

S100A11, an Dual Mediator for Growth Regulation of Human Keratinocytes

Masakiyo Sakaguchi,* Hiroyuki Sonegawa,* Hitoshi Murata,*[†] Midori Kitazoe,[†] Jun-ichiro Futami,[†] Ken Kataoka,* Hidenori Yamada,[†] and Nam-ho Huh*

*Department of Cell Biology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Shikatachou, Okayama 700-8558, Japan; and [†]Department of Bioscience and Biotechnology, Okayama University Graduate School of Natural Science and Technology, Tsushimanaka, Okayama 700-8530, Japan

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We previously revealed a novel signal pathway involving S100A11 for inhibition of the growth of normal human keratinocytes (NHK) caused by high Ca^{++} or transforming growth factor β . Exposure to either agent resulted in transfer of S100A11 to nuclei, where it induced p21^{WAF1}. In contrast, S100A11 has been shown to be overexpressed in many human cancers. To address this apparent discrepancy, we analyzed possible new functions of S100A11, and we provide herein evidence that 1) S100A11 is actively secreted by NHK; 2) extracellular S100A11 acts on NHK to enhance the production of epidermal growth factor family proteins, resulting in growth stimulation; 3) receptor for advanced glycation end products, nuclear factor- κ B, Akt, and cAMP response element-binding protein are involved in the S100A11-triggered signal transduction; and 4) production and secretion of S100A11 are markedly enhanced in human squamous cancer cells. These findings indicate that S100A11 plays a dual role in growth regulation of epithelial cells.

INTRODUCTION

S100 proteins are small molecules (10~12 kDa) with two EF-hands, a canonical hand at the C terminus and a modified, S100-specific hand at the N terminus (Donato 2001; Heizmann *et al.*, 2002; Marenholz *et al.*, 2004). Binding of Ca^{++} to EF-hand motifs changes the conformation and hence the function of S100 proteins (Rety *et al.*, 2000; Dempsey *et al.*, 2003). Although S100 family proteins share such structural features, they show different cell type- and functional state-specific expression profiles in different tissues, and they exhibit distinct functions in diverse physiological and pathological contexts (Donato 2001; Heizmann *et al.*, 2002; Emberley *et al.*, 2004; Marenholz *et al.*, 2004).

S100A11 (S100C) was cloned from the chicken gizzard in 1991 (Todoroki *et al.*, 1991), and it was later shown to bind to annexin A1 (Mailliard *et al.*, 1996), but its function has remained largely unknown. Because S100A11 is encoded in epidermal differentiation complex on chromosome 1 in humans, we studied the biological role of S100A11 in normal human keratinocytes (NHK), and we found that it is an essential element in the signal transduction for high Ca^{++} - and transforming growth factor (TGF) β -induced suppression of the growth of NHK (Sakaguchi *et al.*, 2003, 2004, 2005). On exposure of NHK to either agent, S100A11 was transferred to nuclei, where it induces p21^{WAF1}. Blocking the

S100A11-mediated pathway abrogated the growth suppression by high Ca^{++} and TGF β , even though the canonical nuclear factor of activated T cells (NFAT)1-mediated pathway for high Ca^{++} and the Smad-mediated pathway for TGF β remained intact. Thus, high Ca^{++} and TGF β have a common S100A11-mediated pathway in addition to unique pathways for exhibiting an inhibitory effect on the growth of NHK, and both pathways are indispensable (Sakaguchi *et al.*, 2005).

Conversely, S100A11 has been shown to be overexpressed in many different types of human cancer, including those derived from the esophagus (Ji *et al.*, 2004), stomach (Oue *et al.*, 2004), colorectum (Tanaka *et al.*, 1995; Stulik *et al.*, 1999), pancreas (Ohuchida *et al.*, 2006), thyroid (Torres-Cabala *et al.*, 2004), uterus (Kanamori *et al.*, 2004), and soft tissues (Schaefer *et al.*, 2004). Although the widely observed up-regulation of S100A11 indicates that S100A11 may be involved in growth enhancement, malignant progression of cancer cells, or both, no mechanistic interpretation has been provided. If S100A11 plays a role solely in a growth-suppressive pathway (Sakaguchi *et al.*, 2005), its up-regulation in cancer cells might simply be a compensatory response to enhanced growth of malignant cells. It is more likely, however, that S100A11 is involved in yet-unknown mechanisms featured in cancer cells.

Some S100 family members are known not only to exert their function intracellularly but also to be secreted and act on cell surface receptors. S100A8 and S100A9 are secreted by inflammatory cells, such as neutrophils, activated monocytes, and macrophages, and they act as chemotactic molecules for those cells (Roth *et al.*, 2003; Ryckman *et al.*, 2003; Foell *et al.*, 2004). Serum levels of S100A8 and S100A9 have been shown to be increased in chronic inflammatory diseases, including rheumatoid arthritis (Liao *et al.*, 2004), multiple sclerosis (Bogumil *et al.*, 1998), Crohn's disease (Luger-

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Address correspondence to: Nam-ho Huh (namu@md.okayama-u.ac.jp).

Abbreviations used: NHK, normal human keratinocytes; RAGE, receptor for advanced glycation end products.

ing *et al.*, 1995), and connective tissue diseases (Kuruto *et al.*, 1990). S100B is abundantly produced and secreted by astrocytes, and it functions as a trophic factor for neurons and glial cells in autocrine and paracrine manners (Adami *et al.*, 2001). Increased serum level of S100B has been observed in various inflammatory, ischemic, and psychiatric diseases of the human brain (Rothermundt *et al.*, 2003). Recently, Cecil *et al.* (2005) reported that S100A11 was secreted from and induced hypertrophic change in human chondrocytes.

In the present study, we examined possible new roles of S100A11 in an alternative pathway of growth regulation of NHK. We found that S100A11 is secreted from NHK and acts on the cells to promote cell growth via induction of epidermal growth factor (EGF). Thus, S100A11 functions as a dual mediator for growth regulation of NHK.

MATERIALS AND METHODS

Cells and Chemicals

Neonatal human epidermal keratinocytes (KURABO, Osaka, Japan) were cultured in EpiLife medium with HKGS Growth Supplement (Cascade Biologies, Portland, OR). A431, BSCC-93, and HSC-5 were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM with 10% fetal bovine serum (FBS). For A431, the medium was supplemented with 4 mM L-glutamate and 4.5 g/l glucose. Inhibitors for EGF receptor (AG1478), nuclear factor (NF)- κ B (NF- κ B activation inhibitor IV) and Akt (protein kinase B) were purchased from Calbiochem (San Diego, CA). Histamine, substance P, and recombinant human TGF β were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human EGF, interferon (IFN) α , tumor necrosis factor (TNF)- α , and interleukin (IL)-6 were provided by Peprotech (London, England). Recombinant proteins of human IL-1F9, IL-8/CXCL8, CXCL1, and soluble receptor for advanced glycation end products (RAGE) (sRAGE/Fc chimera), neutralizing mouse antibodies against human EGF and RAGE, and control mouse immunoglobulin (Ig)G (R&D Systems, Minneapolis, MN) were obtained from the designated sources.

Recombinant Protein Preparation

S100A11 cDNA was obtained by RT-PCR and integrated into pET3a (Novagen, Madison, WI). S100A11 protein was purified by differential precipitation using successively polyethylenimine and ammonium sulfate followed by ion exchange column chromatography. Dimer S100A11 was prepared by incubating S100A11 in a HEPES buffer, pH 7.5, at 4°C for 2 wk, and the resulting dimer was fractionated by gel filtration. For biotinylation of S100A11, 3-fold excess moles of Biotin-(AC₅)₂sulfo-OSu (Dojindo, Gaithersburg, MD) was reacted with S100A11 at room temperature for 2 h, and the nonreacted reagent was inactivated with Nap-5 (GE Healthcare Bio-Sciences, Little Chalfont, Buckinghamshire, United Kingdom). About 0.95 molecules of biotin was conjugated per S100A11 molecule as assayed by the HABA method (Pierce Chemical, Rockford, IL). Labeling of S100A11 by Cy3 was performed using a Cy3 antibody labeling kit (GE Healthcare Bio-Sciences).

cDNAs of S100A2 and S100A6 and cDNA of S100A10 were cloned into pGEX2T and pGEX6P1 vectors, respectively (GE Healthcare). The glutathione transferase (GST)-fused recombinant proteins were purified using a Sephadex 4B column after cleaving with Thrombin or PreScission protease (GE Healthcare Bio-Sciences) under conventional conditions.

Western Blot Analysis

Western blot analysis was performed under conventional conditions. Antibodies used are listed in Supplemental Material. The membranes were then treated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Cell Signaling Technology, Danvers, MA), and positive signals were finally visualized by a chemiluminescence system (ECL Plus; GE Healthcare Bio-Sciences).

Rabbit anti-human S100A11 antibody was raised in our laboratory using S100A11 dimer as an immunogen. The purified antibody was confirmed to react with monomer S100A11 and dimer S100A11 and also to neutralize S100A11 (Supplemental Figure S9).

Subfractionation of cells was performed using a subfractionation kit (NEPER nuclear and cytoplasmic extraction reagent kit; Pierce Chemical). The plasma membrane fraction was prepared under conditions similar to those described previously (Sarkadi *et al.*, 1992).

Northern Blot Analysis

Northern blot analysis was performed under conventional conditions. Twenty micrograms of total RNAs was electrophoresed, transferred to Nytran Plus nylon membranes (GE Healthcare Bio-Sciences), and probed with a

502-base pair fragment of EGF cDNA amplified using forward (5'-atgctgctcactcttatcat-3') and reverse (3'-ctggatcaactgctacattt-5') primers.

Arrays

Extracts prepared from NHK cultured with or without S100A11 were analyzed using a proteome profiler array (Human Phospho-MAPK Array; R&D Systems) and a cytokine antibody array (Human Cytokine Antibody Array IV; RayBiotech, Norcross GA).

A Method to Fish Promoter-binding Proteins

We newly developed a method to identify proteins binding to a long region of a given promoter (Supplemental Figure S5). Nuclear extracts preabsorbed with an excess amount of streptavidin agarose were incubated in a buffer [0.1% Triton X-100, 60 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 50 μ g/ml poly(dI-dC), 1 mM dithiothreitol (DTT), 5% glycerol, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 200 μ g/ml phenylmethanesulfonyl fluoride, and 20 mM HEPES, pH 7.4] at 4°C for 120 min with a biotinylated 2081-base pair genomic fragment of the human EGF promoter that was prepared using forward (5'-biotin-atctgggcaactgtagatga-3') and reverse (3'-agggtgagatgattgacc-biotin-5') primers. The resulting protein-primer complex was isolated with streptavidin agarose (Invitrogen, Carlsbad, CA) and then subjected to Western blot analysis. Oligomers used for competitive experiments for which results were shown in Figure 4C were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed under conditions similar to those described previously (Sakaguchi *et al.*, 2005). Rabbit antibodies against human cAMP response element-binding protein (CREB) and c-Jun were obtained from Santa Cruz Biotechnology. Forward (5'-agcgagttatctctcttggcagct-3') and reverse (3'-acagagcaaggcaaggcttagaga-5') primers were used to amplify a 290-base pair fragment covering an AP1 site of the EGF promoter (-167 to +122).

Small Interfering RNAs (siRNAs)

siRNAs against human CREB (predesigned siRNA for CREB) and control siRNA (Silencer Negative control 2 siRNA) were purchased from Ambion (Austin, TX). siRNAs were transfected using Lipofectamine 2000 (Invitrogen).

Luciferase Assay

Genomic fragments of 2081 and 779 base pairs of the human EGF promoter were inserted into pGL4.14-Basic (Promega, Madison, WI) at Kpn1 and Xho1 sites. For more detailed conditions, see Supplemental Material. Nucleotide sequences of the constructs were confirmed by sequencing.

The plasmids were cotransfected with a pTracer-EF/lacZ vector (Invitrogen) into NHK by using Trans IT-keratinocyte transfection reagent (Mirus, Madison, WI). Luciferase activity of cell extracts was measured with a Luciferase reporter assay system (LucLite; Packard Bioscience, Groningen, The Netherlands). The luciferase activities were normalized to β -galactosidase activity derived from a cotransfected plasmid.

RESULTS

Secretion of S100A11 by NHK

At first, we examined possible secretion of S100A11 by NHK. NHK were incubated in EpiLife without HKGS. Addition of FBS, EGF, and IL-1F9 dose-dependently enhanced production and secretion of S100A11 (Figure 1). Similar amounts of S100A11 protein were recovered from the medium with those from the cells when analyzed 24 h after replacement of the medium. Calcium, IL-8, CXCL1, and TGF β enhanced production but not secretion of S100A11 (Supplemental Figure S1A). IL-6, TNF- α , IFN α , substance P, and histamine showed no appreciable effects (Supplemental Figure S1B). Secretion of S100A11 was not due to decomposition of NHK, because no appreciable increase in the release of lactate dehydrogenase from treated NHK was observed (data not shown).

Enhancement of the Growth of NHK by Exogenous S100A11

To study functions of secreted S100A11, we prepared extremely pure recombinant S100A11 protein mainly using ion-exchange column chromatography (Supplemental Fig-

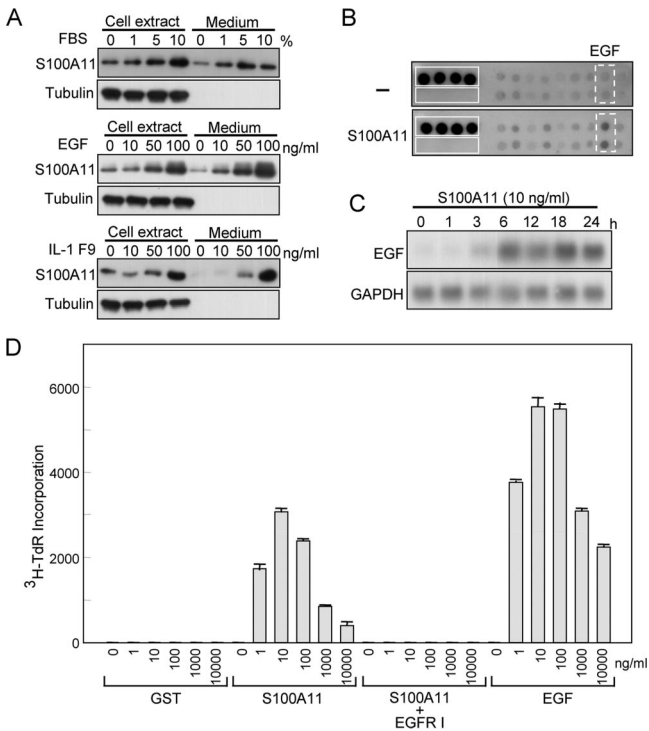


Figure 1. Secretion of and growth stimulation by S100A11 in NHK. (A) Production and secretion of S100A11. NHK were cultured in EpiLife medium without HKGS but with designated additives for 24 h. Equivalent aliquots of whole protein preparations from the cell extract, and the culture medium was applied for Western blot analysis. Tubulin was used as a control for the applied amount of protein preparations. (B) Screening for cytokines induced by S100A11 (10 ng/ml; 24 h) by using a human cytokine antibody array. Dotted squares, EGF; solid squares, positive and negative controls. (C) Induction of EGF by S100A11 in NHK determined by Northern blot analysis. NHK were cultured in HKGS-free EpiLife 24 h before addition of S100A11. (D) Growth stimulation of NHK by exogenous S100A11. NHK were cultured in HKGS-free EpiLife 24 h before addition of S100A11 and incubated for a further 24 h with the designated cytokines. [³H]Thymidine (1 μCi/ml) was added to the medium 1 h before harvest, and radioactivity in an insoluble fraction was counted. GST was used as a control. Epidermal growth factor receptor (EGFR) I, 10 μg/ml an inhibitor for the EGF receptor AG1478.

ure S2). Addition of the purified S100A11 to the HKGS-free culture of NHK remarkably stimulated DNA synthesis even at a concentration as low as 1 ng/ml (Figure 1D). GST produced in the same *Escherichia coli* system and EGF were used as negative and positive controls, respectively.

Screening for cytokines induced by exogenous S100A11 by using a cytokine antibody array resulted in identification of EGF (Figure 1B). Induction of the EGF gene was confirmed by Northern blot analysis (Figure 1C). The amount of EGF protein secreted over a period of 48 h by NHK exposed to 10 ng/ml S100A11 was determined by enzyme-linked immunosorbent assay to be 17.5 pg/ml in 10 ml of HKGS-free EpiLife medium, whereas EGF level in the medium of untreated NHK was below the detection limit (< 5 pg/ml). An inhibitor of the EGF receptor abrogated the growth stimulation of NHK exerted by S100A11 (Figure 1D). In addition to EGF, betacellulin, epiregulin, heparin binding (HB)-EGF, and amphiregulin (although delayed in comparison with the other ligands) but not TGFα were induced by S100A11 among known ligands to the EGR receptor (Sup-

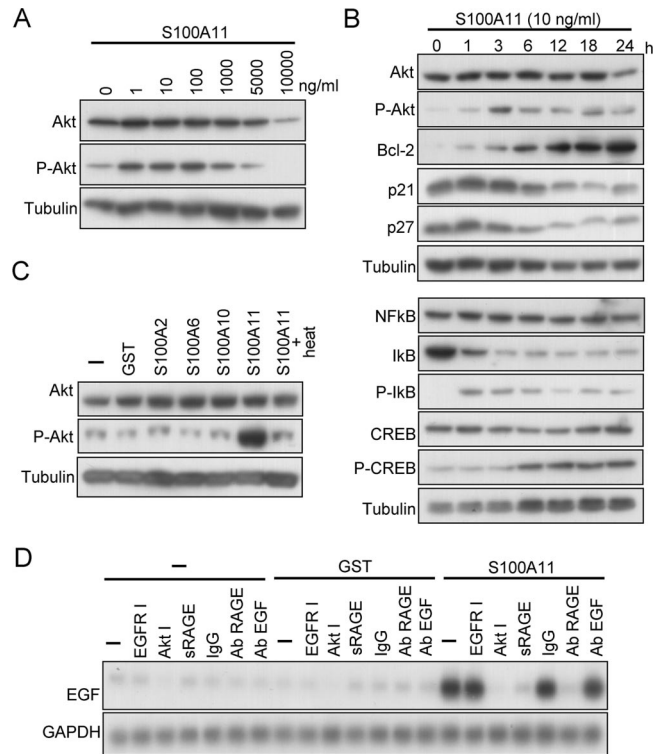


Figure 2. Activation of Akt by S100A11 in NHK. (A) Induction of phosphorylation of Akt. NHK were cultured in HKGS-free EpiLife 24 h before addition of S100A11 protein and incubated for a further 24 h before harvest. Cell extracts were analyzed by Western blot analysis. (B) Time course of activation of Akt and related proteins by S100A11. The experiment was performed under conditions similar to those described in A except for incubation times after addition of S100A11. (C) Specificity in Akt activation among S100 family members. The experiment was performed under conditions similar to those described in A. The recombinant proteins were added at 10 ng/ml. S100A11 was heated at 100°C for 20 min. (D) Inhibition of EGF induction by S100A11 by various agents demonstrated by Northern blot analysis. The experiment was performed under conditions similar to those described in A except for incubation time of 6 h after addition of S100A11 or GST at 10 ng/ml.

plemental Figure S3). These results indicate that exogenous S100A11 stimulates the growth of NHK mainly through activation of the EGF receptor. We hence studied the intracellular signal transduction pathway for the induction of EGF, a representative mediator for growth stimulation by exogenous S100A11.

Akt, an Essential Element for EGF Induction by S100A11

To gain insight into intracellular signaling of the EGF induction by S100A11, we screened protein kinases that were activated in NHK exposed to S100A11 by using a phospho-protein antibody array. We found that phosphorylation of Akt was induced by S100A11 (Supplemental Figure S4), and this was confirmed by Western blot analysis (Figure 2A). Even 1 ng/ml S100A11 increased the phosphorylation level of Akt, and concentrations higher than 5 μg/ml showed a suboptimal effect. The phosphorylation level of Akt was elevated at 1 h and further elevated at 3 h after exposure of NHK to S100A11 (Figure 2B). The enhanced phosphorylation state of Akt was sustained up to 24 h. It should also be noted that antiapoptotic Bcl-2 was substantially up-regulated, whereas negative growth regulators p21^{WAF1} and

p27^{KIP1} were down-regulated. Induction of phosphorylation of Akt seemed specific to S100A11 among S100 family members, because S100A2, S100A6, and S100A10 showed no effect (Figure 2C). Heating inactivated S100A11 and resulted in loss of its capacity to induce phosphorylation of Akt, excluding the possibility of involvement of contaminated LPS that is heat-stable in nature. Activation of Akt is essential for the EGF induction by S100A11 because an Akt inhibitor abrogated it (Figure 2D).

Function of RAGE as a Receptor for S100A11

RAGE is a multiligand receptor binding to at least some S100 family members, including S100A11 (Marenholz *et al.*, 2004; Cecil *et al.*, 2005). RAGE was expressed in NHK as demonstrated by Western blot analysis, and the protein level was enhanced by S100A11 (Figure 3A). Addition of a NF- κ B inhibitor resulted in an undetectable level of RAGE, indicating that NF- κ B is needed not only for induction of RAGE by S100A11 but also for constitutive expression of RAGE. Blocking of RAGE function by either soluble RAGE or an antibody against RAGE completely suppressed the induction of EGF (Figure 2D) and phosphorylation of Akt (Figure 3B) by S100A11. Neither an EGF receptor inhibitor nor an antibody against EGF showed any effect on phosphorylation of Akt, indicating that activation of Akt is upstream to the action of EGF. S100A11 is known to form covalently linked dimers or multimers, and this process facilitates the binding to RAGE (Marenholz *et al.*, 2004; Moroz *et al.*, 2002). We

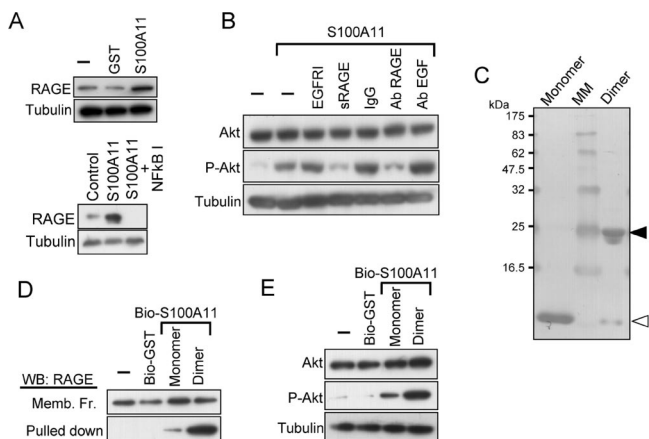


Figure 3. Involvement of RAGE in signal transduction for S100A11. (A) Expression of RAGE in NHK as detected by Western blot analysis. Top, NHK were precultured in HKGS-free EpiLife for 24 h. Cells were harvested 6 h after addition of S100A11 or GST (10 ng/ml). Bottom, after being precultured as described above, NHK were incubated with an inhibitor of NF- κ B (0.5 μ g/ml) followed by exposure to S100A11 at 10 ng/ml for 16 h. (B) Abrogation of S100A11-induced Akt activation by functional blocking of RAGE. EGFR I, 10 μ g/ml AG1478; sRAGE, 1 μ g/ml soluble RAGE; antibody (Ab), 20 μ g/ml antibody. NHK were pre-cultured in HKGS-free EpiLife for 24 h. The agents were added 1 h before the addition of S100A11 and harvested 6 h later. (C) Preparation of monomer S100A11 and dimer S100A11 (see *Materials and Methods*). The membrane was stained with Coomassie Brilliant Blue. MM, molecular markers. White arrowhead, monomer; black arrowhead, dimer. (D) Binding of S100A11 monomer and S100A11 dimer to RAGE. Biotinylated proteins (0.1 nmol) were incubated with 1 mg of membrane preparation of NHK, pulled down with a streptavidin agarose, and analyzed for RAGE by Western blot analysis. (E) Phosphorylation of Akt by S100A11 monomer and S100A11 dimer (10 μ g/ml). The experiment was performed under conditions similar to those described in B.

prepared monomer and dimer preparations of S100A11 as described in *Materials and Methods* (Figure 3C). The S100A11 dimers bound to RAGE more readily and enhanced phosphorylation of Akt to a higher level than did the monomers (Figure 3, D and E).

Mechanisms of Transcriptional Activation of EGF by S100A11

To explore mechanisms of transcriptional activation of the EGF gene, we first pulled down proteins bound to the EGF promoter by a newly developed method that enables fishing for proteins binding to a long region covering 2085 base pairs (see *Materials and Methods* and Supplemental Figure S5). CREB, simian virus 40 promoter factor 1, and NF- κ B were detected among candidate transcription factors examined (Figure 4A). NF- κ B was probably activated by S100A11

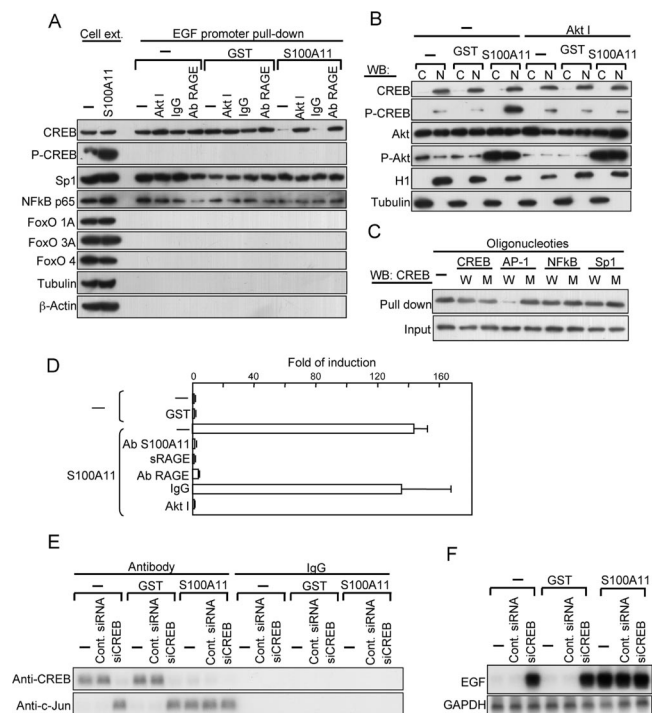


Figure 4. Transcriptional activation of EGF gene by S100A11. (A) Transcription factors bound to the EGF promoter identified by a newly developed method. NHK were precultured in HKGS-free EpiLife for 24 h. An Akt inhibitor and anti-RAGE antibody were added 1 h before the addition of S100A11 or GST and harvested 6 h later. Cell extracts were incubated with a biotinylated EGF promoter fragment (2085 base pairs), and proteins pulled down with streptavidin beads were analyzed by Western blot analysis. (B) Copresence of activated Akt and phosphorylated CREB in nuclei of NHK exposed to S100A11. Histone H1 (H1) and tubulin were used as indicators for nuclei and cytoplasm, respectively. (C) Competitive inhibition of binding of CREB to the EGF promoter by transcription factor-binding elements. Wild-type (W) or mutant (M) oligomers were added to the incubation mixture under conditions similar to those described in A. (D) A luciferase assay for the seventh AP-1 site of the EGF promoter (see Supplemental Figure S7). The seventh AP-1 site was responsive to S100A11 (10 ng/ml), and this was abrogated by blocking of the function of S100A11 (with an antibody, 20 μ g/ml), RAGE (with soluble RAGE at 1 μ g/ml), and Akt (with an inhibitor at 10 μ M). (E) Chromatin immunoprecipitation assay for proteins bound to the seventh AP-1 site described in D. (F) Effect of down-regulation of CREB by siRNA on induction of the EGF gene examined by Northern blot analysis.

through phosphorylation and subsequent degradation of inhibitor of nuclear factor- κ B (I κ B) (Figure 2B). Exposure of NHK to S100A11 changed the binding mode of only CREB, i.e., liberated from the EGF promoter, and this was associated with phosphorylation of CREB (Figures 2B and 4A). CREB is one of the direct substrates of phosphorylation by Akt (Du and Montminy, 1998). In NHK, activated Akt was partly present together with CREB in nuclei, and the Akt inhibitor blocked phosphorylation of CREB induced by S100A11 (Figure 4B and Supplemental Figure S6). Binding of CREB to the EGF promoter region was inhibited by an activator protein (AP)-1 element (Figure 4C), indicating that CREB binds to AP-1 sites of the promoter. The EGF promoter contains 7 AP-1 sites. The proximate seventh site was shown by a luciferase assay to be critical for the induction by S100A11 (Supplemental Figure S7). Transcriptional activation via the 7th AP-1 site by S100A11 was completely suppressed by application to NHK of an antibody against S100A11, soluble RAGE, an antibody against RAGE, or an Akt inhibitor (Figure 4D). A chromatin immunoprecipitation assay revealed that on exposure to S100A11 CREB was liberated from the default binding site of the EGF promoter in NHK (Figure 4E). c-Jun showed a reciprocal binding behavior to the site. Down-regulation of CREB by siRNA (Supplemental Figure S8) induced transcription of the EGF gene by itself (Figure 4F). These results indicate that liberation of CREB from the 7th AP-1 site through phosphorylation triggers transcriptional activation of the EGF gene by AP-1.

Growth Regulation via Endogenous S100A11 in NHK and Squamous Cell Carcinomas (SSCs)

Finally, we examined involvement of endogenous S100A11 in growth regulation of NHK and squamous carcinoma cell lines A431, BSCC-93, and HSC-5. Growing squamous carcinoma cell lines produced and secreted larger amounts of S100A11 than did NHK regardless of the culture medium, i.e., EpiLife or DMEM (Figure 5A). EGF, HB-EGF, and epiregulin were constitutively expressed at higher levels in BSCC-93 cells than in NHK (cultivated with Ca⁺⁺ concentration of 0.03 or 1.5 mM) as determined by Northern blot analysis. (C) Down-regulation of constitutively active Akt in BSCC-93 cells by a neutralizing antibody against S100A11 (20 μ g/ml; 24 h) as shown by Western blot analysis. (D) Down-regulation of constitutive EGF family mRNA levels by an anti-S100A11 antibody (20 μ g/ml; 24 h). (E) Compromise of the growth of NHK and SSCs by an anti-S100A11 antibody. Increase in cell number over a 4-d culture period with or without the antibody (20 μ g/ml added at 0 and 2 culture days) was determined. (F) Schematic presentation of the ambivalent role of S100A11 in growth regulation of NHK.

Ambivalent Nature of S100A11 as a Mediator for Growth Regulation

As described previously (Sakaguchi *et al.*, 2003, 2004, 2005), S100A11 functions as an essential mediator of high calcium and TGF β for growth suppression of NHK. Conversely, our results demonstrated a growth enhancing function of secreted S100A11. The ambivalent pathways are schematically summarized in Figure 5F.

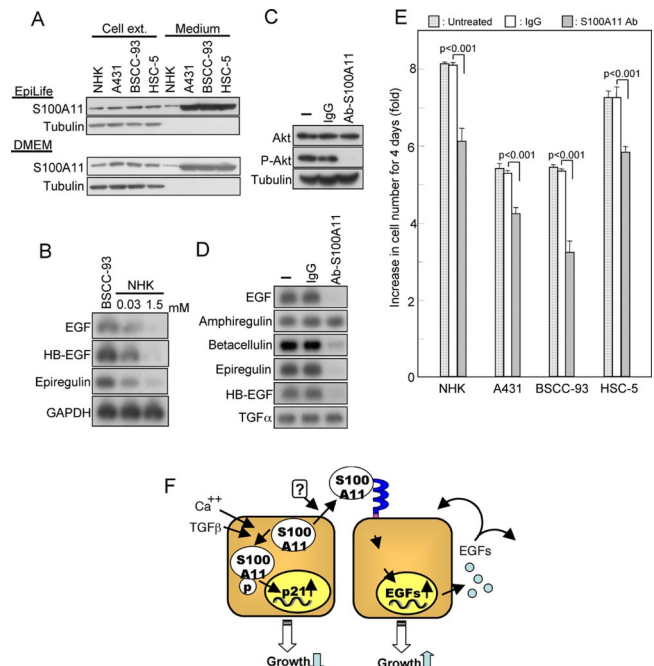


Figure 5. S100A11 in SSC cell lines. (A) Production and secretion of S100A11 in SSCs (A431, BSCC-93, and HSC-5) determined under conditions similar to those described in the legend to Figure 1A. (B) Higher constitutive expression levels of EGF, HB-EGF, and epiregulin in BSCC-93 cells than in NHK (cultivated with Ca⁺⁺ concentration of 0.03 or 1.5 mM) as determined by Northern blot analysis. (C) Down-regulation of constitutively active Akt in BSCC-93 cells by a neutralizing antibody against S100A11 (20 μ g/ml; 24 h) as shown by Western blot analysis. (D) Down-regulation of constitutive EGF family mRNA levels by an anti-S100A11 antibody (20 μ g/ml; 24 h). (E) Compromise of the growth of NHK and SSCs by an anti-S100A11 antibody. Increase in cell number over a 4-d culture period with or without the antibody (20 μ g/ml added at 0 and 2 culture days) was determined. (F) Schematic presentation of the ambivalent role of S100A11 in growth regulation of NHK.

DISCUSSION

Our previous studies clearly demonstrated that S100A11 functions as an essential mediator for growth suppression of NHK triggered by representative growth inhibitors, high Ca⁺⁺ and TGF β , in cells (Sakaguchi *et al.*, 2003, 2004, 2005). Alternatively, the present study provides evidence that S100A11 enhances growth of NHK through induction of EGF and some other ligands to the EGF receptor when acting on the cells from the outside. Thus, S100A11 plays an ambivalent role with respect to growth regulation of NHK.

Involvement of S100A11 in both growth-suppressive and growth-stimulatory signaling in NHK seems to be paradoxical. We have been learning, however, more and more complex functions of a given protein in multiple biological contexts with recent progress in molecular and cellular biology, and S100A11 is not an exception. S100A11 is constitutively expressed in NHK. Under ordinary culture conditions for NHK, the medium contains EGF. EGF enhanced the production and secretion of S100A11 (Figure 1A), which in turn induced EGF in an autocrine/paracrine manner (Figure 1, B and C). Exposure of growing NHK to either Ca⁺⁺ or TGF β overrides growth of NHK supported by EGF, and S100A11 cell autonomously functions as an indispensable mediator of the growth suppression via phosphorylation and translocation into nuclei (Sakaguchi *et al.*, 2003, 2004, 2005). The

mechanistic link between EGF and ambivalent S100A11 demonstrated in the present study may contribute to more exquisite growth regulation and fine tunings.

Secretion of S100A11 was first described in chondrocytes by Cecil *et al.* (2005). S100A11 was markedly overexpressed and secreted into the surrounding matrix by human chondrocytes in osteoarthritis. In the present study, we showed that S100A11 was also secreted from epithelial cells. S100A11 lacks a classical signal peptide sequence for secretion via the endoplasmic reticulum-Golgi pathway. Although several possible mechanisms have been proposed and partly verified for unconventional secretion routes (Nickel, 2005), the mechanisms by which of S100A11 and other secretory S100 family members are secreted remain to be clarified. In this respect, it is noteworthy that S100A13 was shown to be cosecreted with fibroblast growth factor (FGF)1, both of which lack the conventional signal sequence (Landriscina *et al.*, 2001). An inhibitor of actin stress fiber formation suppressed secretion of FGF1 and hence of S100A13, which is known to bind to the actin fiber (Prudovsky *et al.*, 2002). S100A11 also has a capacity to bind to the actin fiber (Sakaguchi *et al.*, 2000). Phosphorylation of S100A11 does not seem to be involved in the secretion. High Ca^{++} induced phosphorylation of S100A11 (Sakaguchi *et al.*, 2003), but it did not affect its secretion (Supplemental Figure S1A), whereas EGF enhanced the secretion (Figure 1A), but it showed no effect on phosphorylation of S100A11 (data not shown). S100A11 protein was identified in human serum by condensation using affinity chromatography and detection by Western blotting (data not shown), indicating that secretion of S100A11 takes place *in vivo* and is not merely an *in vitro* phenomenon.

RAGE is a multiligand receptor that belongs to the immunoglobulin superfamily. RAGE was first identified as a receptor for glycation end products of proteins (Schmidt *et al.*, 1992), but it was soon found to bind diverse ligands, including amyloid β (Yan *et al.*, 1996), HMGB1 (amphoterin) (Hori *et al.*, 1995), and some S100 family members (Donato, 2003; Hofmann *et al.*, 1999). Cecil *et al.* (2005) showed that exogenous S100A11 acted on articular chondrocytes to increase the production of type X collagen and CXCL8/IL8 in culture and that addition of an anti-RAGE antibody abrogated the action of S100A11. Abrogation of the effect of exogenous S100A11 by a neutralizing antibody was also observed in the present study (Figures 3B and 4A). In addition, it was shown that RAGE was coprecipitated with S100A11 from extracts of NHK with a higher efficiency for dimerized S100A11, a functionally more active form than monomeric S100A11 (Figure 3D). Although binding to the common receptor RAGE, different S100 family members seem to exert distinct biological effects. Heterodimeric S100A8 and S100A9, candidate ligands for RAGE (Ehlermann *et al.*, 2006), neither induced EGF nor activated Akt, but they induced cytokines such as IL-8, IL-1F9, and TNF- α in NHK (data not shown). Hsieh *et al.* (2004) showed that intracellular translocation of S100A4, S100A12, S100A13, and S100B was brought about by application of the respective proteins onto endothelial cells. Although those S100 proteins seemed to share a common receptor, RAGE, intracellular translocation was observed in an individual protein-specific manner. The underlying mechanisms of the differential signal transduction by RAGE remain to be elucidated.

NF- κ B is a well-known immediate mediator for RAGE-triggered signaling (Huttunen *et al.*, 1999). In accordance with this, phosphorylation and subsequent degradation of I κ B were observed within 1 h in NHK exposed to S100A11 (Figure 2B). Activation of Akt by phosphorylation was es-

essential for the induction of EGF (Figures 2D and 4A). The phosphorylation level of Akt was appreciably elevated at 1 h and further elevated at 3 h after exposure of NHK to S100A11 (Figure 2B). The enhanced phosphorylation state of Akt was sustained up to 24 h. Transient and sustained modes of Akt activation have been shown to often lead to distinct outcomes in various biological contexts (Foulstone *et al.*, 1999). The products of phosphatidylinositol 3-kinase (PI3K) are absolutely necessary for activation of Akt (Alessi *et al.*, 1996), and PI3K is activated by RAGE via generation of reactive oxygen species (Alessi *et al.*, 1996; Xu and Kyriakis, 2003).

To identify transcription factors involved in induction of the EGF gene, we developed a new method to fish proteins binding *in vitro* to a long region of the EGF promoter (Supplemental Figure S5). The method is facile and costless, and it can be applied for any promoters. Although the length of the target promoter region is restricted by size amplifiable by PCR, usually 2~3 kb because of the necessity of a substantial amount of DNA, longer regions (>10 kb) may be analyzed by mixing flanking segments amplified separately. It is also possible to screen unknown promoter-binding proteins by combination with mass spectrometry. The electrophoretic mobility shift assay, another analytical method for *in vitro* protein-promoter binding, is usually used for a specific short segment of the promoter. The chromatin immunoprecipitation assay is a strong tool for analyzing *in vivo* binding of a given protein to a defined region of the promoter. It is also possible to screen unknown binding regions in genomic DNA when combined with a microarray (chromatin immunoprecipitation on chip; Wu *et al.*, 2006).

S100A11 was produced and secreted at higher levels in squamous cell carcinoma cells than in NHK (Figure 5A). As expected, higher mRNA levels of EGF, HB-EGF, and epiregulin were observed in BSCC-93, A431, and HSC-5 cells than in NHK (Figure 5B; data not shown). If the S100A11-EGF family member axis constitutively functions in squamous carcinoma cells, sequestering of endogenous S100A11 would result in compromise of the pathway. When a neutralizing anti-S100A11 antibody was added to the culture medium, the constitutively higher phosphorylation state of Akt in BSCC-93 cells was almost completely suppressed (Figure 5C), and mRNA levels of EGF, betacellulin, epiregulin and HB-EGF were markedly reduced (Figure 5D). Addition of the anti-S100A11 antibody resulted in a decrease in the number of NHK and squamous carcinoma cells after 4 d of culture by 30~40% (Figure 5E). The observed partial growth suppression was probably due to the fact that, among the EGF family members, TGF α and amphiregulin were hardly affected by S100A11 (Figure 5D) and the fact that some growth-stimulating pathways other than the S100A11-EGF family member axis also play significant roles. The advantageous effect for cancer cells of overexpressed S100A11 and hence activation of RAGE, however, is not necessarily restricted to growth stimulation. S100A11 induced expression of Bcl-2, a representative antiapoptotic protein (Figure 2B). RAGE-mediated signaling has been shown to confer cells resistance to induction of apoptosis in different biological systems (Huttunen *et al.*, 2000; Arumugam *et al.*, 2004). S100P, another ligand for RAGE, was shown to be overexpressed in pancreatic cancer (Crnogorac-Jurcevic *et al.*, 2003; Sato *et al.*, 2004) and to promote growth, survival, and invasion of pancreatic cancer cells (Arumugam *et al.*, 2005). Angiogenesis, which is essential for growth and metastasis of cancer cells, has been shown to be promoted by ligands for the EGF receptor directly and/or via induction of vascular endothelial growth factor and its receptor (Goldman

et al., 1993; Tsai *et al.*, 1995). Recently, Gupta *et al.* (2007) showed that epiregulin is one of the genes that facilitate the assembly of new tumor blood vessels and thus promote lung metastasis. Together, the results suggest that overexpression of S100A11 and eventual activation of EGF family members contribute to facilitation of growth, survival, invasion, and metastasis because S100A11 and EGF family members compose a positive feedback loop. This process could be a target for exploitation of new therapeutic measures.

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