Srcasm Modulates EGF and Src-kinase Signaling in Keratinocytes*

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Understanding the molecular mechanisms that regulate cellular proliferation and differentiation are fundamental biological questions important within metazoans, and tyrosine kinases play critical roles in regulating these processes (1–3). The Src family tyrosine kinases (SFKs) have been implicated in promoting differentiation in a number of cell types such as skin and lens keratinocytes, oligodendrocytes, and endometrial cells (3–8). In addition, increased activity of the SFKs has been associated with a variety of epithelial tumors, including colonic adenocarcinoma, mammary carcinoma, and murine cutaneous squamous cell carcinoma (9–13). Given the importance of SFKs in cellular physiology, cells have evolved a variety of mechanisms to regulate their activity.

Structural analyses have shown that SFKs can form an inactive “closed” configuration, with the SH2 domain bound to the phosphorylated C-terminal tyrosine (Tyr-527) and the SH3 domain associated with a polyproline motif in the linker region; this configuration prevents phosphorylation at tyrosine 416 in the activation loop rendering the kinase inactive (14, 15). Given these structural data, it has been hypothesized that molecules containing ligands for the SH2 and SH3 domains of SFKs may disrupt the SH2-dependent and SH3-dependent intramolecular interactions and promote opening of the “closed” configuration, phosphorylation of tyrosine 416, and activation of the kinase (14, 15). Theoretically, if a molecule contained a stronger ligand for the SFK SH2 domain than the C-terminal pTyr-527 motif, then such a molecule could activate Src kinases regardless of Csk activity (the kinase that induces Tyr-527 phosphorylation), thereby independently promoting important regulatory signals (2, 16). Some SFK-activating molecules have been identified, including FAK and Sin; however, these molecules do not contain the highest affinity ligands for the SFK SH2 domain, a pYEEI motif (17–19).

The Src-activating and signaling molecule (Srcasm) is a recently described activator and substrate of Src-family tyrosine kinases (SFKs). When phosphorylated at specific tyrosines, Srcasm associates with Grb2 and p85, the regulatory subunit of phosphoinositide 3-kinase; however, little is known about the role of Srcasm in cellular signaling. Data presented here demonstrate that epidermal growth factor (EGF) receptor signals promote the tyrosine phosphorylation of endogenous and adenovirally transduced Srcasm in keratinocytes, and that increased levels of Srcasm activate endogenous SFKs, with a preference for Fyn and Src. In addition, Srcasm potentiates EGF-dependent signals transmitted by SFKs in keratinocytes. Tyrosine phosphorylation of Srcasm is dependent on growth factors and the activity of EGFR and SFKs. Increased Srcasm expression enhances p44/42 mitogen-activated protein kinase activity and Elk-1-dependent transcriptional events. Elevated Srcasm levels inhibit keratinocyte proliferation while promoting specific aspects of keratinocyte differentiation. Lastly, Srcasm levels are decreased in human cutaneous neoplasia. Collectively, these data demonstrate that Srcasm plays a role in linking EGF receptor- and SFK-dependent signaling to differentiation in keratinocytes.

The abbreviations used are: SFK, Src family kinase; Ad, adenovirus; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; KGF, keratinocyte growth factor (also known as FGF-7); MAP, mitogen-activated protein; PHK, primary human keratinocyte; SCC, squamous cell carcinoma; SCIS, squamous cell carcinoma in situ; Srcasm, Src activating and signaling molecule; Top-o, transforming growth factor α; BrdUrd, bromodeoxyuridine; aa, amino acids(s); PBS, phosphate-buffered saline; HA, hemagglutinin; h, human; m.o.i., multiplicity of infection; DAPI, 4′,6-diamidino-2-phenylindole.

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by cloning hemagglutinin-tagged Srcasm (HA-Srcasm) into a shuttle vector containing a cytomegalovirus promoter. The expression cassette was excised and ligated into an adenoviral backbone vector (pAdX), and the virus was amplified then purified by CsCl gradient centrifugation. The control adenovirus (Ad-Con) contains the green fluorescent protein coding region driven by a bacterial LacZ promoter.

Pharmacological Manipulations—Ad-Srcasm at an m.o.i. of 200. Cells were deprived of growth factors for 24 h and then stimulated with EGF 10 ng/ml for 5 min. Some cells were pre-treated with a Src family tyrosine kinase inhibitor, PP2 (10 μM), or its negative control, PP3 (10 μM), an EGFR tyrosine kinase inhibitor, AG112 (20 μM), or its negative control, AG9 (20 μM), for 30 min before EGF stimulation. Lysates were subjected to immunoprecipitation with α-Srcasm antibody (clone 3F10, Roche Molecular Biochemicals) was used at 1/1000 for Western blotting and at 2 μg/ml for immunoprecipitation. 

**Primary Human Keratinocyte Culture, Infections/Transfections, and Pharmacological Manipulations—** Cultures of neonatal human keratinocytes were obtained from foreskins. After isolation, the cells were cultured in MCDB-153 medium, supplemented with 0.1 mM ethanolamine (Sigma), 0.1 mM O-phosphoethanolamine (Sigma), 10 μg/ml hEGF (Invitrogen), 5 × 10^-7 M hydrocortisone (Sigma), 5 μg/ml insulin (Sigma), bovine pituitary extract (BPE) (150 μg/ml), 100 units/ml penicillin, and 100 mg/liter streptomycin, 70 μg/ml calcium, and maintained at 37 °C with an atmosphere of 5% CO₂. Growth factor depletion was conducted in MCDB base lacking insulin, EGF, and BPE. All keratinocytes analyzed were less than passage 4. Keratinocytes at the indicated m.o.i. under the culture conditions described above and usually analyzed or manipulated 16 h after infection.

Luciferase assays utilized the PathDetect Elk-1 trans-reporting assay (Stratagene) according to the manufacturer’s recommendations. Cells were transfected in MCDB (without penicillin/streptomycin) using a ratio of 1 μg of DNA to 5 μl of Lipofectamine (Invitrogen). hEGF (10 or 100 ng/ml, Invitrogen), hTGF-α (0.1 ng/ml, Sigma #T7924), hKGF (10 ng/ml, Research Diagnostics Inc.) for the indicated times. Some cells were incubated with Src family kinase-selective inhibitor PP2 (10 μM), or its negative control PP3 (10 μM), or specific EGFR kinase inhibitor AG112 (20 μM) or its negative control AG9 (20 μM), or the MEK 1/2 inhibitors U0126 (10 μM) or its negative control U0124 (10 μM) 60 min before cell lysis (all from Calbiochem).

**Antibodies—** Phosphotyrosine (Upstate Biotechnology, 4G10) was used at 1/1000 for Western blotting. Phosphotyrosine immunoprecipitation of endogenous Srcasm was performed overnight at 4 °C using 1.5 mg of protein lysate and 2.5 μg of antibody; phosphotyrosine immunoprecipitation for transduced Srcasm was performed using 0.5 mg of lysate for 15 min with 1.5 μg of antibody using the Catch and Release Reversible Immunoprecipitation system (Upstate Biotechnology). High affinity α-HA antibody (clone 3F10, Roche Molecular Biochemicals) was used at 1/1000 for Western blotting and at 2 μg/ml for immunoprecipitation. 

Activated Src family kinase antibody (phosphor-Tyr-416) (Cell Signaling) was used at 1/1000 for Western blotting, α-p44/42 MAP kinase antibody and α-phospho-p44/42 MAP kinase antibody (Cell Signaling) were used at 1/1000 to detect total or phosphorylated p44/42 MAP kinase. Fyn (SC-16), Src (SC-19), and Yes (SC-14, all from Santa Cruz Biotechnology) were all used at 1:1000 for Western blotting, and 3 μg was used for immunoprecipitation. Src2 antibody (SC-8056) that detects Src, Fyn, and Yes was used at 1:500. Srcasm antibody is a polyclonal antibody that was generated by incubating rabbits with three purified glutathione S-transferase fusion proteins spanning murine Srcasm (aa 1–200, aa 150–400, and aa 389–474). For Western blotting, the Srcasm antisera was diluted 1:500. For endogenous Srcasm immunoprecipitation, IgG was purified from the crude serum via Protein A affinity chromatography; 6 μg of this IgG fraction was used for each immunoprecipitation. The specificity of the α-Srcasm antibody was demonstrated by Western blotting COS protein lysates overexpressing Srcasm, and preincubating the antisera with the 1 μM Srcasm fusion proteins for 30 min at room temperature ablates detection of Srcasm (Fig. 8A).

**Immunoblotting and Immunoprecipitation—** Cell lysates were prepared using radioimmune precipitation assay lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 5 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 1 μg/ml aprotinin, 100 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO₃). Tissue samples of normal skin and squamous cell...
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Srcasm increases cellular tyrosine phosphorylation and activates SFKs in keratinocytes. A, EGF treatment and Srcasm overexpression increase protein tyrosine phosphorylation. PHKs infected with Ad-Con or Ad-Srcasm (m.o.i. 200) were subjected to EGF stimulation. Lysates were analyzed by Western blotting with a-phosphotyrosine, a-Srcasm, or a-b-actin (loading control) (n = 2). B, srcasm activates endogenous SFKs. PHKs were subjected to no virus, control adenovirus, or Srcasm adenovirus at an m.o.i. of 200. Some cells were treated with hEGF, 100 ng/ml for 15 min. Cell lysates were subjected to SDS-PAGE followed by Western blotting with the following antibodies: a-activated src kinase (pY416), a-SFK (Src2), and a-Srcasm. Data representative of four experiments. C, Srcasm activates endogenous SFKs in a dose-dependent manner. PHKs were cultured in MCDB complete media and infected with Ad-Con or Ad-Srcasm at the indicated m.o.i. Cell lysates were analyzed as in B (n = 2). D, association of activated SFKs and Srcasm by immunofluorescence. PHKs cultured in 2-well chamber slides were infected with Ad-Con or Ad-Srcasm at an m.o.i. of 200. Some cells were treated with EGF, 100 ng/ml for 5 min. Cells were double-stained a-p416 (green) and a-HA (red). DAPI was used for nuclear staining (magnification, ×600).

In vitro Kinase Assays—PHKs were infected with Ad-Con or Ad-Srcasm at m.o.i. 200 and cultured in MCDB complete media. Sixteen hours post-infection, cells were lysed in radioimmunoprecipitation assay buffer, and 1 mg of cell lysate was subjected to immunoprecipitation with 3 µg of a-Fyn, a-Src, or a-Yes for 16 h at 4 °C. The immunoprecipitated Fyn, Src, and Yes were subjected to no virus, control adenovirus, or Srcasm adenovirus at m.o.i. 200 and cultured in MCDB complete media. Sixteen hours post-infection, cells were lysed in radioimmunoprecipitation assay buffer, and 1 mg of cell lysate was subjected to immunoprecipitation with 3 µg of a-Fyn, a-Src, or a-Yes for 16 h at 4 °C. The immunoprecipitated Fyn, Src, and Yes were subjected to in vitro kinase assays according to manufacturer's specifications (Src Assay kit #17-131, Upstate Biotechnology). Phosphocellulose filters with bound 32P-labeled substrate were assessed by liquid scintillation using Econofluor-2 (Packard Instrument Co., Cat. no. 6NE9699) in a Beckman LS-6500 instrument. Background cpm activity (from a no kinase control reaction) was subtracted from all values. cpm values of assays of SFKs from control cells was set equal to 1; values of assays from Ad-Srcasm cells were divided by the corresponding value from control infected cells to obtain -fold stimulation. Data presented are from two independent experiments.

Luciferase Assays—Human primary keratinocytes were plated in complete MCDB media, allowed to reach 50% confluency, and then transfected with plasmids from the Pathdetect trans-reporting system (Stratagene), specific for assaying Elk-1-dependent transcription from serum-response elements. Primary keratinocytes plated at 5 × 10^4 per 35-mm well were transfected using Lipofectamine (5 µl/µg of DNA) with 0.5 µg of pFA2-E1K1 and 1.0 µg of pFR-Luc. Approximately 16 h after transfection, cells were not infected or infected with Ad-Srcasm or Ad-Con at an m.o.i. of 200. After 18 h of infection, some cells were stimulated for 5 h with 100 µg/liter hEGF. Luciferase activity in equivalent amounts of cell lysates was determined using the firefly luciferase assay system (Promega) in a Monolight 3096 luminometer (BD Biosciences) equipped with Simplicity 2.0 software. Relative luciferase units reflect the numerical values obtained from the luminometer; values represent the averages of three experiments with the standard deviation indicated by bars. Equivalent amounts of cell lysate were counterstained using DAPI. Imaging was performed on an IX-81 inverted fluorescence scope under oil coupled with a Cooke Sensicam digital camera. Where indicated, deconvolved, confocal images (nearest neighbors algorithm) and image overlay were performed using Slidebook, version 4, at ×600 magnification.
subjected to Western blotting to detect Srcasm.

Flow Cytometry Proliferation Assay—Sub-confluent PHKs infected with Ad-Con or Ad-Srcasm at an m.o.i. of 200. Cellular SFKs were immunoprecipitated with specific antibodies to Fyn, Src, or Yes. The immunoprecipitates were split, subjected to SDS-PAGE, followed by Western blotting with α-pY416 and α-SFK. Data are representative of two experiments. B, in vitro kinase assays show Srcasm preferentially activates Fyn and Src. Fyn, Src, and Yes were immunoprecipitated from PHKs transduced with Ad-Con or Ad-Srcasm (m.o.i. 200). Immunoprecipitates were subjected to an in vitro kinase assay (see “Experiment Procedures”). The -fold stimulation of Fyn, Src, or Yes kinase activity from Ad-Srcasm cells relative to control cells is shown (n = 2). C, Srcasm preferentially localizes with Fyn and Src by immunofluorescence. PHKs were cultured in 2-well chamber slides and infected as in panel A. Cells were double-stained to detect Srcasm (red) and Fyn, Src, or Yes (green). DAPI was used for nuclear staining (magnification, ×600).

subjected to Western blotting to detect Srcasm.

Flow Cytometry Proliferation Assay—Sub-confluent PHKs infected with Ad-Srcasm or Ad-Con (m.o.i. 200) 18 h prior were labeled with 10 μM BrdUrd for 3 h under standard MCDB culture conditions (see above). Then, cells were harvested and stained with fluorescein isothiocyanate conjugated anti-BrdUrd antibody and 7-amino-actinomycin D following the BrdUrd flow kit instruction (BD Pharmingen); the analysis parameters used are those recommended by the manufacturer. The stained cells were analyzed by FACScan machine (BD Pharmingen); the cell populations were sorted into G0/G1 (gate R2), G2/M (gate R3), and S phase (gate R4) according to the manufacturer’s specifications. In Fig. 6C, subconfluent PHKs were infected as above and deprived of growth factors for 16 h, and then some cells were stimulated with EGF (10 ng/ml) for the indicated times. These cells were collected, washed in PBS, and fixed in 70% ethanol, overnight at 4 °C. Cells were pelleted then washed in PBS with 2% fetal calf serum, and subjected to passage through a 0.2-μm filter. Cells were treated with RNase A and stained with propidium iodide. Cells were analyzed on a FACScan machine using ModFit LT 3.1 (Verity Software House) to determine S-phase fraction. Data are representative of two experiments.

BrdUrd Labeling and Filaggrin Blotting—Cells were cultured in 2-well Lab-Tek chamber slides (Nalge Nunc International) and infected with Ad-Srcasm or Ad-Con (m.o.i. 200) in MCDB complete media. 24 h after infection, cells were labeled with BrdUrd (10 μM) for 3 h and then fixed and permeabilized with 50% acetic acid, 70% ethanol, pH 2, for 20 min at 4 °C. Cells were stained for BrdUrd, filaggrin, or Srcasm as indicated under “Immunofluorescence Analysis.” Cells stained for filaggrin and Srcasm were fixed and permeabilized also as indicated under “Immunofluorescence Analysis.”

For filaggrin Western blotting, PHKs were plated on collagen-coated dishes and infected with Ad-Srcasm or Ad-Con (m.o.i. 200). Cells were cultured in MCDB complete media. At the times indicated (post-infection), cells were lysed in radioimmune precipitation assay, and equivalent protein amounts were subjected to Western blot analysis.

Immunohistochemical Detection of Srcasm in Formalin-fixed Biopsy Specimens—Formalin-fixed, paraffin-embedded tissue samples of actinic keratosis, squamous cell carcinoma, in situ, invasive squamous cell carcinoma and unremarkable skin were collected from the dermatopathology archives of the University of Pennsylvania Department of Dermatology with Internal Review Board approval under protocol 704450. Biopsy specimens containing unremarkable epidermis adjacent to lesional skin were selected as normal skin provides an internal control. For immunohistochemical staining, tissue samples were blocked for 1 h at room temperature with 10% horse serum, then incubated for 1 h with affinity-purified rabbit polyclonal anti-Srcasm antibody (4 μg/ml). The antibody was affinity-purified by incubating anti-sera with glutathione S-transferase immobilized on polyvinylidene difluoride membranes followed by the Srcasm fusion proteins immobilized on polyvinylidene difluoride membranes. The anti-Srcasm antibody was eluted and handled as previously discussed (22). Control
Srcasm modulates p44/42 MAP kinase activation in keratinocytes. A, primary keratinocytes were cultured in MCDB with additives (see "Materials and Methods") and then stimulated with 100 ng/ml EGF for the indicated times. Cell lysates were subjected to Western blotting with the following antibodies: activated p44/42, p44/42, and Srcasm (n = 3). B, Srcasm-dependent activation of p44/42 MAP kinase necessitates activity of EGFR, SFKs, and MEK 1/2. Primary keratinocytes were infected with Ad-Con or Ad-Srcasm at an m.o.i. of 200. After deprivation of growth factors for 24 h, cells were stimulated with 10 ng/ml EGF for 5 min. Some cells were pre-treated with PP2 (10 μM), PP3 (10 μM), AG112 (20 μM), AG9 (20 μM), U0126 (10 μM), or U0124 (10 μM) for 30 min before EGF stimulation. Cell lysates were subjected to Western blotting with the following antibodies: activated p44/42, p44/42, and Srcasm (n = 2). In C, upper panel, increased Srcasm expression correlates with activated p44/42 under culture conditions but not with short-term EGF stimulation. Primary keratinocytes cultured in chamber slides were infected with Ad-Con or Ad-Srcasm at an m.o.i. of 200. Some cells were treated with EGF, 100 ng/ml for 5 min. Cells were double-stained to detect phosphorylated p44/42 (green) and HA-tagged Srcasm (red). DAPI was used for nuclear staining. Lower panel, as above but with no DAPI staining to highlight nuclear localization of activated p44/42 MAP kinases (magnification, ×600).

The behavior of adenovirally transduced HA-Srcasm was examined in PHKs. PHKs transduced with HA-Srcasm adenovirus and cultured in complete media exhibited readily detectable levels of tyrosine-phosphorylated Srcasm (Fig. 1C), whereas PHKs deprived of growth factors (EGF, insulin, and bovine pituitary extract) for 24 h contained very low levels of tyrosine-phosphorylated Srcasm. PHKs stimulated with EGF demonstrated a rapid and significant increase in tyrosine-phosphorylated Srcasm (Fig. 1C); these results for transduced Srcasm parallel those for endogenous Srcasm.

RESULTS

EGFR Ligands and SFKs Promote Tyrosine Phosphorylation of Srcasm in Primary Human Keratinocytes—Because EGF stimulation of cells can activate Src kinases, the effect of EGF on the tyrosine phosphorylation of endogenous Srcasm was evaluated in primary human keratinocytes (Fig. 1) (23, 24). Immunoprecipitation with α-Srcasm followed by Western blotting with α-phosphotyrosine demonstrated increased levels of Srcasm tyrosine phosphorylation in EGF-treated keratinocytes (Fig. 1A). Similarly, immunoprecipitation with α-phosphotyrosine from EGF-treated cells followed by Western blotting using α-Srcasm demonstrated more phospho-Srcasm present in EGF-treated cells (Fig. 1B). Parallel experiments with TGF-α and KGF were performed; TGF-α treatment of keratinocytes increased tyrosine phosphorylation of Srcasm, whereas KGF treatment did not (Fig. 1B). These results show that EGF and TGF-α, both EGFR ligands, induce tyrosine phosphorylation of endogenous Srcasm.

Because Srcasm can activate SFKs in vitro, the effect of...
Srcasm on endogenous keratinocyte SFK activation was evaluated. In non-infected PHKs, the levels of activated SFKs were low (Fig. 2B); similar results were seen with control adenovirus. In contrast, infection of the keratinocytes with Ad-Srcasm led to significantly higher levels of activated SFKs (Fig. 2B). Increased levels of Srcasm also potentiated the ability of EGF to activate Src-kinases (Fig. 2B). These results show that increased levels of Srcasm are associated with activation of endogenous SFKs in PHKs and that increased Srcasm level can lead to higher levels of activated SFKs secondary to EGF treatment.

The ability of Srcasm to activate SFKs is dose-dependent; the level of activated SFKs is proportional to the level of cellular Srcasm (Fig. 2C). Association of increased Srcasm levels with SFK activation was evaluated at the cellular level via immunofluorescence. Cells containing higher amounts of Srcasm have higher levels of activated SFKs (Fig. 2D).

**Srcasm Differentially Activates SFKs in Primary Keratinocytes**—Because keratinocytes express Fyn, Src, and Yes, the effect of increased Srcasm levels on each SFK family member was evaluated. Immunoprecipitation of Fyn from keratinocytes containing higher levels of Srcasm followed by Western blotting to assess levels of activated kinase demonstrated increased Fyn activation when compared with control cells (Fig. 3A). Activation of Src was also seen in similar experiments. Interestingly, Yes showed decreased activation in Ad-Srcasm-infected cells relative to controls (Fig. 3A).

The effect of increased Srcasm levels on SFK activity was also evaluated using in vitro kinase assays. Both Fyn and Src showed increased kinase activity in PHKs infected with Ad-Srcasm compared with control cells (Fig. 3B). The kinase activity of Yes was mildly decreased in Ad-Srcasm-infected PHKs compared with control cells (Fig. 3B). Using two different experimental methods, increased Srcasm levels are associated with increased Fyn and Src activity but with decreased Yes activity.

Immunofluorescence studies demonstrated more prevalent co-localization of Fyn and Src with Srcasm, as indicated by increased yellow color in the merged confocal images (Fig. 3C). In contrast, relatively little co-localization of Yes and Srcasm was seen in similar experiments (Fig. 3C). Therefore, the degree of cellular co-localization between Srcasm and Fyn, Src, or Yes appears to correlate with the ability of Srcasm to activate these kinases.

**Srcasm Modulates p44/42 MAP Kinase Activation in Primary Keratinocytes**—EGF stimulation of primary human keratinocytes leads to the activation of ERK2 kinase (p44 MAP kinase) via Ras (25). Given that Srcasm can amplify some EGF-dependent signals, the effect of Srcasm on p44/42 MAP kinase activation was evaluated in primary keratinocytes (23). Keratinocytes with increased Srcasm levels demonstrated a significant increase in basal p44/42 MAP kinase activity when compared with control cells (Fig. 4A). In fact, the level of p44/42 MAP kinase activation in Ad-Srcasm cells mirrors that seen in EGF-stimulated control cells (Fig. 4A). At early time points of EGF stimulation, keratinocytes with elevated levels of Srcasm maintained a high level of p44/42 MAP kinase activation similar to that in control cells, confirming that the level of p44/42 MAP kinase activation already was at near-maximal levels in the unstimulated Ad-Srcasm-infected cells. However, by 10 min, EGF stimulation led to a marked decrease in p44/42 activation in cells with high levels of Srcasm, whereas levels of activated p44/42 were elevated and stable in control cells. After 30 min of EGF stimulation, the levels of p44/42 activation dropped in control cells (data not shown). Based on these observations, increased Srcasm levels activate p44/42 MAP kinases in unstimulated keratinocytes, and, in the same cells, EGF stimulation promotes a rapid deactivation of these kinases. Therefore, Srcasm has the ability to modulate p44/42 MAP kinase activity in keratinocytes.

The Srcasm-dependent activation of p44/42 MAP kinases was investigated using pharmacological inhibitors of EGFR, SFKs, and MEK 1/2. Preincubation of keratinocytes with AG12, an EGFR kinase inhibitor, resulted in a decrease in the Srcasm-dependent activation of p44/42 MAP kinases (Fig. 4B). In a similar manner, preincubation of keratinocytes with PP2, an inhibitor of SFKs, and UO126, a MEK 1/2 kinase inhibitor, ablated the Srcasm-dependent activation of p44/42 MAP kinases. These results demonstrate that Srcasm mediates p44/42 MAP kinase activation through a pathway dependent on the activity of EGFR, SFKs, and MEK 1/2.

The relationship between Srcasm levels, p44/42 MAP kinase activation, and EGF treatment was investigated at the single cell level using immunofluorescence. Keratinocytes infected with control adenovirus demonstrated very weak staining for activated p44/42 MAP kinase (Fig. 4C), while control cells stimulated with EGF exhibited stronger staining for activated p44/42 MAP kinase. In contrast, cells with higher levels of Srcasm demonstrated markedly increased staining for activated p44/42 MAP kinase prior to stimulation. EGF treatment of these cells led to decreased levels of activated p44/42 MAP kinase; however, in cells with lower levels of Srcasm staining, EGF induced high levels of activated p44/42 MAP kinase staining (Fig. 4C). Prominent nuclear localization of activated p44/42 MAP kinase was seen in keratinocytes over expressing Srcasm prior to EGF stimulation or in control cells stimulated with EGF (Fig. 4C, lower panel). EGF treatment of Ad-Srcasm cells resulted in a marked decrease of activated p44/42 MAP kinase in the nucleus. The results of the immunofluorescence studies confirm at the single cell level that Srcasm stimulates p44/42 activity under basal conditions and promotes rapid deactivation of these kinases secondary to EGF stimulation.
Based on these data, Srcasm transmits signals from EGFR and SFKs that can regulate the activity of p44/42 MAP kinases.

**Srcasm Promotes Elk-1-dependent Transcription**—Elk-1 is a transcription factor of the ETS family that can be phosphorylated by kinases, including activated p44/42 MAP kinases (26). Subsequently, phosphorylated Elk-1 can promote transcription from serum-response elements (27–29). Because Srcasm can activate p44/42 MAP kinases, its ability to promote Elk-1-dependent transcription was evaluated in primary keratinocytes. In this transcriptional assay, preliminary experiments demonstrated that EGF stimulation resulted in consistent EGF-inducible luciferase activity and that EGF stimulation for 5 h resulted in higher levels of p44/42 activation in Ad-Srcasm-infected PHKs relative to control cells (data not shown). Uninfected keratinocytes transfected with the Elk-1 plasmid and reporter plasmid demonstrated only low levels of luciferase activity basally or after EGF stimulation (Fig. 5). Infection of keratinocytes with control adenovirus increased luciferase activity compared with uninfected cells under both conditions. Cells infected with Ad-Srcasm demonstrated the highest levels of luciferase activity, and the EGF-dependent induction of luciferase activity in these cells was significantly greater than in uninfected cells or control infected cells. These data suggest that Srcasm can promote transcriptional events downstream of the EGF-RAS-MAP kinase pathway involving Elk-1, and Srcasm may potentiate the effect of EGF on Elk-1-dependent transcription in primary keratinocytes.

**Increased Srcasm Expression in Keratinocytes Inhibits DNA Synthesis and Promotes Differentiation**—Increased Fyn activity in primary murine keratinocytes inhibits cell proliferation and promotes the expression of differentiation markers such as transglutaminase and filaggrin (6, 30). Because Srcasm can promote activation of SFKs in human keratinocytes, including Fyn, the effect of Srcasm on cell proliferation and differentiation was evaluated. Keratinocytes infected with control or Srcasm adenovirus were labeled with BrdUrd and 7-amino-actinomycin D and subjected to flow cytometric analysis to determine the S-phase fraction (data not shown). Ad-Srcasm-infected PHKs had significantly lower S-phase fractions than control cells (Fig. 6, A and B). The ability of increased Srcasm levels to decrease the S-phase fraction of PHKs was tested under a variety of growth conditions. Under both growth factor depletion and EGF stimulation, Ad-Srcasm-infected PHKs had significantly lower S-phase fractions than control cells (Fig. 6C). These data confirm...
that Ad-Srcasm-infected PHKs have a decreased S-phase fraction compared with control cells.

Decreases in the percentage of keratinocytes in S-phase could correlate with initiation of differentiation; therefore, the effect of increased Srcasm on keratinocyte differentiation was assessed. The effect of increased Srcasm on filaggrin expression and BrdUrd labeling was characterized using immunofluorescence. Immunofluorescence staining for BrdUrd and Srcasm demonstrated that keratinocytes with higher levels of Srcasm did not contain nuclear BrdUrd, whereas cells with lower levels of Srcasm had nuclear BrdUrd (Fig. 7A, left panel). In similar experiments, keratinocytes expressing the filagrin precursor protein did not demonstrate nuclear staining for BrdUrd, while cells with little filaggrin staining did (Fig. 7A, middle panel). Keratinocytes with increased Srcasm levels demonstrated higher levels of the filagrin precursor protein (Fig. 7A, right panel). Therefore, increased Srcasm levels correlate with decreased BrdUrd labeling and increased filagrin expression in keratinocytes.

Western blotting of lysates from keratinocytes infected with the Srcasm adenovirus demonstrated increased levels of filaggrin at 3 and 5 days post-infection relative to control cells (Fig. 7B). Interestingly, the levels of cellular Srcasm also increased in a parallel manner (Fig. 7B). Together, these data demonstrate that increased Srcasm levels inhibit keratinocyte proliferation and promote aspects of the keratinocyte differentiation program such as increased filagrin expression.

**Srcasm Protein Levels Decrease in Human Cutaneous Neoplasia**—Because increased levels of Srcasm inhibit cell proliferation and promotes keratinocyte differentiation program, the level of Srcasm was evaluated in human cutaneous neoplasia where keratinocyte differentiation is disrupted. This question was addressed by immunohistochemistry using affinity-purified rabbit polyclonal α-Srcasm on sections representing the various stages of human cutaneous neoplasia and by Western blotting of lysates from tissue samples (31). The specificity of α-Srcasm was demonstrated by detecting increased levels of Srcasm in COS cell lysates overexpressing Srcasm, and blocking detection by preincubating the antisera with Srcasm fusion proteins (Fig. 8A). Staining with purified IgG from pre-immune serum (Fig. 8B) or secondary antibody alone (data not shown) did not demonstrate significant specific epidermal staining. Staining with pre-immune IgG did demonstrate nonspecific staining of the stratum corneum, which is commonly seen but was not seen with the affinity purified antibody (Fig. 8, C–F).

A total of 17 actinic keratoses (early dysplasia), 8 squamous cell carcinomas in situ, and 12 squamous cell carcinoma (SCC) biopsy samples were examined. For each experiment, two biopsies of normal skin were used as controls. In addition, nearly all lesional samples examined contained adjacent portions of normal epidermis on the same tissue profile that served as internal controls. When compared with normal skin samples and perilesional skin, there were readily detectable decreases in Srcasm staining in 15/17 actinic keratoses, 7/8 squamous cell carcinoma in situ, (SCIS), and 10/12 invasive SCC (Figs. 8 C–E). Those lesions that did not show a decrease in Srcasm staining showed similar levels of Srcasm as did the controls (data not shown). The relative decrease of Srcasm staining between lesional and normal epidermis was more readily detected in SCCs than in SCIS and actinic keratoses; however, this difference cannot be quantified using immunohistochemistry. In addition, lysates derived from cutaneous SCC contain much lower levels of Srcasm compared with control cells.

**Fig. 7.** Srcasm promotes keratinocyte differentiation by decreasing BrdUrd staining and increasing filaggrin expression. A, primary human keratinocytes cultured in 2-well chamber slides were labeled with 10 μM BrdUrd for 3 h and double-stained with the following: left panel, α-BrdUrd (red) and α-Srcasm (green); middle panel, α-BrdUrd (red) and α-filaggrin (green). Keratinocytes cultured on chamber slides were also stained with α-Srcasm (red) and α-filaggrin (green), confocal image in the right panel. DAPI is the nuclear counterstain used. B, Western blot analysis correlates increased levels of Srcasm with increased levels of filaggrin. Keratinocytes were infected with Ad-Con or Ad-Srcasm at an m.o.i. of 200. Cells were lysed at the indicated times post-infection. Lysates were subjected to Western blot analysis using α-filaggrin, α-Srcasm, and α-β-actin.
amounts of Srcasm than lysates from unremarkable skin (Fig. 8G). These data suggest that decreased Srcasm levels are associated with cutaneous keratinocytic neoplasia.

**DISCUSSION**

This report provides novel data regarding how Srcasm influences important regulatory signaling pathways involving the EGFR, SFKs, and p44/42 MAP kinases, and it provides insight into how Srcasm may affect the biology of primary human keratinocytes. Previous work has shown that Srcasm can activate SFKs and that it is an *in vitro* substrate of these kinases (20). Once tyrosine-phosphorylated, Srcasm can associate with important cell signaling molecules, including SFKs, Grb2, or p85, the regulatory subunit of phosphoinositide 3-kinase (20). Given these characteristics, Srcasm may activate SFKs and help transmit signals from these kinases to other intracellular signaling pathways. Until now, it was not known what signaling pathways are influenced by Srcasm or how Srcasm is regulated by specific cell signaling pathways.

The EGFR signaling pathway is important for regulating cell growth and differentiation in keratinocytes and other epithelial cells. A large body of work supports the hypothesis that signaling through EGFR promotes keratinocyte proliferation and is important in keratinocytic neoplasia (32–36). In addition, signaling through EGFR may also promote differentiation in keratinocytes cultured on specific substrates (37). One consequence of EGFR stimulation is activation of SFKs (38). Because activation of these kinases represents an important step in transmitting the EGF-dependent signal, it was important to determine if Srcasm was involved with EGFR signaling in keratinocytes (39, 40). EGFR and TGF-α stimulation of keratinocytes promote tyrosine phosphorylation of Srcasm, and increased Srcasm levels are associated with higher levels of SFK activation. Therefore, Srcasm is a component of the EGFR signaling pathway that activates SFKs and may promote SFK-dependent signaling in keratinocytes. Because tyrosine phosphorylation of Srcasm was dependent on both EGFR and SFK activity, other tyrosine kinases activated by EGFR, including Janus kinases, probably do not efficiently phosphorylate Srcasm (41).

Originally, Srcasm was isolated using Fyn as the bait for a yeast two-hybrid screen of a keratinocyte library (20). Therefore, it was not surprising to find that Srcasm can activate Fyn in keratinocytes; however, it is intriguing that Srcasm preferentially activates Fyn and Src over Yes in human keratinocytes (Fig. 3). This observation implies that Srcasm may preferentially promote Fyn- and Src-dependent signals in keratinocytes. In primary murine keratinocytes, enhanced Fyn activity is associated with differentiation; increased calcium exposure,
which induces differentiation, preferentially activates Fyn but not Src or Yes (6, 42). Fyn activity is important also for proper expression of filaggrin and transglutaminase and decreased DNA synthesis, suggesting that increased Fyn activity is associated with keratinocyte differentiation (6, 30). It appears that increased Srcasm levels promote human keratinocyte differentiation by decreasing DNA synthesis and promoting filaggrin expression (Figs. 6 and 7). Additional studies in other cell lines will reveal if Srcasm can promote differentiation in a variety of cells.

An important question in cell biology regards the means by which growth factor tyrosine kinases and non-receptor tyrosine kinases are linked to the p44/42 MAP kinase pathway. In keratinocytes, activation of the EGFR has been linked to activation of p44/42 MAP kinases (43). However, the mechanism by which SFKs are integrated into the EGFR-MAP kinase pathway has not been well defined. Srcasm appears to link SFKs to the EGFR-MAP kinase pathway, because increased Srcasm levels were associated with higher levels of activated SFKs and p44/42 MAP kinases, and this activation was minimized by inhibitors of EGFR and SFKs (Fig. 4). Also, the observed Srcasm-dependent activation of p44/42 MAP kinases was abrogated by U0126, a MEK 1/2 inhibitor. Therefore, Srcasm appears to function at an important signaling nexus linking EGFR and SFKs with MEK 1/2 and p44/42 MAP kinases.

Modulation of p44/42 MAP kinase activity is critical for regulating cell proliferation and differentiation (25). The ability of Srcasm to modulate p44/42 MAP kinase activity correlates well with a role in promoting keratinocyte differentiation. The EGFR-induced inactivation of p44/42 MAP kinases in cells overexpressing Srcasm is an interesting observation because of the difference between short-term activation and long-term activation of this pathway. Interestingly, persistent EGF signaling, which activates p44/42 MAP kinases, can decrease proliferation of neoplastic cells, whereas transient activation of the same pathway promotes proliferation (44).

To transiently deactivate p44/42 MAP kinase in the early stages of EGF stimulation, Srcasm may activate/recruit a phosphatase, because levels of total p44/42 MAP kinase remain stable. An alternative interpretation is that the fraction of activated p44/42 MAP kinases may be small compared with the total cellular pool of p44/42 MAP kinases; therefore, degradation of activated p44/42 MAP kinases by a Srcasm-promoted proteasome-dependent mechanism cannot be completely excluded. It will be interesting to determine the mechanism by which increased Srcasm levels promote EGFl-dependent transient deactivation of p44/42 MAP kinases and if this phenomenon occurs in transformed cells.

Decreased Srcasm levels were seen in lesions of human keratinocytic neoplasia (Fig. 8). This observation raises the hypothesis that Srcasm expression may be down-regulated in cutaneous neoplasia. It is well known that uncontrolled Src kinase activity can lead to neoplasia in a variety of cell types (1). In fact, overexpression of Src in murine keratinocytes enhances tumor promotion and malignant progression (45). One hypothesis stemming from these observations is that Srcasm levels may be important for directing SFK signaling through the proper pathways. Recent studies have shown that the GAT domain of Srcasm and TOM1 may play a role in binding ubiquitinated proteins and promoting ubiquitination (46–48). It will be important to determine how the GAT domain of Srcasm regulates SFK function. Further studies, including the use of transgenic mice overexpressing Src kinases and Srcasm, will clarify the roles of these molecules in cutaneous neoplasia.

The data presented begin to construct a signaling paradigm for Srcasm in which this molecule transmits signals from the EGFR and SFKs to important cell regulatory signaling pathways (Fig. 9). Srcasm activates the p44/42 MAP kinase/Elk-1 pathway in a manner dependent on the activity of the EGFR receptor, SFKs, and MEK 1/2 kinases. Therefore, Srcasm ap-
pears to modulate a key growth regulatory signaling pathway in keratinocytes. Additional work to characterize the role of Srcasm in modulating the EGF-RAS-MAP kinase pathway may provide new insights on how this critical pathway can be regulated. Genetically engineered mice overexpressing or lacking SFKs and Srcasm should provide novel insights about Srcasm function and how it mediates aspects of SFK signaling.

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