Inhibition of the Prohormone Convertase Subtilisin-Kexin Isoenzyme-1 Induces Apoptosis in Human Melanoma Cells

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Prohormone convertases (PCs) are endoproteases that process many substrates in addition to hormone precursors. Although overexpression of PCs is linked to carcinogenesis in some solid tumors, the role of subtilisin-kexin isoenzyme-1 (SKI-1) in this context is unknown. We show that SKI-1 is constitutively expressed in human pigment cells with higher SKI activity in seven out of eight melanoma cell lines compared with normal melanocytes. SKI-1 immunoreactivity is also detectable in tumor cells of melanoma metastases. Moreover, tissue samples of the latter display higher SKI-1 mRNA levels and activity than normal skin. From various stimuli tested, 12-O-tetradecanoylphorbol-13-acetate and tunicamycin affected SKI-1 expression. Importantly, SKI-1 inhibition by the cell-permeable enzyme inhibitor decanoyl-RRLL-chloromethylketone (dec-RRLL-CMK) not only suppressed proliferation and metabolic activity of melanoma cells *in vitro* but also reduced tumor growth of melanoma cells injected intracutaneously into immunodeficient mice. Mechanistic studies revealed that dec-RRLL-CMK induces classical apoptosis of melanoma cells *in vitro* and affects expression of several SKI-1 target genes including activating transcription factor 6 (ATF6). However, *ATF6* gene silencing does not result in apoptosis of melanoma cells, suggesting that dec-RRLL-CMK induces cell death in an ATF6-independent manner. Our findings encourage further studies on SKI-1 as a potential target for melanoma therapy.

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INTRODUCTION

Prohormone convertases (PCs) are evolutionary conserved serine proteases that were initially characterized as enzymes processing precursor hormones such as proopiomelanocortin or proenkephalin (Seidah and Chrétien, 1999a; Seidah, 2011). They include PC1/3, furin, PC4, PC5/6, paired basic amino acid cleaving enzyme 4, PC7, subtilisin-kexin isoenzyme-1 (SKI-1), and PC subtilisin/kexin 9. The first seven of these PCs cleave substrates within the motif (K/R)–$(X)_n$–(K/R)↓, where $n=0$, 2, 4, or 6 and X is any amino acid except Cys. SKI-1 cleaves at the consensus motif (R/K)–*X*–(hydrophobic)–*X*, where *X* is variable (Pasquato *et al.*, 2006), whereas PC subtilisin/kexin 9 cleaves behind VFAQ↓ (Benjannet *et al.*, 2004).

SKI-1 belongs to the most recently discovered PCs (Seidah *et al.*, 1999b). It regulates cartilage development (Schlombs *et al.*, 2003), bone mineralization (Gorski *et al.*, 2009), and processing of viral glycoproteins, e.g., those of Lassa virus (Lenz *et al.*, 2001). SKI-1 activates the sterol regulatory element binding proteins 1 and 2 (Espenshade *et al.*, 1999); hence, it is a key regulator of lipid metabolism. Accordingly, pharmacological inhibitors are being developed for the future treatment of dyslipidemia (Seidah and Prat, 2007). The essential role for SKI-1 is highlighted by genetic ablation studies. Here, global SKI-1 deficiency leads to early embryonic death of mice (Yang *et al.*, 2001).

In this study, we investigated the expression, regulation, and function of SKI-1 in normal human melanocytes (NHMs) and melanoma cells. Our study was inspired by increasing numbers of reports that indicate a role for PCs, especially furin and paired basic amino acid cleaving enzyme 4, in tumor progression and metastasis of some solid tumors (Bassi *et al.*, 2005). In this context, it was shown that PC1/3, PC2,

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Spencer JD (2007) H_2O_2-mediated oxidation affects POMC processing and POMC-derived pepides in the human epidermis using vitiligo as a model for oxidative stress. PhD thesis, University of Bradford

Abbreviations: Ab, antibody; AP-1, activator protein-1; ATF6, activating transcription factor 6; CDDE, cell death detection ELISA; dec-RRLL-CMK, decanoyl-RRLL-chloromethylketone; GRP78, glucose-regulated protein 78; MSH, melanocyte-stimulating hormone; NHM, normal human melanocyte; PC, prohormone convertase; SKI-1, subtilisin-kexin isoenzyme-1; siRNA, small interfering RNA; TF, transcription factor; TPA, 12-O-tetradecanoylphorbol-13-acetate

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and furin are expressed in NHMs (Peters *et al.*, 2000; Spencer *et al.*, 2008). However, little is known about PCs and their potential role in malignant transformation of melanocytes, respectively. Here, we show that SKI-1 is constitutively expressed in pigment cells. Using a small-peptide SKI-1 inhibitor, we were able to pinpoint an essential role of this enzyme in the growth and survival of melanoma cells. On the basis of our observations, we suggest a biological role of SKI-1 in melanoma development. Moreover, our data support SKI-1 as a potential target for melanoma treatment.

RESULTS

Detection of SKI expression and activity in human pigment cells *in vitro*

We first examined the expression of SKI-1 in NHMs and human melanoma cell lines. The latter included WM35 (radial growth phase) and WM902B (vertical growth phase of a primary melanoma); IPC298, A375, G361 (advanced primary melanomas); and SK-Mel-30, IGR37, and WM9 (metastases).

Real-time reverse transcriptase-PCR (RT-PCR) analysis revealed similar mRNA levels of SKI-1 in NHMs and melanoma cells (Figure 1a). The identity of the amplification product in one representative melanoma cell line (IPC298) was confirmed by DNA sequencing and was found to be identical to the mRNA sequence of SKI-1, as deposited in the NCBI (data not shown). Western immunoblotting confirmed these findings. Here, a single band of ~100 kDa was detected. It comigrated with the positive control (HepG2), indicating fully processed SKI-1 (Figure 1b). Notably, SKI-1 protein was also detected in conditioned media of melanoma cells, as shown by one representative melanoma cell line (Figure 1c). Immunofluorescence analysis demonstrated that SKI-1 immunoreactivity is detectable in NHMs as a faint cytoplasmic staining. A speckled immunostaining was also seen in nuclei of NHMs, albeit this staining was more prominent in SK-Mel-30 melanoma cells. By contrast to PC1/3 and PC2 (Peters *et al.*, 2000), SKI-1 did not colocalize in NHMs with the premelanosomal marker NKI/beteb (Figure 1d). In melanoma cells, SKI-1 further colocalized with some melanosomes (Figure 1e). Negative controls consisting of omission of the first antibody (Ab) did not show any staining (data not shown).

To clarify whether the detected SKI-1 in pigment cells is active, we established SKI-1 enzyme assays using the synthetic fluorogenic substrate Ac-RSLK-AMC. Interestingly, SKI-1 activity in conditioned media (Figure 1f), but not in total cell lysates (Supplementary Figure S1 online), was significantly elevated in seven out of the eight melanoma cell lines compared with NHMs.

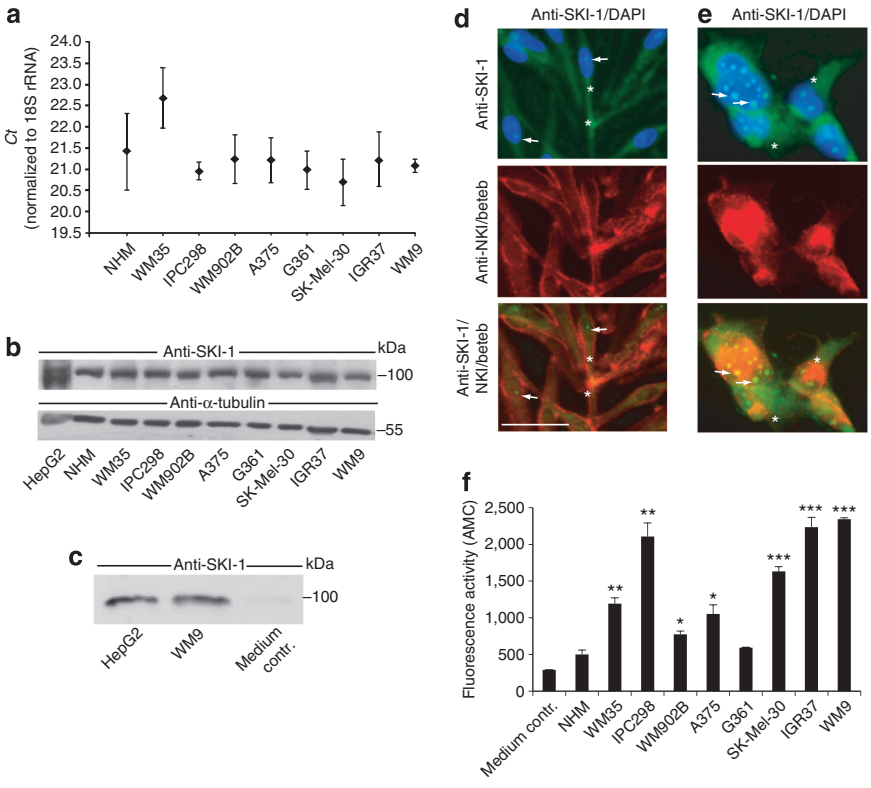

Figure 1. Subtilisin-kexin isoenzyme-1 (SKI-1) expression and activity in human pigment cells *in vitro*. Real-time reverse transcriptase-PCR of SKI-1 in normal human melanocytes (NHMs) and melanoma cell lines; $n = 3$ (**a**). SKI-1 expression in cell lysates of NHMs and melanoma cell lines (15 μg per lane), as shown by western immunoblotting (**b**). Results are based on triplicate experiments. Detection of SKI-1 in conditioned media of melanoma cells as shown by western immunoblotting (40 μg per lane) (**c**). Subcellular localization of SKI-1 in NHMs (**d**) and SK-Mel-30 melanoma cells (**e**), as shown by immunofluorescence analysis. Note cytoplasmic (asterisks) and nuclear SKI-1 immunostaining (arrows). Results are based on triplicate experiments. Bar = 30 μm. SKI-1 activity in conditioned media of NHMs and melanoma cell lines (**f**). Normalized conditioned media were incubated for 3 hours with Ac-RSLK-AMC. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

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