate extent. Similar induction was observed in HaCaT cells in a time-dependent manner (Fig. 6 B).

To confirm the role of S100C/A11 on the induction of p21$^{CIP1/WAF1}$, anti-S100C/A11 antibody was introduced into HaCaT cells by the aid of Chariot or polyethyleneimine (PEI). The polyclonal antibody that we used was specific as shown in Fig. 6 C, and was demonstrated to recognize the NH_2-terminal pepA and COOH-terminal pepF of S100C/A11 (unpublished data). Binding of the introduced antibody to endogenous S100C/A11 was demonstrated by immunoprecipitation (Fig. 6 D). Introduction of the antibody inhibited the high Ca^{2+}-induced phosphorylation of S100C/A11 (Fig. 6 E). Fab fragments of the anti-S100C/A11 antibody also reduced the phosphorylation of S100C/A11 in HaCaT cells (Fig. S8, available at http://www.jcb.org/cgi/content/full/jcb.200304017/DC1). The induction of p21$^{CIP1/WAF1}$ by TAT-S100C/A11 was dose-dependently abrogated by the antibody introduced into the cells (Fig. 6 F). The decrease in the amount of p21$^{CIP1/WAF1}$ protein due to the action of anti-S100C/A11 antibody was associated with the recovery of cdk2 activity as assayed in vitro (Fig. 6 F). Inhibition of cdk2 activity by S100C/A11 introduced into HaCaT cells was dose- and time-dependent. Finally, anti-p21$^{CIP1/WAF1}$ antibody (but not anti-p16^{INK4a} antibody) completely abrogated the growth inhibition by S100C/A11 as assayed for [^{3}H]TdR incorporation or for cdk2 activity (Fig. 6, G and H), indicating

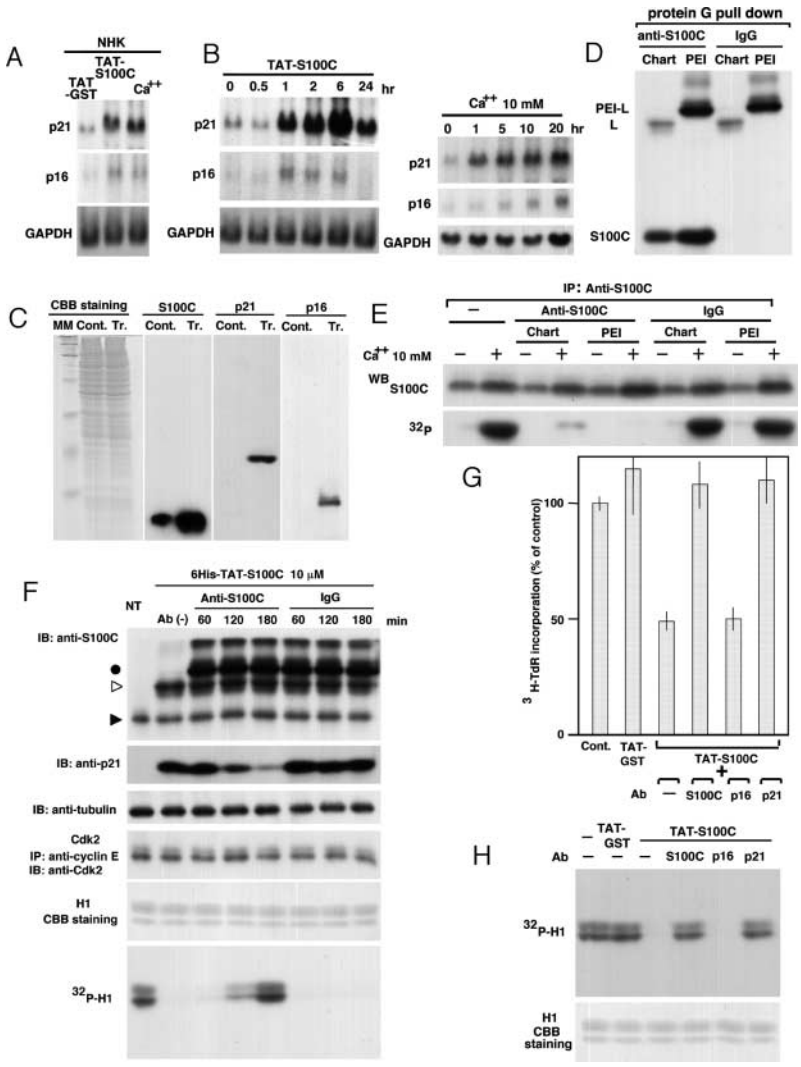

Figure 6. **Induction of Cdk inhibitors and concomitant inhibition of Cdk2 by S100C/A11.** (A) Induction of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ by TAT-S100C/A11 or high Ca^{2+} applied to NHK cells for 6 h as assayed by Northern blot analysis. (B) Time-dependent induction of $p21^{CIP1/WAF1}$ and $p16^{INK4a}$ by TAT-S100C/A11 or high Ca^{2+} in HaCaT cells. (C) Specificity of antibodies used. HaCaT cells with (Tr.) or without (Cont.) transfection of each construct were analyzed by Western blotting. MM, molecular marker. (D) Binding of anti-S100C/A11 antibody to S100C/A11 in HaCaT cells. Anti-S100C/A11 antibody and the control IgG were introduced into the cells by Chariot (Chart) or polyethyleneimine (PEI). 2 h later, fractions of cell extracts bound to protein G were immunoblotted using anti-S100C/A11 antibody. L, light chain of the antibodies; PEI-L, light chain of the antibodies conjugated with PEI. (E) Blocking of the phosphorylation of S100C/A11 by anti-S100C/A11 antibody introduced into cells. The antibody and IgG were introduced as in D. Anti-S100C/A11 antibody was added to the cell extracts to pull down S100C/A11 in the IgG-treated group as well. The antibodies were recovered by protein A and the bound proteins were analyzed by Western blotting using anti-S100C/A11 antibody (top) or by autoradiography (bottom). (F) Induction of $p21^{CIP1/WAF1}$ protein by TAT-S100C/A11 was blocked by anti-S100C/A11 antibody introduced into HaCaT cells. TAT-S100C/A11 was applied 6 h before the application of the antibody. At various times after applying the antibodies, cell extracts were immunoblotted using anti-S100C/A11 antibody (closed circle, L-chain; open arrowhead, His_6-TAT-S100C; closed arrowhead, endogenous S100C/A11), anti-$p21^{CIP1/WAF1}$, or anti-tubulin. The extracts were immunoprecipitated with anti-cyclin E antibody and blotted with anti-Cdk2 antibody. Amounts and radioactivity of histone H1, the substrate of in vitro Cdk2 kinase assay, were shown. (G) Anti-$p21^{CIP1/WAF1}$ antibody blocked growth inhibition by S100C/A11. HaCaT cells were incubated with the indicated antibodies with Chariot. 2 h later, TAT-S100C/A11 was applied and the cells were cultivated for 6 h before harvest. (H) Under experimental conditions similar to G, anti-$p21^{CIP1/WAF1}$ antibody (but not anti-$p16^{INK4a}$ antibody) blocked the inhibition of Cdk2 activity by TAT-S100C/A11.

that $p21^{CIP1/WAF1}$ is the principal effector protein of the S100C/A11-mediated growth inhibition of human keratinocytes. Specificity of anti-$p21^{CIP1/WAF1}$ and anti-$p16^{INK4a}$ antibodies was confirmed as shown in Fig. 6 C.

In nuclei, S100C/A11 competitively binds to nucleolin and liberates Sp1 and Sp3, which eventually induce $p21^{CIP1/WAF1}$

Next, we addressed the question of how S100C/A11 in the nuclei induces the effector $p21^{CIP1/WAF1}$. Screening using Signal Transduction AntibodyArray™ (Hypromatrix) resulted in identification of Sp1 as a protein that binds to nucleolin, and the binding was inhibited by the addition of S100C/A11(TD) (Fig. 7 A). Anti-Sp2 antibody (but not anti-Sp3 antibody) was also on the membrane, but showed a negative result. Binding of Sp1 to nucleolin was confirmed by applying the extract of HaCaT cells onto GST-nucleolin beads (Fig. 7 B). Sp1 did not bind directly to S100C/A11.

To examine whether nucleolin binds to Sp1 in the cells, we performed a coprecipitation assay (Fig. 7 C). Sp1 and S100C/A11 were detected in the precipitates prepared using anti-nucleolin antibody, whereas nucleolin, but not S100C/A11, was detected in the precipitates prepared using anti-Sp1 antibody. These results indicate that binding among the proteins actually takes place in vivo, and that S100C/A11 does not bind to Sp1. In HaCaT cells cultivated in the presence of 1.5 mM Ca^{2+}, nucleolin bound to Sp1, but not to S100C/A11. When the Ca^{2+} concentration was increased to 10 mM, S100C/A11 instead of Sp1 was detected in the immunoprecipitates prepared using anti-nucleolin antibody, and the bound S100C/A11 was phosphorylated. When the extract was treated with alkaline phosphatase in advance, the amount of S100C/A11 bound to nucleolin was dramatically reduced and the binding of Sp1 was recovered concomitantly. Immunoprecipitation with anti-Sp1 antibody confirmed that the stimulation with high Ca^{2+} liberated Sp1 from nucleolin.

Regions of nucleolin and Sp1 involved in the interaction were determined using recombinant partial proteins (Fig. 7 E). The COOH-terminal region of Sp1 (segment 3) includ-

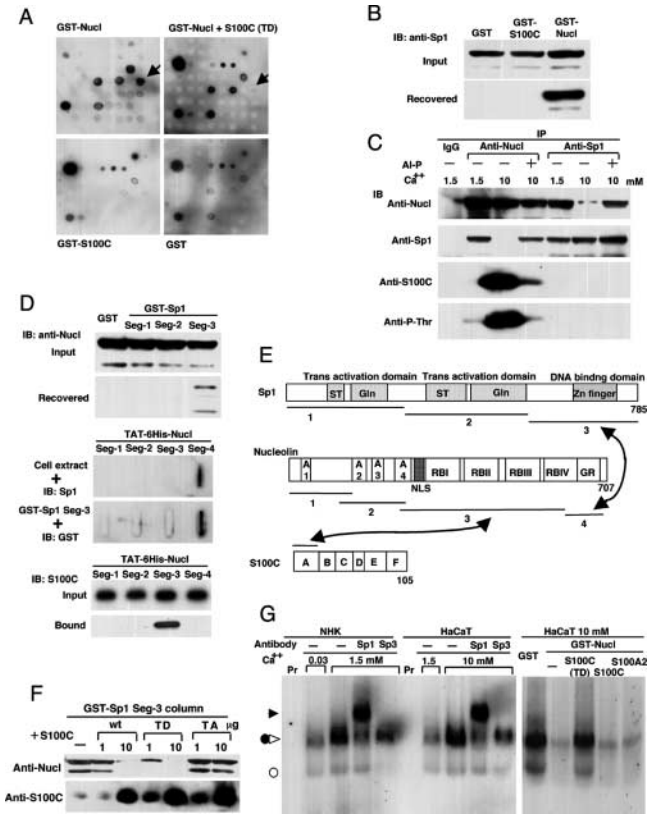

Figure 7. **Induction of p21$^{CIP1/WAF1}$ via Sp1 by S100C/A11.** (A) HaCaT cell extract was applied onto Signal Transduction AntibodyArray™, followed by incubation with either GST-nucleolin alone or GST-nucleolin mixed with S100C/A11(TD), and was visualized with HRP-conjugated anti-GST antibody. The arrows indicate spots showing positive binding to nucleolin and specific abrogation of the binding by the presence of S100C/A11(TD). The spot is anti-Sp1 antibody. Incubation with either GST-S100C/A11 or GST was used as a background control. (B) HaCaT cell extract was incubated with beads conjugated with GST, GST-S100C/A11, or GST-nucleolin. Sp1 in the extract bound to nucleolin, but not to S100C/A11. (C) HaCaT cell extracts were immunoprecipitated with anti-nucleolin or anti-Sp1 antibody, followed by blotting with indicated antibodies. A part of the extracts was treated with alkaline phosphatase before immunoprecipitation. (D) Binding regions of Sp1 and nucleolin were determined using recombinant partial proteins indicated in E. Sp1: Seg-1, residues 1–260; Seg-2, residues 261–540; Seg-3, residues 541–785. Nucleolin: Seg-1, residues 1–141; Seg-2, residues 142–270; Seg-3, residues 271–643; Seg-4, residues 644–707. Top: nucleolin in the HaCaT cell extract bound to Seg-3 of Sp1. Middle: Sp1 in the cell extract and recombinant Sp1 Seg-3 bound to Seg-4 of nucleolin. Bottom: S100C/A11 was recovered only from beads conjugated with Seg-3 of nucleolin. (E) Schematic representation of interacting segments of Sp1, nucleolin, and S100C/A11. (F) Interaction among Sp1, nucleolin, and S100C/A11. HaCaT cell extracts were applied onto an immobilized recombinant Sp1 Seg-3 column and the bound fractions were analyzed by Western blotting using anti-nucleolin antibody. (G) A gel shift assay performed using the GC-rich regions of the human p21$^{CIP1/WAF1}$ promoter as probes. Left: nuclear extracts were mixed with the probes with or without an antibody against Sp1 or Sp3. Expected positions of shifted bands (open arrowhead for Sp1 and open circle for Sp3) and super-shifted bands by the antibodies (closed arrowhead for Sp1 and closed circle for Sp3) are indicated. Right: under similar conditions, the band shift was abrogated by addition of GST-nucleolin. The amount of the shifted bands reduced by nucleolin was recovered when S100C/A11(TD) (but not wild-type S100C/A11 or S100A2) was added into the reaction tube. Amounts of recombinant proteins, 1 μg.

ing a zinc-finger domain was shown to be responsible for the binding to nucleolin (Fig. 7 D, top). In turn, Sp1 in the cell extract bound to segment 4 of nucleolin immobilized on a membrane (Fig. 7 D, middle). Recombinant Sp1 segment 3 also bound to the nucleolin segment 4, indicating direct interaction between the two proteins. S100C/A11 was recovered only from beads conjugated with segment 3 of nucleolin (Fig. 7 D, bottom). When segment 3 of nucleolin was introduced into HaCaT cells, the Ca^{2+}-induced p21$^{CIP1/WAF1}$ protein level was decreased and the activity of cdk2 was recovered within 8 h, despite continuous cultivation at 10 mM Ca^{2+} (unpublished data). This is possibly due to a dominant-negative effect of the peptide through sequestering S100C/A11 by binding. The interacting regions of the proteins are depicted in Fig. 7 E.

Binding of nucleolin in cell extracts to Sp1 (segment 3) was dose-dependently inhibited by recombinant S100C/A11 (Fig. 7 F). S100C/A11(TD) showed a stronger effect, whereas S100C/A11(TA) did not affect the binding between nucleolin and Sp1 (segment 3).

Because Sp3 is also known to activate the p21$^{CIP1/WAF1}$ promoter (Prowse et al., 1997), we examined possible interaction with nucleolin and S100C/A11 and found that (1) Sp3 can bind to nucleolin segment 4 via its COOH-terminal zinc-finger domain; and (2) the binding of Sp3 to nucleolin was inhibited by S100C/A11 (unpublished data).

A gel shift assay was performed using GC-rich regions of the p21$^{CIP1/WAF1}$ promoter, the well-known Sp1/Sp3-binding sites (see Materials and methods). As shown in Fig. 7 G, exposure of NHK and HaCaT cells to high Ca^{2+} resulted in an increase in the intensity of the shifted bands by Sp1 and Sp3, which were further retarded by the addition of anti-Sp1 or -Sp3 antibody. Under similar experimental conditions in which HaCaT cells were cultivated in a high Ca^{2+} medium, addition of GST-nucleolin into the incubation mixtures inhibited the extent of the band shift as compared with GST (Fig. 7 G, right). This was possibly due to sequestration of Sp1 by exogenous nucleolin. The inhibition of the band shift by GST-nucleolin was abrogated by the ^{10}Thr phosphorylation equivalent form S100C/A11(TD), whereas wild-type S100C/A11 and S100A2 showed no effect. We found that the interaction of the proteins were dose-dependent, and addition of an excess amount of wild-type S100C/A11 showed recovering effects similar to S100C/A11(TD) (unpublished data). These results indicate the competitive nature of the binding between S100C/A11 and Sp1/Sp3 to nucleolin. We performed a series of luciferase assays using a plasmid containing the p21$^{CIP1/WAF1}$ promoter region (Nakano et al., 1997) and obtained essentially the same results as those obtained by using the gel shift assay (unpublished data). Deletion of the Sp1/Sp3-binding sites in the promoter abrogated the induction of luciferase activity by S100C/A11.

Biological relevance of the S100C/A11 pathway as a mediator of the growth inhibition of keratinocytes

The findings described in the previous paragraphs indicate that S100C/A11 is involved in growth inhibition of human keratinocytes induced by high Ca^{2+} (Fig. 8 A). Under low Ca^{2+} conditions, S100C/A11 is present mostly in the cytoplasm. The unphosphorylated S100C/A11 partly binds to

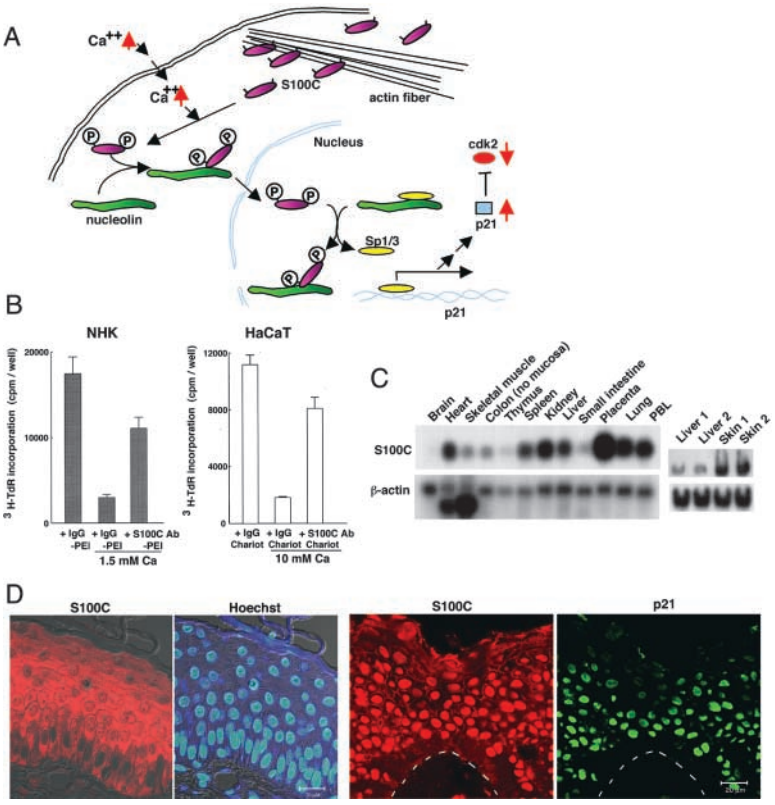

Figure 8. **An S100C/A11-mediated pathway for inhibition of cell growth and implication of its biological significance.** (A) A schematic diagram of the S100C/A11-mediated pathway revealed in the present paper. (B) Significance of the S100C/A11-mediated pathway in Ca^{2+}-induced growth inhibition of human keratinocytes. Anti-S100C/A11 antibody was introduced 2 h before increasing the Ca^{2+} concentrations, and the cells were cultivated for a further 10 h. (C) Northern blot analysis of various human tissues for S100C/A11 expression. PBL, peripheral blood leukocytes. (D) Immunostaining of normal human epidermis for S100C/A11 and $p21^{CIP1/WAF1}$ protein in paraffin (left) and frozen (right) sections. The nuclei of cells in the basal layer were specifically free from S100C/A11 protein (left). In the double-staining picture, note that $p21^{CIP1/WAF1}$ was detected only in the nuclei that were positive for S100C/A11 (right). Dotted line indicates the boundary between the epidermis and dermis. Bars, 20 μm.

actin fiber via its COOH-terminal region (unpublished data). An increase in intracellular Ca^{2+} results in activation of an as-yet-unidentified protein kinase(s) that phosphorylates S100C/A11 at ^{10}Thr and ^{94}Ser. Phosphorylated S100C/A11 binds to nucleolin via its NH_2-terminal region and is translocated to nuclei. In the nuclei, S100C/A11 competes with Sp1 and Sp3 for binding to nucleolin, and the resulting free Sp1 and Sp3 induce $p21^{CIP1/WAF1}$.

A question that arises is to what extent the pathway is biologically relevant. To address this question, anti-S100C/A11 antibody was introduced into NHK and HaCaT cells by PEI and Chariot, respectively. The introduction efficiency was confirmed to be >70% by immunostaining. The introduced antibody inhibited the high Ca^{2+}-induced phosphorylation of S100C/A11 (Fig. 6 E). High Ca^{2+} suppressed DNA synthesis to ~17% of the control level, and the antibody recovered the DNA synthesis to 64 and 72% of the control level in NHK and HaCaT cells, respectively, indicating that S100C/A11 is a principal (if not sole) mediator of growth regulation (Fig. 8 B). The incomplete recovery may be either due to incomplete blockage of S100C/A11 function by the introduced antibody or due to the presence of some additional pathway mediating high Ca^{2+}-induced growth inhibition.

S100C/A11 was found to be expressed at various levels in many human tissues (Fig. 8 C), among which skin showed a particularly high level of S100C/A11 expression. When normal human skin tissues were stained with anti-S100C/A11 antibody, S100C/A11 protein was detected in epidermal keratinocytes (Fig. 8 D). Nuclei of cells in the basal layer were consistently negative for S100C/A11 protein, whereas nuclei of cells in the suprabasal layers were positive. $p21^{CIP1/WAF1}$ protein was not detected in the basal layer, but appeared in suprabasal cell nuclei in which S100C/A11 was present. Among 21 nuclei counted in the basal layer, 20 and 1 nuclei were negative and positive for both S100C/A11 and $p21^{CIP1/WAF1}$, respectively. In the suprabasal layer, 89 nuclei were positive for S100C/A11 and $p21^{CIP1/WAF1}$, whereas 11 nuclei were positive for S100C/A11, but negative for $p21^{CIP1/WAF1}$. No S100C/A11-negative nuclei expressed $p21^{CIP1/WAF1}$. These results indicate that S100C/A11 not only mediates high Ca^{2+}-induced growth inhibition of human keratinocytes in culture, but most likely plays a key role in the growth regulation in vivo as well.

Discussion

We have demonstrated that an S100C/A11-mediated pathway plays a key role in high Ca^{2+}-induced growth inhibition of human keratinocytes in culture. This finding has substantial biological significance for the following reasons: (1) high Ca^{2+} is a physiological inducer of growth arrest and differentiation of human keratinocytes in culture, and probably in vivo as well; (2) prior introduction of anti-S100C/A11 antibody resulted in recovery of the high Ca^{2+}-induced growth inhibition of human keratinocytes by ~70%, indicating that S100C/A11 is a key (if not the sole) mediator of growth regulation; and (3) immunostaining of the human epidermis for S100C/A11 and $p21^{CIP1/WAF1}$ showed a picture consistent with the model presented as a summary of the results of the present report using cultured human keratinocytes (Fig. 8 A).

Phosphorylation and nuclear translocation of S100C/A11

Endogenous S100C/A11 was phosphorylated at ^{10}Thr and ^{94}Ser in HaCaT cells exposed to high Ca^{2+} (Fig. 3 D),

whereas only ^{10}Thr was shown to be the major target of phosphorylation in confluent normal human fibroblasts (Sakaguchi et al., 2000). The phosphorylation of S100C/A11 at ^{10}Thr stimulated binding to nucleolin via its NH_2-terminal region, this being a prerequisite for nuclear translocation of S100C/A11 (Fig. 5, C–F). In human keratinocytes, some of the cytoplasmic S100C/A11 proteins were colocalized with actin fibers. We found that S100C/A11 binds to actin through the COOH-terminal region and that phosphorylation of ^{94}Ser reduced the affinity (unpublished data). The liberation of S100C/A11 from actin may facilitate the association with nucleolin and the eventual nuclear translocation of S100C/A11.

Ca^{2+} binds to S100C/A11 and induces its conformational changes (Rety et al., 2000). The binding of Ca^{2+} to S100C/A11 appears to be critical for the S100C/A11-mediated growth inhibition because interference of the binding either by amino acid substitution or with a membrane-permeable Ca^{2+} chelator (BAPTA-AM) inhibited phosphorylation, binding to nucleolin, and nuclear translocation of S100C/A11 (Fig. 4, A and C; Fig. 5 E). Cellular kinase activity for S100C/A11 as assayed in vitro using S100C/A11 or pepA was induced by high Ca^{2+} in NHK and HaCaT cells. The kinase activity was detected in a cytosolic-soluble fraction, but not in an insoluble or nuclear fraction, of HaCaT cells exposed to high Ca^{2+}. When the cell extract was applied onto a cation exchange column, the activity was eluted with 160–180 mM KCl (unpublished data). Further characterization of the kinase(s) is now in progress in our laboratory.

Interaction among nucleolin, S100C/A11, and Sp1/Sp3

Nucleolin was first identified as a protein involved in ribosomal assembly and maturation, but was later found to have diverse biological functions (Srivastava and Pollard, 1999). Although nucleolin is mainly localized in the nucleus, it shuttles between the nucleus and cytoplasm and is even present on the cell surface. When we microinjected wheat germ hemagglutinin into HaCaT cells, which is known to generally inhibit nuclear import of proteins (Finlay and Forbes, 1990), nucleolin was diffusely distributed in the cells (unpublished data). Recently, Shibata et al. (2002) reported that nucleolin mediates a signal from the cell surface to the nucleus. Nucleolin inhibited transcriptional activation by A-Myb through specific binding (Ying et al., 2000). Heat shock or irradiation was shown to mobilize nucleolin from nucleoli to promote the formation of a nucleolin–p53 complex (Daniely et al., 2002). In the present work, we showed that S100C/A11 and Sp1/Sp3 compete for binding to nucleolin. This is the first report of nucleolin providing a site for switching transcriptional activation through protein–protein interaction. Unexpectedly, it was found that S100C/A11 and Sp1/Sp3 bind to different regions of nucleolin (Fig. 7 E). Binding of S100C/A11 may change the conformation of nucleolin so that it can no longer hold Sp1 and Sp3. Although nucleolin is notoriously sticky, the interaction with Sp1/3 and S100C/A11 is unlikely to be nonspecific because (1) only the specific regions of the respective proteins show the binding capacity; and (2) other members of S100, such as S100A2 and S100A6, did not bind to nucleolin.

Transcriptional activation of $p21^{CIP1/WAF1}$ by Sp1 and Sp3

Sp1/krupple-like factors act as ubiquitous and tissue-restricted transactivators in many different contexts. Sp1 and Sp3 were shown to activate the $p21^{CIP1/WAF1}$ promoter (Prowse et al., 1997). In the gel shift assay, the shifted bands shown in Fig. 7 G were further retarded by incubation with anti-Sp1 and anti-Sp3 antibody. We performed a series of luciferase assays using the plasmid containing the human $p21^{CIP1/WAF1}$ promoter sequence of 2.4 kb (Nakano et al., 1997). Application of TAT-S100C/A11 induced the activity of the $p21^{CIP1/WAF1}$ promoter by more than 10-fold in HaCaT cells in an Sp1/Sp3 site-dependent manner (unpublished data). This induction was abrogated by pretreatment of the cells with TAT-nucleolin segment 3, but not TAT-nucleolin segment 4. These results indicate that $p21^{CIP1/WAF1}$, the effector protein of S100C/A11-mediated growth inhibition (Fig. 6 G), is transcriptionally activated by Sp1 and Sp3 in human keratinocytes when exposed to high Ca^{2+}. This induction must be p53-independent because HaCaT cells are known to have only mutated p53 genes (Lehman et al., 1993).

Biological effects of $p21^{CIP1/WAF1}$ in human keratinocytes

There remains little doubt that $p21^{CIP1/WAF1}$, when expressed, inhibits the growth of keratinocytes through inactivating Cdks (Gartel et al., 1996). The results of immunostaining for $p21^{CIP1/WAF1}$ (Fig. 8 D) suggest that $p21^{CIP1/WAF1}$ is involved in growth arrest and differentiation of human epidermal keratinocytes in vivo. Various differentiation factors for human keratinocytes, including high Ca^{2+} (Todd and Reynolds, 1998), TGFβ (Datto et al., 1995), and TPA (Todd and Reynolds, 1998), are known to induce $p21^{CIP1/WAF1}$.

In the human epidermis, $p21^{CIP1/WAF1}$ protein was detected in the lower and middle suprabasal layers, but not in the uppermost layers (Fig. 8 D; Ponten et al., 1995). This seems reasonable because overexpressed $p21^{CIP1/WAF1}$ was shown to inhibit terminal differentiation of mouse keratinocytes at later stages (Di Cunto et al., 1998). Alternatively, $p21^{CIP1/WAF1}$ may simply not be needed in irreversibly arrested cells.

Regulation of growth and differentiation in keratinocytes

TGFβ transduces signals via Smad proteins. In HaCaT cells treated with TGFβ, Smad3 and Smad4 were found to cooperate with Sp1 for transcriptional activation of the $p21^{CIP1/WAF1}$ promoter (Pardali et al., 2000). When we treated HaCaT and NHK cells with TGFβ, S100C/A11 was translocated to nuclei (unpublished data). Thus, S100C/A11 may be a partial converging point of high Ca^{2+} and TGFβ.

The proposed S100C/A11-mediated pathway for Ca^{2+}-induced growth arrest (Fig. 8 A) has partly revealed the thus-far-unknown link from the initial events triggered by high Ca^{2+} to the induction of Cdk inhibitors, particularly $p21^{CIP1/WAF1}$. However, we still have little information on how S100C/A11 is phosphorylated upon stimulation by high Ca^{2+}. The specific phosphorylation observed in the present paper in terms of culture conditions and substrates (Fig. 3, B and D) provides an opportunity for the identification of the involved protein kinase(s). Furthermore, the

function of S100C/A11 for liberation of Sp1 and Sp3 in nuclei was born by residues 1–23 of the NH_2-terminal peptide. Considering the ubiquitous expressions of nucleolin and Sp1/Sp3 and the large variety of promoters that are regulated by Sp1 and Sp3, further narrowing down of the functional structural domain of the peptide and screening for chemicals that have similar structure may be a promising venture eventually leading to pharmacologically useful substances.

Materials and methods

Cells

NHK cells (Kurabo Industries, Ltd.) were cultured in Defined Keratinocyte-SFM (GIBCO BRL) with Defined Keratinocyte-SFM Growth Supplement (GIBCO BRL). HaCaT cells, a gift from Dr. Fusenig (German Cancer Research Center, Heidelberg, Germany), were cultured in DME with 10% FBS. For monitoring DNA synthesis, 1 μCi/ml tritiated thymidine ([^{3}H]TdR; American Radiolabeled Chemicals) was added to the cultures 1 h before cell harvest. An anti-BrdU antibody (Neomarkers) was used to stain BrdU-incorporated cells. Subfractionation of cells was performed as described previously (Lindeman et al., 1997). Imaging analysis of the intracellular Ca^{2+} level was performed as reported previously (Yamamoto et al., 2000).

Determination of phosphorylation sites

To determine the phosphorylation sites of endogenous S100C/A11, the protein was collected from HaCaT cells cultivated in the low or high Ca^{2+} medium using an affinity column with anti-S100C/A11 antibody. After further purification by PAGE, the protein was cleaved with cyanogen bromide and the peptides were fractionated by HPLC. Four peptides (Fig. 3 D) were identified by partial sequencing. pep1 was further cleaved with endoproteinase Asp-N (Roche). The purified p1-1 and p4 were subjected to NH_2-terminal sequencing on a peptide sequencer (model 491; Applied Biosystems). Phosphorylated amino acid residues were run through the column, and thus disappeared from the expected position of unphosphorylated residues.

Introduction of proteins into cells

Peptides and S100C/A11 protein with relatively low molecular sizes were introduced into cells by the aid of the protein transduction domain of TAT protein (YGRKKRRQRRR) from human immunodeficiency virus (Schwarze et al., 1999). A Chariot kit (active motif) and cationic PEI-conjugated S100C/A11 antibody were used to introduce antibodies into HaCaT and NHK cells, respectively. To functionally inactivate proteins within the cells, rabbit anti–human S100C/A11, mouse anti–human $p16^{INK4a}$ (Ab-4, clone 16P04; Neomarkers), and mouse anti-$p21^{CIP1/WAF1}$ (Ab-11, clone CP74: Neomarkers) antibodies were used. The anti-S100C/A11 antibody detects only a single band in Western blot analysis using cells irrespective to Ca^{2+} levels in the media (Fig. 6 C). Efficacy for neutralization of the target proteins $p16^{INK4a}$ and $p21^{CIP1/WAF1}$ by the antibodies was confirmed by in vitro phosphorylation assay.

Antibodies and plasmid constructs

Antibodies and plasmid constructs used in the present work are listed in the Online supplemental material section.

siRNA

An optimal target site of nucleolin mRNA for siRNA was determined as 5′-GACAGTGATGAAGAGGAGG-3′ by a program provided by QIAGEN, and the synthetic siRNA was purchased from the same company. siRNA against GAPDH mRNA (Ambion) was used as a control. The siRNA was transfected to the logarithmically growing HaCaT cells using LipofectAMINE™ 2000 (Invitrogen).

Cdk2 kinase assay

An in vitro cdk2 kinase assay was performed using immunoprecipitates with anti–human cyclin E antibody and histone H1 as a substrate as described previously (Ohashi et al., 1999).

Antibody array

Signal Transduction AntibodyArray™ (Hypromatrix) was used under the conditions indicated by the manufacturer. In brief, the antibody membrane was incubated with 1-mg HaCaT cell extracts, followed by the application of 10-μg full-length recombinant proteins (GST, GST-S100C/A11, GST-nucleolin, or GST-nucleolin + S100C/A11(TD)). After washing, the bound proteins were detected using HRP-conjugated anti-GST antibody (Santa Cruz Biotechnology, Inc.).

Gel shift assay

A gel mobility shift assay was performed as described by Nakano et al. (1997). We used GC-rich regions located between 120 and 94 bp (A), 93 and 74 bp (B), and 73 and 47 bp (C) of the human $p21^{CIP1/WAF1}$ promoter as probes. The ^{32}P-labeled probes (A + B + C; 10^4 cpm) were mixed with 4 μg crude nuclear extracts of NHK or HaCaT cells, incubated for 20 min at RT, and electrophoresed in a 5% polyacrylamide gel under nondenaturing conditions. For a super-shift experiment, 1 μg mouse anti–human Sp1 antibody (Santa Cruz Biotechnology, Inc.) or rabbit anti–human Sp3 antibody (Santa Cruz Biotechnology, Inc.) was added to the reaction mixture.

Acquisition and processing of images

For GFP-labeled vital cells, images were acquired by a fluorescent microscope (IX71–22FL/PH; CCD camera, DP50; objective lens, LCPlan F1 40×; Olympus) and processed using Adobe Photoshop® 6.0. For fixed cells labeled with FITC, Alexa® 488, or TRITC, a laser-scanning microscope (Axioplan 2; objective lens, Plan-Apochromat® 63× 1.4 oil DC and Plan-Neofluar® 40×/0.75; Carl Zeiss MicroImaging, Inc.) was used.

Online supplemental material

Fig. S1 shows dose-dependent inhibition of DNA synthesis by TAT-S100C. Fig. S2 shows expression of variant S100C/A11 proteins. Fig. S3 shows effects of high Ca^{2+} on endogenous S100C/A11 levels. Fig. S4 depicts intracellular localization of nucleolin. Fig. S5 shows necessity of nucleolin for nuclear translocation of S100C/A11. Fig. S6 depicts binding of S100C/A11 pepA with nucleolin. Fig. S7 shows proteins recovered by protein G from HaCaT cells applied with anti-S100C antibody or IgG. Fig. S8 shows Fab of anti-S100C/A11 antibody and reduced phosphorylation level of S100C/A11 induced by high Ca^{2+}. Fig. S9 depicts induction of $p21^{CIP1/WAF1}$ by variant S100C/A11 proteins. Fig. S10 shows interaction among nucleolin, Sp1, and S100C/A11. Fig. S11 shows competition of S100C/A11 peptides with Sp1 and Sp3 for binding to nucleolin. Fig. S12 shows the dominant-negative effect of nucleolin Seg-3 for the induction of $p21^{CIP1/WAF1}$ by TAT-S100C/A11. The supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200304017/DC1.

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