Stromal-derived factor-1α induces a non-canonical pathway to activate the endocrine-linked Tac1 gene in non-tumorigenic breast cells

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Abstract

The chemokine Stromal-derived factor-1α (SDF-1α) interacts with seven transmembrane (TM) G-protein-coupled receptor (GPR), CXCR4. SDF-1α is linked to inflammation, chemotaxis, cancer metastasis, and hematopoiesis. Tachykinin (Tac1) peptides bind seven transmembrane (TM), GPR and are involved in tumor promotion. SDF-1α regulates Tac1 expression in non-tumorigenic breast cells through a bimodal pattern with repression at high levels through nuclear factor-kappa B (NFκB) activation. This study focuses on the mechanism of activation at low SDF-1α in MCF12A non-tumorigenic breast cells. Reporter gene assays with the 5’ flanking region of Tac1 (exon 1 omitted) and co-transfection with the repressor of cAMP response element (CREB) (ICER), and transfection with the CRE sites mutated, verified critical roles for CRE sites in SDF-1α-mediated Tac1 activation. Western blots and functional assays with specific inhibitors indicated that SDF-1α phosphorylated CREB (P-CREB) via Gαi-2-PI3K-protein kinase C (PKC)ζ-p38-extracellular signal-regulated kinase (ERK) and no evidence of cAMP–PKA pathway. This observation is different from previous studies that reported CREB-phosphorylated PKA pathway in the activation of Tac1 in bone marrow stromal cells. This suggests cell specificity in Tac1 expression. In conclusion, this study reports on a non-canonical pathway in Tac1 activation by SDF-1α. This finding is significant, since Tac1 is relevant to breast cancer metastasis, to bone marrow where stromal cells have a significant facilitating function.

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Introduction

Stromal-derived factor (SDF-1), which belongs to the CXC chemokine family, functions as a chemoattractant for immune and hematopoietic cells (Ganju et al. 1998). Through alternative splicing, two SDF-1 variants are produced, α and β forms (De La Luz Sierra et al. 2004). Each variant binds to the G-protein-coupled seven transmembrane CXCR4 receptor (Juarez et al. 2004). Activation by SDF-1α leads to intracellular calcium mobilization and signaling linked to cell motility (Wang et al. 2000).

SDF-1α levels are elevated in several organs, including the bone marrow, although its concentrations are compartmentalized (Lapidot et al. 2005). SDF-1α levels are relatively high close to the endosteum of bone marrow, which has been attributed to its constitutive production by the endogenous stromal cells (Kucia et al. 2005). Bone marrow shows a gradient pattern in SDF-1α levels across the cavity (Jung et al. 2006). This pattern is indirectly proportional to the level of hypoxia (Ceradini et al. 2004, Jung et al. 2006). Through multiple mechanisms, SDF-1α is involved in cellular movement in and out of the bone marrow toward other organs and also in the peripheral circulation (Ceradini et al. 2004, Kucia et al. 2005, Lapidot et al. 2005, Jung et al. 2006). Membrane-bound SDF-1α is partly responsible for retention of cells in the bone marrow, including hematopoietic stem cells, which express its cognate receptor, CXCR4 (Gazitt 2004).

Tac1 (preprotachykinin-A) is an evolutionary conserved single copy gene that is ubiquitously expressed (Greco et al. 2004). Tac1 encodes several peptides belonging to the tachykinin family, of which substance P and neurokinin-A are its major products (Singh et al. 2000, Bellucci et al. 2002, Greco et al. 2004). The tachyklinins interact with varying affinities to three neurokinin (NK) receptors such as NK1, NK2, and...
NK3 (Kang et al. 2004). NK receptors belong to the group of G-protein-coupled seven TM receptors (Bandari et al. 2005). In healthy cells, NK1 and NK2 exhibit intracellular crosstalk that appears to regulate the functions of each other (Kang et al. 2004).

Tac1, NK1, and NK2 are co-expressed in several cancers, including breast cancer (Singh et al. 2000, Rao et al. 2004, Bigioni et al. 2005, Jung et al. 2006). In several cases, cause–effect relationships have been demonstrated between Tac1 expression and tumorigenesis (Aalto et al. 1998, Moharita et al. 2004, Oh et al. 2004, Rao et al. 2004, Bigioni et al. 2005). In addition, experimental studies have demonstrated a facilitating role for Tac1 in the entry of breast cancer cells into bone marrow (Oh et al. 2004, Rao et al. 2004). Tac1 peptides have also been shown to mediate functions amenable to cancer development. These include protection from radiation damage, hypoxia, apoptosis, enhanced proliferation, and induction of cytokines with angiogenic functions and growth-promoting properties (Fan et al. 1993, Rameshwar & Gascon 1996, Aalto et al. 1998, Qian et al. 2001b). These studies linking Tac1 to breast cancer are important when one considers this type of cancer as hormone dependent. In fact, we have previously shown an inductive role for two neurohormones, prolactin and adrenocorticotropin, in the expression of Tac1 and one of its receptor, neurokinin-1 (Maloof et al. 2001).

SDF-1α has a major role in cancer metastasis due to its chemoattractant property for CXCR4-expressing cancer cells (Muller et al. 2001). The density of CXCR4 on breast cancer cells is proportional to the invasiveness of the cancer (Kato et al. 2003, Mori et al. 2004, Smith et al. 2004). Tac1 appears to be involved in the production of SDF-1α since its level is decreased in Tac1 knockdown breast cancer cells (Oh et al. 2004). The Tac1 gene has been linked to stress, which has been considered as a risk for breast cancer recurrence (Palesh et al. 2007, Ziemsen & Kern 2007). In addition, Tac1 and its receptors, NK1 and NK2, are expressed in breast cancer cells (Singh et al. 2000, Oh et al. 2004, Rao et al. 2004). Furthermore, their expressions appear to be linked to the invasiveness of the cancer cells lines (Castro et al. 2005). Thus, an understanding of the molecular pathway by which Tac1 is regulated would provide insights on the role of the endocrine-linked Tac1 to breast cancer metastasis, and perhaps relapse. We report on the mechanism by which SDF-1α induces Tac1 expression in non-tumorigenic breast cells. This model has been selected to avoid confounds of multiple mutations in breast cancer cells. Using reporter gene assays with the 5′ flanking region of Tac1, we have identified stimulation through Gα2 activation of the MAPK cascade, but not the expected PKA phosphorylation of CRE-binding proteins (Qian et al. 2001a, Jung et al. 2006).

Materials and methods

Reagents and antibodies

FCS, anti-diphosphorylated ERK1/2, and -actin mAb were purchased from Sigma. Recombinant human (rh) SDF-1 was purchased from R&D Systems (Minneapolis, MN, USA). Anti-PKCζ, anti-PKCζ, and horse radish peroxidase (HRP)-conjugated goat anti-murine IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-CREB, anti-phospho-CREB, anti-ATF-2, HRP-conjugated goat-anti rabbit IgG, and PI3K inhibitor (LY294002) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to G-proteins were purchased as a kit of multiple anti-G-proteins from Biomol (Plymouth Meeting, VA, USA). cAMP inhibitor (cAMPS-Rp, triethylylammonium salt) was purchased from Tocris Bioscience (Ellisville, MO, USA). PKA inhibitor (H-89), p38 inhibitor (2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-diarylpyrazol-3-one), ERK inhibitor (3-(2-aminoethyl)-5-((4-ethoxyphenyl) methylene)-2,4-thiazolidinedione, HCl), AKT inhibitor (1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate), and C-Jun N-terminal kinases (JNK) inhibitor (anther [1,9-cd]pyrazol-6(2H)-one 1,9-pyrazolanthrone) were purchased from EMD Biosciences (San Diego, CA, USA). Myristoylated PKC inhibitor was purchased from Promega. Anti-PKCζ and anti-PKCζ were purchased from Santa Cruz Biotechnology. Anti-MEKK1/2 antibody was purchased from Upstate (Charlottesville, VA, USA).

Cell lines

MCF12A cells were purchased from American Type Culture Collection, ATCC (Manassas,VA, USA). The cell line was cultured according to ATCC instructions.

Vectors

pGL3-basic containing inserts of the 5′ flanking regions of Tac1 were previously described (Qian et al. 2001a). The basic form is under the control of the inserted promoter as compared with the control promoter, which is under the control of SV40. Tac1/1.2 was previously referred to as PPT-I/1.2. The designation is changed in order to maintain consistency with the evolving consensus for referring to the family of genes, of which there are currently four members. Tac1/1.2 includes the upstream region of intron 1, exon 1, and sequences that flank the 5′ region of exon 1. The other insert, Tac1/N0 was previously referred to as PPT-I/N0. This insert represents only the upstream region of exon 1. Mutated inserts were constructed using the site directed mutagenesis kit from Stratagene (La Jolla, CA, USA), as previously described (Qian et al. 2001a). The expression vector containing...
the ICER II-γ coding sequence (pSV2ICER II-γ) is under the control of the SV40 promoter, which has previously been described (Qian et al. 2001a).

Transfection and reporter gene assays

MCF12A cells were transfected with Effectene reagent (Qiagen). Transfectants were stimulated with SDF-1α ranging between 5 and 100 ng/ml in sera-free media. In parallel studies, MCF12A cells were co-transfected with pSV2ICER II-γ and PPT-I/N0, then stimulated with 20, 50, or 100 ng/ml SDF-1α. In inhibitor studies, transfectants were stimulated with 50 ng/ml SDF-1α in the presence or absence of cAMP, PKA, AKT, LY294002, PKC, JNK, ERK, or MAPK (p38) inhibitors. After 16 h, cells were collected in 30 μl lysis buffer (Promega) and then subjected to freeze-thaw cycles in a dry ice/ethanol bath. Cell-free lysates were obtained by centrifugation at 4000 × g for 5 min at 4 °C. Luciferase activities were quantitated with the lysates using the Luciferase Assay System (Promega) and then presented as luciferase activity per microgram of total protein. The activities subtracted the luciferase background obtained with vector alone, which were <5 RLU. Total protein was determined with a kit purchased from Bio-Rad DC protein assay method.

Western analysis

MCF12A cells were unstimulated or stimulated with SDF-1α (20, 50, or 100 ng/ml). In parallel studies, MCF12A cells were stimulated with SDF-1α (50 ng/ml) and/or the PI-3K inhibitor LY294002 (50 μM) or pertussis toxin (100 ng/ml). After 1 h, nuclear proteins were extracted using the N-extract kit (Sigma) and the total protein concentrations were determined as described above. Extracts (15 μg) were analyzed by western blots using 12% SDS-PAGE, and the proteins were transferred onto polyvinylidene difluoride membranes (Perkin–Elmer Life Sciences, Boston, MA, USA). The membranes were incubated overnight with primary antibody. Except for anti-actin, rabbit was the source of all primary antibodies. Primary antibodies were detected by 2-h incubation with HRP-conjugated IgG. All primary and secondary antibodies were used at final dilutions of 1/1000 and 1/2000 respectively. HRP was developed with chemiluminescence detection reagent (Perkin–Elmer Life Sciences).

Data analyses

Statistical evaluations of the data were done with ANOVA and Tukey–Kramer multiple comparisons test. A value of \(P<0.05\) was considered significant.

Results

The effects of SDF-1α on Tac1 expression follow a bell-shaped pattern with high levels repressing its activity through NFκB activation and low levels activating its expression (Corcoran et al. 2007). The repression of Tac1 activity at high SDF-1α concentration by NFκB activation involves the untranslated exon 1 (Corcoran & Rameshwar 2007). An understanding of SDF-1α-mediated stimulation of Tac1 requires studies that omit exon 1. This was addressed with a reporter gene construct with 740 bp upstream of exon 1, Tac1/N0 (Qian et al. 2001a). This region has two CRE-binding sites that have previously been reported to activate Tac1 (Qian et al. 2001a). It is also possible that the CRE-binding sites might be responsive to downstream events by G-protein activation linked to CXCR4 (Roland et al. 2003).

ICER in Tac1/N0 activation

The first set of studies focused on reporter gene activity by Tac1/N0 in which we blunted the CRE-binding sites with the negative regulator of CRE sites, early inducible cAMP repressor, ICER (Rosenberg et al. 2002, Servillo et al. 2002). MCF12A cells were co-transfected with pGL3-Tac1/N0 and pSV2ICER II-γ, an ICER expression vector, which negatively regulate activators of CRE-binding proteins (Rosenberg et al. 2002, Servillo et al. 2002). Transfectants were stimulated with concentration ranges of SDF-1α that have been shown to exhibit activation effects on endogenous Tac1 expressions, and reporter gene activity in the presence of exon 1 (20 and 50 ng/ml; Corcoran & Rameshwar 2007). To compare the stimulatory effects, we performed parallel studies with one concentration that inhibited endogenous Tac1 expression, including reporter gene activity when exon 1 (100 ng/ml) was present (Corcoran & Rameshwar 2007). Controls included unstimulated transfectants or cells transfected with pGL3 alone. As expected (Corcoran & Rameshwar 2007), there were dose-dependent effects between luciferase activities and SDF-1α levels (Fig. 1A, hatched bars). However, in transfectants with pSV2ICER II-γ, there was significant (\(P<0.05\)) reduction in luciferase activities (Fig. 1A, open bars). The results showed negative effects of pSV2ICER II-γ on the activation of Tac1/N0.

Effects of CRE-binding sites on the activation of PPT-I/N0 by SDF-1α

The next set of studies sought to determine whether one or both CRE sites were involved in SDF-1α-mediated activation of PPT-I/N0. We therefore transfected MCF12A with Tac1/N0 wild type or mutated at the first, second, or both CRE sites (CRE-Mut I, CRE-Mut II, or CRE-Mut I and II). The effectiveness of mutation was
previously reported by gel shift assays (Qian et al. 2001a). To remain focused, we selected 50 ng/ml SDF-1. As expected, there was significant increase in luciferase activity for cells transfected with wild-type CRE (Fig. 1B, open bars). Single mutation at either CRE sites significantly (*P<0.05) reduced luciferase activities (Fig. 1B, two middle bars). However, the blunting effect was more pronounced for CRE-Mut I than CRE-Mut II (Fig. 1B). Interestingly, the stimulation of transfectants with double CRE mutations led to significant (**P<0.05) increase in luciferase activities (Fig. 2B, hatched bar). These were highly significant findings that are discussed.

While single mutation of either CRE-Mut I blunted the response to SDF-1, CRE-Mut II was less efficient at inhibiting the effect of SDF-1.

**CREB activation in SDF-1-α-stimulated MCF12A**

Based on a role for CRE-binding proteins in the activation of Tac1/N0 (Fig. 1A and B), we next confirmed that the reporter gene activity correlates with the presence of CRE-binding proteins. To address this question, we studied cell extracts for two of the major CRE-binding proteins, phospho (P)-CREB and ATF-2 (Rosenberg et al.).

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**Figure 1** Role of CRE-binding sites in SDF-1-α-mediated activation of PPT-I/N0. (A) MCF12A was transfected with pGL3/Tac1/N0 and/or pSV2ICER II-γ. After 24 h, transfectants were stimulated for 16 h with different concentrations of SDF-1. Luciferase activities were determined and presented as mean RLU ± S.D., n=4. *P<0.05 versus Tac1/N0 alone. (B) MCF12A was transfected with pGL3-PPT-I/N0, wild-type CRE sites; CRE-mutant I, CRE-mutant II, or CRE-mutants I and II. Transfectants were stimulated with 50 ng/ml SDF-1. The results are presented as mean RLU ± S.D., n=5. (C) MCF12A cells were unstimulated or stimulated with SDF-1 (20, 50, or 100 ng/ml). After 1 h, whole cell extracts were analyzed by western blots with anti-P-CREB. Membranes were stripped and reprobed consecutively with anti-CREB, anti-P-ATF-2, and β-actin. Representative blots are shown for four different experiments. (D) The CREB and P-CREB bands were normalized with β-actin and then presented as the fraction of total P-CREB/total CREB (mean ± S.D., n=4). *P<0.05 versus transfectants without ICER.
Extracts were obtained from MCF12A stimulated for 1 h with SDF-1α at 20, 50, or 100 ng/ml. The stimulation time was determined in time-course studies (not shown). Representative of four western blots, performed for total CREB, P-CREB, and ATF-2, shows increase in nuclear P-CREB at stimulations by 20 and 50 ng/ml SDF-1α, and decrease in its phosphorylation at 100 ng/ml SDF-1α (Fig. 1C). Normalization was done by stripping of membranes and then reprobing with anti-β-actin (Fig. 1C, bottom row). The changes in P-CREB are presented as a fraction of normalized total CREB (Fig. 1D, n=4). While SDF-1α at 20 and 50 ng/ml showed four- and sixfold increase in P-CREB, at 100 ng/ml SDF-1α, there was significant (P<0.05) decrease in P-CREB (Fig. 1D). In summary, the results show an increase in P-CREB at low levels of SDF-1α and a reduction at higher concentration.

cAMP–PKA in SDF-1α-linked P-CREB

Since the experimental evidence indicates that SDF-1α stimulation leads to increased P-CREB, and also shows a role for CRE-binding sites in the activation of Tac1/N0 (Fig. 1), we next performed studies to identify the candidate molecules upstream of P-CREB. The studies focused on cAMP–PKA pathway due to its involvement in Tac1 activity in bone marrow stromal cells (Qian et al. 2001a). MCF12A was transfected with pGL3-Tac1/N0 and then stimulated with SDF-1α at 50 ng/ml in the presence or absence of the cAMP inhibitor, cAMPS-Rp (10 μM) or PKA inhibitor H-89 (5 μM). The concentrations of inhibitors were determined in dose–response curves (not shown). Interestingly, each inhibitor resulted in significant (P<0.05) increase in luciferase activities (Fig. 2A). While this indicates that neither cAMPs nor PKAs are upstream mediators of P-CREB, it brings up an important observation that cAMP–PKA could cause exacerbated Tac1 activity.

PI3K in SDF-1α-mediated activation of PPT-I/N0

SDF-1α activation of CXCR4 has been shown to involve Gαi, which has been reported to inhibit cAMP activation (Goichberg et al. 2006). Studies with cAMP and PKA inhibitors rule out these messengers as upstream mediators in the activation of Tac1/N0 (Fig. 2A). We therefore examined alternative pathways that could lead to P-CREB, which has been reported to occur through PI3K-AKT pathway (Du & Montminy 1998, Perkinton et al. 2002). MCF12A was transfected with pGL3-Tac1/N0 and then stimulated with SDF-1α at 50 ng/ml in the presence or absence of 5 μM PI3K or AKT inhibitor. Inhibitor concentrations were determined in dose–response studies (not shown). While there was significant decrease (P<0.05) in reporter gene activity with PI3K inhibitor, there was significant (P<0.05) increase in AKT inhibitor (Fig. 2B). This suggests the involvement of PI3K, but not AKT in the activation of Tac1/N0. Thus, the studies suggest that PI3K activation is upstream of activator of CREB.

Mediators downstream of PI3K

The results in Fig. 2B implicate PI3K as a mediator between SDF-1α stimulation and phosphorylation of CREB. We propose PKC as a mediator since it has been shown to activate CREB through the MAPK cascade.
(Greco et al. 2006). We therefore examined secondary messengers downstream of PI3K in the activation of Tac1/N0 by selecting PKC, MAPK (p38), ERK, and JNK, based on their involvement in other studies (Perkinton et al. 2002). In general, PI3K activates PKC, which in turn activates Raf-1 (Takeda et al. 1999). The latter activates MEKK1/2 (Takeda et al. 1999). At this point, there are three potential targets of MEKK1/2 such as p38, ERK, and JNK (Kito et al. 2000). We first transfected MCF12A with pGL3-Tac1/N0 and then stimulated the transfectants with 50 ng/ml SDF-1α in the presence or absence of PKC (10 μM), ERK (5 μM), JNK (1 μM), or p38 (1 μM) inhibitors. Their respective concentrations were determined in dose–response curves (not shown). After 16 h, cell extracts were quantified for luciferase activity. The results showed significant ($P<0.05$) decrease in luciferase activities in the presence of PKC, ERK, and p38 (MAPK) inhibitors (Fig. 3A), and significant ($P<0.05$) increase with JNK inhibitor (Fig. 3A). The results indicate the involvement of PKC-p38 and ERK activation, but not of JNK.

Functional assays in the presence of specific inhibitors indicated that Tac1 activation involved PKC-MEKK1/2-ERK1/2 signaling pathway. We therefore performed Western analyses for the phosphorylated activators. MCF12A cells were stimulated with 20, 50, or 100 ng/ml SDF-1α. Unstimulated cells served as controls. After 1 h, cell fractions were analyzed by Western analyses. PKCζ has been implicated in ERK1/2 activation and was therefore selected as the focused isoform (Short et al. 2006). The results shown in Fig. 3B indicate activated PKCζ in membrane fractions (PKCζ M, row 1) whereas the inactivated forms are detected in the cytosolic fraction (PKCζ C, row 2). When the bands in the top row are normalized with β-actin (Fig. 3B, last row), the level of activated PKCζ is proportional to the SDF-1α concentration. We next studied whether a similar relationship existed for the classical PKC isoform, PKCζ. Unlike PKCζ, PKCζ showed no change with SDF-1α stimulation (Fig. 3B, rows 3 and 4). Since PKCζ is an upstream kinase of MEKK1/2 and ERK1/2, we examined nuclear extracts for phospho-MEKK1/2 or ERK1/2 and found that phosphorylation of both was directly proportional to SDF-1α concentration (Fig. 3B, second and third panels from the bottom). In summary, the studies in the section indicate activation of PKCζ, MEKK1/2, and ERK1/2 by SDF-1α in MCF12A cells.

**Gz subunit in SDF-1α stimulation**

SDF-1α stimulation led to the activation of CREB and ATF-1 in MCF12A (Fig. 1C). Furthermore, the CRE-binding sites in Tac1 are relevant to its stimulation (Fig. 1A). However, neither cAMPs nor PKAs appear to be mediators between SDF-1α stimulation and the activation of the 5′ flanking region of Tac1 (Fig. 2A). Although SDF-1α has been reported to activate CXCR-4 through Gzq and Gzα (Sotsios et al. 1999, Rochdi & Parent 2003), it is unclear which Gz subunit is activated in our model system. We stimulated MCF12A with 50 or 100 ng/ml SDF-1α. After 30 min, 1 h, and 3 h, cell extracts were prepared and analyzed for activated Gz-protein subunits by Western blot. The data, representative of four blots, showed undetectable Gzq (Fig. 4A, third row from the bottom). Since Gzq has been shown to activate cAMP and PKA, the observation correlates.
with the lack of cAMP–PKA activation (Fig. 2A). Interestingly, G\(_{\alpha_1}\) became undetectable following stimulation with 50 ng/ml but increased after 3 h (Fig. 4A, second row). If the bands for G\(_{\alpha_1}\) are subtracted from those for G\(_{\alpha_1}/G_{\alpha_2}\) (Fig. 4A, top row), it could be deduced that G\(_{\alpha_2}\) is increased at 50 ng/ml up to 3 h. This increase corresponds to the enhanced activity of Tac1/N0 at 50 ng/ml SDF-1\(_{\alpha}\) (Fig. 1A). G\(_{\alpha_3}\) and G\(_{\alpha_q}\) were unchanged in unstimulated and stimulated MCF12A (Fig. 4A, rows 3–5).

**Role of G\(_{\alpha_2}\) subunits in SDF-1\(_{\alpha}\)-mediated activation of PPT-I/N0**

The experimental evidence suggests that G\(_{\alpha_2}\) could be a key factor following stimulation of MCF12A with SDF-1\(_{\alpha}\) (Fig. 4A). To ascertain that its activation is not an artifact, we repeated the experiments described for Fig. 4A in the presence or absence of pertussis toxin, which is an inhibitor of G\(_{\alpha_4}\). The results showed that undetectable G\(_{\alpha_1}\) in cells stimulated in the presence of

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**Figure 4** Role of activated G-proteins in MCF12A cells stimulated SDF-1\(_{\alpha}\). (A) MCF12A cells were unstimulated or stimulated with SDF-1\(_{\alpha}\) (50 or 100 ng/ml). At different times, whole cell extracts were analyzed by western blots with anti-G\(_{\alpha_1}\), anti-G\(_{\alpha_1}\) and 2, anti-G\(_{\alpha_3}\), anti-G\(_{\alpha_3}\) and G\(_{\alpha_0}\), anti-G\(_{\alpha_q/1}\), anti-G\(_{\alpha_s}\), anti-G\(_{\beta}\), and anti-\(\beta\)-actin. Multiple probings were done by consecutively stripping and reprobing of membranes. (B) MCF12A cells were unstimulated or stimulated with 50 ng/ml SDF-1\(_{\alpha}\) in the presence or absence of LY294002 or pertussis toxin. After 3 h, extracts were analyzed by western blot with anti-G\(_{\alpha_1}\), anti-G\(_{\alpha_1}\), anti-G\(_{\alpha_3}\), anti-G\(_{\alpha_3}\), anti-G\(_{\alpha_3}\) and G\(_{\alpha_0}\), anti-G\(_{\alpha_q/1}\), anti-G\(_{\alpha_s}\), anti-G\(_{\beta}\), and anti-\(\beta\)-actin. Membranes were stripped and reprobed consecutively. (C) MCF12A was transiently transfected with pGL3-Tac1/N0. After 16 h, transfectants were stimulated with 50 ng/ml SDF-1\(_{\alpha}\) and/or 100 ng/ml pertussis toxin.
pertussis toxin (Fig. 4B, second row, lane 3). When the normalized densities of Gz1,2 are subtracted from Gz1 and Gz2,2 (Fig. 4B, rows 1 and 2), there was a fivefold reduction in Gz2,2 (Fig. 4B, row 1, lane 3). This indicates that SDF-1α activates Gz2,2 which is partially sensitive to pertussis toxin.

We next determined which G-protein subunit was expressed if we block downstream PI3K. To this end, we repeated the experiments described for Fig. 4A, in the presence or absence of the PI3K inhibitor, LY294002. The results showed a slight increase in the band density for Gz2,2 (Fig. 4B, lanes 1 and 2). This increase is consistent with the signaling pathway being blocked to cause an accumulation of activated Gz2,2. The slight accumulation of Gz2,2 is not an artifact for all G-protein subunits since we observed a reduction in Gzq, in the presence of both LY294002 and pertussis toxin (Fig. 4B, row 5). A point worth mentioning is that down-regulation of Gzq, both in the presence of pertussis toxin and accumulation in the presence of LY294002, signifies that it is not the major signaling molecule but rather Gz2,2 could be the predominant subunit.

The next set of studies determined whether the loss in Gz, protein when cells were stimulated in the presence of pertussis toxin (Fig. 4B, rows 1 and 2) affected the activity of Tac1/N0. To address this question, MCF12A cells were transfected with Tac1/N0. After 16 h, the transfectants were stimulated with 50 ng/ml SDF-1α, in the presence or absence of 100 ng/ml pertussis toxin. At 16 h of stimulation, luciferase activities were quantitated in the presence or absence of 100 ng/ml pertussis toxin. At 16 h of stimulation, luciferase activities were quantitated in the presence or absence of the PI3K inhibitor, LY294002. As we observed a reduction in Gz2,2 in the presence of both LY294002 and pertussis toxin (Fig. 4B, row 5). A point worth mentioning is that down-regulation of Gzq, both in the presence of pertussis toxin and accumulation in the presence of LY294002, signifies that it is not the major signaling molecule but rather Gz2,2 could be the predominant subunit.

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**Discussion**

This study dissects the mechanism by which SDF-1α activates the 5′ flanking region of Tac1 in non-tumorigenic MCF12A. The role of SDF-1α on the regulation of Tac1 is complex, depending on the concentration of SDF-1α and the activator sequences within Tac1. SDF-1α induces the activation of the 5′ flanking region of Tac1 in a dose-dependent manner, only if the upstream sequence of exon 1 is absent (Corcoran & Rameshwar 2007). While relatively low concentrations (20–50 ng/ml) of SDF-1α stimulate the 5′ flanking region of Tac1 and induce its expression, higher concentration (100 ng/ml) mediates inhibition (Corcoran & Rameshwar 2007). In the absence of exon 1 (Tac1/N0), there is a dose-dependent effect between SDF-1α and Tac1 activity (Corcoran et al. 2007). NFκB-binding region found within exon 1 of the Tac1 gene has been shown to mediate gene repression at high concentrations of SDF-1α (Corcoran & Rameshwar 2007). Since this study sought to determine the stimulatory pathway of SDF-1α in Tac1 activation, we focused on the PPT1/N0 gene construct.

The CRE-binding regions of Tac1 are relevant to gene activation in bone marrow cells (Qian et al. 2001a). In this report, overexpression of the negative regulator of CRE-binding proteins, ICER inhibited the activation of Tac1/N0 despite stimulation with SDF-1α (Fig. 1A). This indicates a role for CRE region within Tac1 in SDF-1α-mediated stimulation of PPT1/N0. These studies were supported by functional assays with PPT1/N0 in which the CRE sites were mutated (Fig. 1B). Interestingly, mutation of CRE II showed a trend toward Tac1/N0 activation, independent of SDF-1α (Fig. 1B). On the other hand, double mutation of the CRE region showed a decrease in baseline luciferase activity as compared with wild-type CRE (Fig. 1B). However, despite the double mutations, we observed an increase in luciferase activity upon SDF-1α stimulation (Fig. 1B, extreme right hatched bar). Other transcription factor-binding sites in Tac1/N0 might answer this unexpected increase in luciferase with the double mutants. Ongoing studies have found a stimulatory role for an overlapping Smad (small mothers against decapentaplegic) site following CRE II mutation (unpublished).

Computer analysis of Tac1/N0 for transcription factor motifs has indicated consensus regions for multiple factors, including a YY1 site ~15 bp upstream of CRE I. In addition, we also observed a SMAD4- and REST-binding sites at 20 and 50 bp upstream of the CRE II site respectively. The transcription factors, especially YY1 and SMAD4, would be difficult to access by their respective binding sites when CRE-binding sites are occupied. While we have shown by electrophoretic mobility shift assay that SMAD4 is an authentic binding site (unpublished), similar functional analyses have not been studied for the YY1 consensus sequence. Thus, the increase in luciferase at baseline and in stimulated cells with CRE double mutants might be explained by the newly exposed YY1 and SMAD sites. This is further supported by the clear repression of SDF-1α activation of Tac1 by ICER, which competes for binding to the CRE site (Fig. 1A; Rosenberg et al. 2002). Single mutants also inhibited Tac1/N0 activation by SDF-1α (Fig. 1B).

Cytokines, such as IL-1α, activate Tac1 via the cAMP pathway (Qian et al. 2001a). CXCR4 generally signals via Gz, with concomitant inhibition of the cAMP activator, cAMP. Other studies have shown that an accumulation of cAMP could negatively affect PI3K but nonetheless stimulate molecules downstream of PI3K, including PKC (Goichberg et al. 2006). Thus, cAMP while inhibiting PI3K could activate PKC through Rap1-GTP (Goichberg et al. 2006). In this report, we have ruled out the involvement of cAMP in Tac1/N0 activation (Fig. 2A). Rather we showed the involvement of PKC, MAPK, and ERK (Fig. 3A).
Indeed, SDF-1α activates PI3K by the activation of Ga2 (Figs 2B and 4). Downstream of PI3K is PKCζ followed by MEKK1/2 – ERK1/2 (Figs 3 and 5). Although MEKK1/2 was determined by western analysis for its phosphorylated forms, and not by function, this sequential was deduced, in combinations with the investigations for cAMP–PKA pathway (Figs 2 and 3). The reported findings on SDF-1α signaling through PI3K–PKC–MAPK pathway to activate Tac1/N0 are consistent with the literature pertaining to pathways in brain (Petit et al. 2005).

The present report has physiological relevance to breast cancer. Both SDF-1α and Tac1 expressions are altered in breast cancer cells as they traverse the bone marrow cavity toward the region of high SDF-1α levels (Rao et al. 2004, Kucia et al. 2005). Thus, an understanding of the relationship between SDF-1α and Tac1 expression in non-tumorigenic cells, as reported in this study, could be extrapolated to an understanding bone marrow micrometastasis of breast and, perhaps other cancers. Based on our study of the SDF-1α/Tac1 pathway in the non-tumorigenic model (Fig. 5) and previous report that breast cancer cells adapt a quiescent phenotype in bone marrow, we propose that as cancer cells enter bone marrow they are likely to follow the pathways shown in this study with non-tumorigenic cells (Fig. 5). As cancer cells move toward the endosteum, where the SDF-1α level is high in the stromal cells and low in the integrating breast cancer cells (Moharita et al. 2006), Tac1 expression would be consistent with the combined levels of Tac1 and the microenvironment, where cytokines such as TGF-β levels are increased in the breast cancer cells (Oh et al. 2004). The various changes in both breast cancer and bone marrow-resident cells ultimately lead to a quiescent

**Figure 5** Summary shown for SDF-1α-mediated induction of Tac1. Shown is the intracellular pathway described in this report following the stimulation of MCF12A by SDF-1α at 50 ng/ml to the activation of Tac1. SDF-1α activates Ga2, which in turn activates PKCζ–MEKK1/2–ERK1/2 (Fig. 3A) resulting in the phosphorylation of CREB, which is repressed by ICER (Figs 1 and 2). Phosphorylation of CREB leads to the activation of the 5’ flanking region of Tac1.
phenotype of the breast cancer cells, without affecting hematopoiesis (Rao et al. 2004). Tac1 appears to be necessary for endothelial–mesenchymal–stromal transition, and efficient entry of breast cancer cells into the bone marrow, in particular in regions close to the endostium (Rao et al. 2004). These studies will require in vivo models to further evaluate how the SDF-1x gradient in bone marrow and the SDF-1x/Tac1 pathway in the non-tumorigenic model could relate to breast metastasis. However, we propose that the regulation of Tac1 by SDF-1x is amenable to breast cancer cells as they move into the bone marrow through a gradient change in SDF-1x.

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