The S100A7-c-Jun Activation Domain Binding Protein 1 Pathway Enhances Prosurvival Pathways in Breast Cancer

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Abstract

S100A7 is among the most highly expressed genes in preinvasive breast cancer, is a marker of poor survival when expressed in invasive disease, and promotes breast tumor progression in experimental models. To explore the mechanism of action, we examined the role of S100A7 in cell survival and found that overexpression of S100A7 in MDA-MB-231 cell lines promotes survival under conditions of anchorage-independent growth. This effect is paralleled by increased activity of nuclear factor-κB (3-fold) and phospho-Akt (4-fold), which are known to mediate prosurvival pathways. S100A7 and phospho-Akt are also correlated in breast tumors examined by immunohistochemistry (n = 142; P < 0.0001; r = 0.34). To explore the underlying mechanism, we examined the role of a putative c-Jun activation domain-binding protein 1 (Jab1)–binding domain within S100A7 using a panel of MDA-MB-231 breast cell lines stably transfected with either S100A7 or S100A7 mutated at the Jab1 domain. Structural analysis by three-dimensional protein modeling, immunoprecipitation, and yeast two-hybrid assay and functional analysis using transfected reporter gene and Western blot assays revealed that the in vitro effects of S100A7 on phospho-Akt and the nuclear factor-κB pathway are dependent on the Jab1-binding site and the interaction with Jab1. Enhanced epidermal growth factor receptor signaling was also found to correlate with the increased phospho-Akt. Furthermore, the Jab1-binding domain is also necessary for the enhanced tumorigenicity conferred by S100A7 expression in murine xenograft tumors in vivo. We conclude that the S100A7-Jab1 pathway acts to enhance survival under conditions of cellular stress, such as anoikis, which may promote progression of breast cancer. (Cancer Res 2005; 65(13): 5696-702)

Introduction

S100A7 is relatively unique among the S100 gene family in terms of structure and its prominent association with early stages of carcinoma (1). Originally associated with abnormally differentiating keratinocytes in psoriasis, “psoriasin” (S100A7) has since been found to be expressed in association with neoplasia in several tissues, including squamous carcinomas in skin (2, 3) and bladder (4), as well as adenocarcinomas of the breast (5, 6). Although the protein is not expressed in normal luminal epithelium, increased expression occurs in parallel with early stages of tumor progression, and S100A7 can be among the most highly expressed proteins in preinvasive carcinoma in situ in both skin (3, 7) and breast (8, 9). With progression to invasive carcinoma, expression is often diminished, but persistent expression can occur in subsets of invasive breast carcinomas where S100A7 is associated with a worse clinical outcome (10).

The action of S100A7 in breast cells may be exerted through an interaction with the multifunctional signaling molecule c-Jun activation domain-binding protein 1 (Jab1). S100A7 expression in the MDA-MB-231 cell model is associated with altered activity of activator protein-1 (AP-1) and hypoxia-inducible factor-1 (HIF-1) transcription factors and their downstream genes, consistent with the downstream pathways believed to be regulated by Jab1 (11). These molecular changes associated with expression of S100A7 are correlated with changes in several biological features in vitro, including decreased adhesion, increased growth, and increased invasiveness. However, the magnitude of these in vitro effects is relatively modest in comparison with a significant overall effect of S100A7 on tumorigenesis in an in vivo xenograft model (11). This suggests that S100A7 may exert other effects that are more important and primarily manifested in the context of the more complex and three-dimensional in vivo environment.

While considering the function of S100A7, we have observed that S100A7 expression can be prominent within areas of tumors that show loss of adhesion and increased apoptosis and necrosis. These are particularly prominent features within high-grade ductal carcinoma in situ (DCIS), but dyskeratosis and loss of cell adhesion are also common features within preinvasive epidermal squamous carcinoma and inflammatory skin lesions, including psoriasis and other idiopathic dermatoses, where S100A7 is highly expressed (12). In breast cells, overexpression is associated with diminished adhesion (11) and S100A7 is induced under conditions of cellular stress, including loss of adhesion (8). In this study, we have examined the relationship between S100A7 and cellular response to stress related to loss of adhesion and the possible role of the S100A7-Jab1 pathway in mediating a prosurvival effect in breast cancer.

Materials and Methods

Mutation of the c-Jun activation domain-binding protein 1–binding site in S100A7 and molecular modeling. The complete open reading frame sequence for S100A7 was cloned previously into the pcDNA3.1 Zeo (Invitrogen, Canada) expression vector (11). Site-directed mutagenesis was done using the Transformer Site-Directed Mutagenesis kit (Clontech, Mountain View, CA) according to the manufacturer’s instructions. The three amino acids (Asp58, Leu80, and Gin89) comprising the putative Jab1-binding domain (13) were altered to encode Gly58, Met80, and Lys89, respectively, using mutagenic oligonucleotides to produce the plasmid

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PCDNA3.1^mut−psor. All mutations were confirmed by DNA sequencing. Three-dimensional models of S100A7 and mutant S100A7 (S100A7^mut) were built based on the X-ray structures (Protein Data Bank codes 3PSR and 2PSR) and compared with the experimental findings (14). CACHe Work-System Pro 6.1.1 (Fujitsu, Beaverton, OR) software was used to substitute the residues and to build the three-dimensional models. The energy of the resulting molecules was optimized using molecular mechanics (MM2) and optimization was continued until the energy change was <0.001 kcal/mol.

Cell culture and stable transfection. Human breast carcinoma cell lines MDA-MB-231 and MDA-MB-468 were cultured as described previously (9). The former cell line is negative for S100A7; whereas the latter expresses S100A7 mRNA and protein [confirmed by reverse transcription-PCR (RT-PCR) and Western blot; data not shown]. MDA-MB-231 cells stably expressing S100A7 were described previously (11). MDA-MB-231 clones stably expressing mutated S100A7 (S100A7^mut) were generated by transfecting the plasmid pCDNA3.1^mut−psor using Effectene transfection reagent (Qiagen, Canada) followed by zeocin selection (Invitrogen). Two MDA-MB-231 clones were isolated that express S100A7^mut (designated as 231-PTM1.2 and 231-PTM1.4; PTM = psoriasin triple mutant). This was determined by Western blot using a specific polyclonal anti-S100A7 antibody (10, 11) directed to the COOH-terminal region of S100A7 that detects both wild-type and mutant proteins.

Anchor-independent growth viability assay. Cells were grown to 40% confluence in 100 mm tissue culture dishes, trypsinized, and resuspended in complete medium. Suspended cells were continuously rocked back and forth in 100 mm sterile bacterial dishes at 40 rpm in a standard tissue culture incubator for 72 hours at 37°C. Nonviable cells were defined according to morphologic assessment of chromatin condensation using fluorescent dyes that bind DNA (15). Briefly, cells are stained for 10 minutes in 5 μL PBS with 5 μg/mL acridine orange (Calbiochem, San Diego, CA) and 5 μg/mL ethidium bromide (Sigma, Canada). A 10 μL aliquot of cells was placed on a microscope slide and viewed with a fluorescent microscope using a wide-band fluorescent filter. Nonviable cells were defined as having condensed chromatin, which lines the nuclear membrane in crescent shapes. The relative viability for each line was calculated and compared with the MDA-MB-231 parental line. The calculated relative viability reflects the proportion of viable cells for 300 cells counted per assay. Triplicate assays were done.

Yeast two-hybrid system. Studies were conducted essentially as described previously (11). The coding region of full-length human S100A7 and the COOH-terminal half (encoding amino acids 43-101) were fused in-frame with the GAL4 DNA-binding domain of the pGBT9 vector (Clontech) to generate pGGBT9-S100A7 and pGGBT9-Cterm-psor, respectively (both containing a frame with the GAL4 DNA-binding domain of the pGBT9 vector; Clontech) and the COOH-terminal half (encoding amino acids 43-101) were fused in-

Tissue microarray and immunohistochemistry. A tissue microarray was constructed based on a cohort of 142 estrogen receptor (ER)−negative invasive carcinomas with ductal or lobular histology selected from the Manitoba Breast Tumor Bank. Duplicate 0.6 mm tissue cores were removed from the central portion of a representative paraffin block from each tumor using a Beecher Instruments (Sun Prairie, WI) tissue arrayer. Immunohistochemical staining for S100A7 and phospho-Akt was done using an automated tissue immunostainer (Ventana Medical Systems, Tucson, AZ) and bulk reagents supplied by manufacturer (10). Primary S100A7 antibody (1:3,000) or phospho-Akt (Ser^197) antibody (1:150) was incubated for 32 minutes. Tumor cell staining was scored as described (10) to generate a semiquantitative immunostaining score. The immunohistochemistry score % positive neoplastic epithelial cells × intensity (%) that ranged from 0 to 300. For categorical statistical analysis, S100A7 immunohistochemistry score of >0 was regarded as positive for S100A7 (10, 16). For phospho-Akt, immunohistochemistry scores of >0, ≥20 (median score), and ≥60 (upper quartile score) were used to define positive status in separate analyses because a wide range of cut points have been used in the literature (17, 18).

Results

S100A7 expression is associated with increased survival under anchorage-independent conditions. We observed that S100A7 expression in some lung and breast tumors can be prominent in apparently detached but morphologically viable tumor cells adjacent to and within regions of necrosis and/or increased apoptosis (Fig. 1A). To determine if S100A7 enhanced viability under...
conditions of decreased cellular adhesion, MDA-MB-231 cells expressing S100A7 were assessed after 72 hours of growth under anchorage-independent conditions (Fig. 1B). Two independent cell clones (231-PTM.2 and 231-PTM.14) were characterized and shown to express comparable levels of S100A7mut to the level of S100A7 in the previously established MDA-MB-231 clone 231-HP2 (Fig. 2A), but both S100A7mut clones showed no difference in growth rate when compared with parental cells (data not shown). The two independent clones constitutively expressing S100A7 (231-HP1 and 231-HP2) had a similar 1.32 ± 0.053-fold increase (mean ± SD, n = 3) and 1.34 ± 0.036-fold increase, respectively, in viability compared with nonexpressing control (231-neo) and MDA-MB-231 parental cells (Fig. 1C). Two clones expressing S100A7mut were also assessed under the same conditions. They exhibited a partial increase in their relative viability, with rates that were less than those shown by S100A7-expressing lines (Fig. 1C). The mean ± SD (n = 3) relative loss of viability for 231-PTM.2 and 231-PTM.14 were 1.174 ± 0.034 and 1.193 ± 0.022, respectively. These data suggest that S100A7 confers a survival advantage in cells subjected to anchorage-independent conditions.

**Mutation of the c-Jun activation domain-binding protein 1–binding domain of S100A7 abrogates the interaction with c-Jun activation domain-binding protein 1.** We have identified previously an interaction between S100A7 and Jab1 and an association between S100A7 expression and alteration of AP-1 and HIF-1 activities. We therefore postulated that the S100A7-Jab1 interaction might be necessary for the effect on survival pathways. Sequence analysis of S100A7 reveals a domain within the COOH-terminal region that is present in other proteins that physically interact with Jab1 (11, 13). To test the role of this putative Jab1-binding domain in mediating the interaction with S100A7, we first did three-dimensional protein modeling of the potential effect of mutation of the three key amino acids within this domain (13) using the known resolved structure for the S100A7 protein (14). Comparison of the three-dimensional models for S100A7 and S100A7mut proteins confirmed that mutation within this domain does not significantly alter the overall protein structure. However, regional changes in conformation are predicted to occur (Fig. 3A). A minimal structural error of 0.1767 as determined by root mean square is calculated when the three residues Asp58/Gly58, Leu80/Met80, and Gin95/Lys95 are superimposed on each other. Similarly, changes occur around the metal sites for Ca2+ and Zn2+ and structural error of 0.3981 is predicted on metal sites when the three metal cations are superimposed (data not shown). These three-dimensional modeling data predict that mutation of the three key amino acids within the Jab1-binding domain would not substantially alter overall protein structure but would result in significant regional changes in conformation.

To next assess the effect of mutation of this domain on the Jab1 interaction, we mutated the putative Jab1-binding domain in both the full-length S100A7 cDNA and a cDNA representing the COOH-terminal portion alone and coexpressed these proteins separately with Jab1 in the yeast two-hybrid assay. We found that in both contexts these mutations abolished the interaction between S100A7 and Jab1 (Fig. 3B). To further test the role of this same domain within human breast cells and to enable subsequent functional analysis, we then developed MDA-MB-231 cells with stable expression of the full-length mutant S100A7 cDNA (S100A7mut). The clones 231-PTM.2 and 231-PTM.14 were then assayed for an interaction between S100A7mut protein and Jab1 by coimmunoprecipitation (Fig. 3C). Under the same conditions whereby endogenous and transgene-derived S100A7 protein could be coimmunoprecipitated with Jab1 from MDA-MB-468 and 231-HP2 cell clones, respectively, mutation of the Jab1-binding domain within S100A7 significantly reduced the association between S100A7mut and Jab1 in the 231-PTM.2 and 231-PTM.14 clones. However, higher levels of expression seen in 231-PTM.14 cells (Fig. 3A) revealed a limited residual interaction (Fig. 3C).

The **S100A7-c-Jun activation domain-binding protein 1 interaction can stimulate activation of Akt and nuclear factor-κB activity.** The activation of the Akt kinase is thought to be a major factor in promoting survival in epithelial cells under anchorage-independent conditions (19). We therefore assessed Akt expression and phosphorylation status in MDA-MB-231 cell clones expressing S100A7 and S100A7mut proteins by Western blot. Total Akt levels were similar in all cell clones; however, a 4.3 ± 0.4-fold increase (mean ± SD, n = 3) in the relative amount of phospho-Akt (Ser473) was observed in both clones expressing S100A7 when
b, relative NF-κB activity in the same panel of MDA-MB-231 cell lines as determined by transient reporter gene transfection assay. Columns, mean of triplicate independent experiments; bars, SD. *P* were determined by *t* test.

An intact c-Jun activation domain-binding protein 1–binding domain in S100A7 is required for enhanced tumorigenesis in vivo. To determine if abrogation of the S100A7–Jab1 interaction affects promotion of tumorigenesis in vivo, the S100A7mut-expressing MDA-MB-231 cell lines were injected into the mammary fat pad of nude mice and the generation of tumors was assessed in comparison with S100A7-expressing lines and control parental cells (Fig. 5). All cell lines generated tumors that were detectable in most animals by 3 weeks. However, S100A7 cells developed larger tumors significantly more rapidly than the S100A7mut cells, which in turn developed tumors similarly to and showed no significant difference from parental controls (Fig. 5). At 6 weeks postinjection, tumors >1.5 cm had developed in 5 of 5 and 3 of 5 231-HP1 and 231-HP2 cell–injected mice, respectively, but in 0 of 5, 1 of 5, and 0 of 5 for 231-PTM2, 231-PTM14, and parental control MDA-MB-231 cell–injected mice. This same difference was reflected in a significantly (*P* = 0.0039) worse overall survival time for animals injected with S100A7 cells compared with parental control cells as shown previously (11). However, tumors derived from both S100A7mut cells showed no significant difference from the parental control (Fig. 5).

S100A7 induction of phospho-Akt is dependent on phosphatidylinositol 3-kinase activity. Akt activation is typically the consequence of extracellular signals that act through a range of cell surface receptors to stimulate PI3K, the upstream positive regulator of Akt. Alternative mechanisms also exist, including reduced activity of PTEN, a negative regulator of Akt. We observed that PTEN levels were unaffected by the expression of S100A7 or S100A7mut (Fig. 2A), but inhibition of PI3K by LY294002 significantly reduced or abolished the expression of phospho-Akt associated with S100A7 (Fig. 6). We next considered the possible role of the EGFR signaling pathway that is one of many pathways that might act to stimulate PI3K, because AP-1 is essential for the transcriptional regulation of the EGFR ligand, EGF (23). We observed an increase in EGF mRNA levels associated with S100A7 but not S100A7mut (Fig. 6B) and this correlated with induction of p21 protein, a downstream marker of EGFR signaling in the same cells. Additional experiments confirmed that all cell clones express EGFR at equivalent levels (Fig. 6C). Treatment of the different cell clones with a specific EGFR inhibitor (BPIQ) revealed that both baseline and induced phospho-Akt amounts in all cell clones could be significantly reduced regardless of whether S100A7 was present or was mutated (Fig. 6D). These data suggest that in the MDA-MB-231 cell line model S100A7 induces phospho-Akt.
through a classic PI3K-dependent pathway. This may in part be attributable to the autocrine effect of AP-1 induction of EGF leading to an increase in the stimulation of EGFR. When EGFR activity is elevated, it promotes the stimulation of PI3K through phosphorylation events, which in turn result in an enhancement in the phosphorylation status of Akt.

Discussion

In neoplasia, S100A7 is expressed in several tumor types. In addition, S100A7 is induced and sometimes highly expressed with abnormal differentiation in inflammatory dermatoses (12) and in response to diverse stress stimuli, including loss of substrate attachment (8) and UV-B radiation (24) in either breast or skin. S100A7 expression is correlated with increased phospho-Akt in human breast tumors. A, histogram representation of the relative levels of phospho-Akt expression (p-Akt immunohistochemistry score) among S100A7-negative (n = 64) and S100A7-positive (n = 78) tumor subgroups as determined by immunohistochemistry in a tumor tissue microarray. B, contingency table analysis of S100A7 and phospho-Akt status. S100A7 and phospho-Akt immunohistochemistry scores of >0 were used to define a tumor as positive and statistical significance was tested by Fisher’s exact test.

Figure 4. S100A7 expression is correlated with increased phospho-Akt in human breast tumors. A, histogram representation of the relative levels of phospho-Akt expression (p-Akt immunohistochemistry score) among S100A7-negative (n = 64) and S100A7-positive (n = 78) tumor subgroups as determined by immunohistochemistry in a tumor tissue microarray. B, contingency table analysis of S100A7 and phospho-Akt status. S100A7 and phospho-Akt immunohistochemistry scores of >0 were used to define a tumor as positive and statistical significance was tested by Fisher’s exact test.

Figure 3. The putative Jab1-binding domain in S100A7 is required for the interaction with Jab1. A, three-dimensional ribbon model to illustrate the overall similarity and regional differences between the predicted molecular structures of S100A7 mutant compared with S100A7 protein. Structures are aligned in the same orientation. B, yeast two-hybrid control plate (left) shows that the presence of both a bait and a prey plasmid are required for growth in the absence of tryptophan and leucine. However, both the full-length and the COOH-terminal half of S100A7 can interact with Jab1 (middle), but mutation of the Jab1-binding domain prevents an association between these proteins as determined by inactivation of the histidine reporter gene resulting in the absence of growth (middle). Right, plate sector key diagram. C, communoprecipitation reveals that although S100A7 and Jab1 interact in human breast cell lines that express endogenous (MDA-MB-468) or transgene-derived S100A7 (231-HP1 and 231-HP2) no significant interaction is seen in cells expressing S100A7 with mutation of the Jab1-binding domain (231-PTM.2 and 231-PTM.14, respectively).
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S100A7- and S100A7mut-expressing cells is attributable to the intact Jab1-binding domain in the former cells. Whereas S100A7-expressing cells were found previously to have a modest 1.3-fold increase in their growth rate in vitro (11), S100A7mut-expressing cells showed no difference in growth rate compared with MDA-MB-231 parental cells (data not shown).

To understand the possible connections between S100A7 expression and survival in breast cancer cells, it may be relevant to consider similar pathways in keratinocytes in the skin under circumstances where S100A7 is also highly expressed. As already noted, these include inflammatory dermatoses (34) and the response to UV-B-related stress (24), resistance to anoikis, and response to wound healing, where altered signaling pathways have been relatively well defined (23, 35–37). In these circumstances, induction of Akt, NF-κB, and AP-1 are often critical components. In breast cells, Akt and NF-κB have also been implicated in cell survival under anoikis conditions (38, 39); however, phosphorylation of Akt may not necessarily be critical for enhanced survival (40).

A link between Jab1 and Akt has not been identified previously. We can speculate that in breast cells S100A7-mediated induction of phospho-Akt is attributable to increased production of AP-1 and possibly also NF-κB-regulated growth factors and cytokines that can act in an autocrine fashion to stimulate PI3K through their cognate receptors. One such pathway, based on our current data, may be the EGF-EGFR-PI3K signaling pathway. A similar autocrine effect has recently been recognized in relation to overexpression of another EGFR ligand in breast cells (41). We have shown here that the in vitro relation between S100A7 and increased phospho-Akt can also be identified in vivo in ERα-negative tumors. Akt activation is known to promote localization of p27 to the cytoplasm (42) and reduced p27 has been observed in ERα-negative tumors (43). We have also shown previously that S100A7 is associated with reduced p27 in DCIS and invasive breast cancer (16).

Jab1 might also influence NF-κB activity directly by binding to the Bcl-3 protein, which enhances p50-Bcl-3-DNA complex formation, thereby potentially influencing NF-κB activity (44). The effect of S100A7 on NF-κB is consistent with the known role of NF-κB in promoting cell survival in breast cancer (45). S100A7 expression is strongly correlated with the ERα-negative phenotype in both DCIS and invasive disease (9). In ERα-negative breast cell lines, a critical role for EGFR-NF-κB signaling has been defined in mediating cell cycle progression (46) and resistance to chemotherapeutic–apoptotic drug treatment (47). ErbB-2 signaling can also alter NF-κB activity, which correlates with reduced apoptosis. More recently, it has also been shown that an ERα-negative subset of invasive tumors shows increased activation of NF-κB. In this small series (45), 56% of ER-negative invasive tumors manifested higher levels of NF-κB activation, which is similar to the frequency of S100A7 expression in the same tumor phenotype.

Promoting cell survival is a role previously suspected for several other S100 family proteins and S100A7 is one of many similar factors that may influence the balance between survival and apoptosis in breast cancer. However, S100A7 is important because, as several groups have confirmed using distinct technical approaches, S100A7 is among the most highly expressed mRNAs and proteins in DCIS and is frequently differentially expressed during the transition to invasive carcinoma in skin and breast (6–8, 48, 49). The S100A7-Jab1 prosurvival pathway therefore has the potential to have a significant impact in promoting breast cancer at a critical stage in tumor progression.

**Figure 5.** S100A7 but not S100A7mut promotes cell growth and enhanced survival in vivo. Survival plot showing that cells expressing S100A7mut show no significant difference from parental control cells (MDA-MB-231) with respect to tumor growth and survival compared with cells expressing S100A7. MDA-MB-231 cell clones were injected into the mammary fat pads of nude mice. Three animal groups represent mice injected with MDA-MB-231 parental cells (n = 5), S100A7mut cells (n = 10), and S100A7 cells (n = 10). Statistical significance was tested by log-rank test.

**Figure 6.** S100A7 activation of Akt is dependent on PI3K and correlates with increased EGFR signaling as indicated by increased EGF and p21. A, Western blot analysis shows that both enhanced expression of phospho-Akt in cells expressing S100A7 and baseline phospho-Akt expression in parental cells and cells expressing S100A7mut can be significantly reduced or eliminated by treatment with a specific PI3K inhibitor, LY294002. B, RT-PCR analysis shows increased levels of EGF mRNA in clones expressing S100A7 but not S100A7mut. C, Western blot analysis shows increased levels of p21 in clones expressing S100A7 but not S100A7mut. D, Western blot showing phospho-Akt levels can be equalized and decreased in all clones regardless of S100A7 expression by pretreatment with the EGFR inhibitor BPIQ. Controls used are GAPDH mRNA and β-actin protein. See Fig. 2 for key to cell lines.
References


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