Neutrophil Gelatinase-associated Lipocalin (NGAL) Expression Is Dependent on the Tumor-associated Sigma-2 Receptor S2RPgrmc1*\[5\]

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**Background:** Tumor cell invasion is important in cancer progression, and the sigma-2 receptor (S2RPgrmc1) contributes to invasion.

**Results:** S2RPgrmc1-knockdown cells had diminished levels of NGAL protein and RNA, corresponding with decreased active NFκB.

**Conclusion:** We propose a model in which S2RPgrmc1 elevates NGAL expression via EGFR and NFκB.

**Significance:** S2RPgrmc1 may be a potent target for inhibiting tumor invasion.

Tumor invasion is a critical step in the spread of cancer. S2R (sigma-2 receptor)/Pgrmc1 (progesterone receptor membrane component 1) is a cytochrome b\(_5\)-related drug-binding orphan receptor essential for tumor formation and invasion. Secretory proteins drive these processes, so we screened for S2RPgrmc1-dependent secreted proteins using antibody arrays. S2RPgrmc1 markedly regulated the expression of NGAL/LCN2 (neutrophil gelatinase-associated lipocalin/lipocalin 2), a secreted glycoprotein that binds to MMP-9 (matrix metalloproteinase 9) and protects it from degradation. S2RPgrmc1 knock-down blocked NGAL/LCN2 expression at the protein and RNA levels and decreased MMP9 activity. NGAL expression was required for MMP-9 activity and tumor formation. S2RPgrmc1 associates with EGFR and increases EGFR levels at the plasma membrane, and the EGFR inhibitors erlotinib and AG1478, as well as Akt and ERK inhibitors, suppressed the NGAL/LCN2 RNA and protein levels. NGAL is transcriptionally regulated by NFκB, and S2RPgrmc1 knock-down decreased the NFκB subunit p65/RelA acetylation, phosphorylation, and activation. In S2RPgrmc1 knock-down cells, p65 acetylation was reversed by inhibitors of histone deacetylase 1, and the inhibitors partially restored NGAL levels. Our results are consistent with a model in which S2RPgrmc1 increases NGAL/LCN2 levels by activating NFκB via EGFR.

Cancers spread to distant sites by tumor invasion, which is driven by the combined action of proteases, adhesion factors, and signaling proteins. Neutrophil gelatinase-associated lipocalin/lipocalin 2 (NGAL/LCN2)\[2\] is an iron-binding protein (1, 2) that complexes and stabilizes the matrix metalloproteinase MMP9 (3, 4), promoting survival (5, 6), and invasion (4, 7, 8). The NGAL-MMP9 complex is expressed in tumors and is detectable in the blood and urine of cancer patients (3, 9). NGAL expression is driven, at least in part, by a pathway consisting of the HER2/neu-phosphotyrosinol 3-kinas-NFκB pathway in breast cancer cells (7, 10), where it profoundly increases tumor formation and invasion (7). NFκB exists in an inactive complex in the cytoplasm, and is activated by acetylation and phosphorylation (11), although the mechanism through which HER2/neu activates NFκB to elevate NGAL is unknown.

S2RPgrmc1 was originally identified as a putative hormone receptor (12–15), but S2RPgrmc1 is related to cytochrome b\(_5\) and binds to heme (14, 16–21). In normal tissues, S2RPgrmc1 increases lipid synthesis via P450 proteins (20, 22–24), while in tumor cells, it has a profound effect on cell signaling (25–27). Specifically, S2RPgrmc1 associates with EGFR in lung and breast cancer cells, where it elevates plasma membrane levels of the receptor (28). Recently, S2RPgrmc1 was also identified as the putative sigma-2 receptor, an intracellular drug-binding protein (29). S2RPgrmc1 also associates with PAIR-BP1 in ovarian cells (30, 31), although the mechanism of this complex is unknown.

S2RPgrmc1 expression is elevated in a broad spectrum of tumors (32–34), where it is variously associated with survival, tumor stage (34), hormone receptor status (25), and hypoxia (35). In lung cancer cell lines, we have shown that S2RPgrmc1 increases proliferation, invasion, tumor growth, and metastatic colonization (26). In ovarian cancer, S2RPgrmc1 promotes tumor formation and suppresses apoptosis (30, 34, 36). The ligand-binding domain of S2RPgrmc1 is attractive as a therapeutic target, and an S2RPgrmc1 inhibitor, AG-205 (26, 37), inhibits cancer cell proliferation (26), destabilizes EGFR (28) and reverses S2RPgrmc1 agonist binding (29).

In the present study, we report that S2RPgrmc1 drives the transcription of NGAL and the activation of the NGAL-MMP9 complex. We show that NGAL is required for tumor formation in lung cancer cells; NGAL transcription requires EGFR, and
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both proteins are activated by S2R\textsuperscript{\textgamma}m1. The results suggest a model in which S2R\textsuperscript{\textgamma}m1 promotes metalloproteinase activity by activating receptor signaling to NFκB.

EXPERIMENTAL PROCEDURES

Cell Lines and Treatments—A549 and NCI-H226 cells were obtained from the ATCC, cultured under the suggested conditions, and their identity was verified by Genetica LLC (Cincinnati, OH). Cells were maintained in DMEM containing 10% serum and antibiotics, except where described. The AG-205 inhibitor (26) has been described. Short hairpin RNA (shRNA) lentiviruses (Sigma-Aldrich) were prepared using the following formula (27), and EGFR was expressed from the plasmid pcDNA3-EGFR, which was a kind gift from Drs. Penni Black of the University of Kentucky and William Pao of Vanderbilt University. Erlotinib (LC Laboratories, Woburn, MA), S2RPgrmc1 (PGR-UK1 (26)), HA (HA11, Covance), MMP9 (#3852, Cell Signaling), and MMP12 (#3852, Cell Signaling) were used as indicated. Conditioned media was generated by incubating cells in serum-free DMEM media and concentrating the media 10-fold using an Amicon Ultracel 10 kDa molecular mass cut-off filter unit (Milipore, Billerica, MA) and sodium butyrate (Sigma) were used as indicated. Conditioned media were resolved on a 10% Novex Zymogram Gel (Invitrogen, Carlsbad, CA). Thermo-Pierce p65/RelA activation was measured using the NFκB p65 chemiluminescent transcription factor assay kit according to the manufacturer’s instructions. Nuclear lysates were prepared using the NE-PER reagent, and antibody incubations were for 1 h, as indicated.

RNA Analysis—For RT-PCR, total RNA was isolated by TRIzol Reagent (Invitrogen; 15596-026) according to manufacturer’s protocols. cDNA synthesis from 2 μg of RNA was carried out using SuperScript II (Invitrogen) with random hexamers as described (38). Semi-quantitative RT-PCR was performed as described (38). Triplicate samples for quantitative PCR were run in an iCycler (Bio-Rad) using the SYBR Green I system (Bio-Rad). ΔCt for each gene was determined after normalization to β-actin, and ΔΔCt was calculated relative to the control. Gene expression values were then expressed as a fold change, calculated by 2^{-ΔΔCt}. The primer sequences were NGAL-F, 5′-TGAGCACAACATAACACAG-3′; NGAL-R, 5′-AGAGATTGGGAGAGCGGGATG-3′; β-actin-F, 5′-CCTCTCCTGGGATGGAGTCC-3′; β-actin-R, 5′-GGAGCAATGATC-TTGTTCCT-3′.

RESULTS

NGAL Expression Is Dependent on S2R\textsuperscript{\textgamma}m1—S2R\textsuperscript{\textgamma}m1 promotes tumor cell migration and metastatic colonization (26), and we probed conditioned media from S2R\textsuperscript{\textgamma}m1-knockdown A549 NSCLC cells (26) for secreted proteins associated with invasion. Using two arrays that included 81 embedded antibodies to secreted proteins, we detected numerous altered proteins (supplemental Fig. S1). NGAL was essentially absent in S2R\textsuperscript{\textgamma}m1-knockdown cells, the 125 kDa complex decreased 8.5-fold in S2R\textsuperscript{\textgamma}m1-knockdown media (Fig. 1D), and we probed conditioned media from S2R\textsuperscript{\textgamma}m1-knockdown cells, the 125 kDa complex decreased 8.5-fold in zymography (Fig. 1E), the lower percent band decreased 4.1-fold. MMP9 bands were essentially unchanged in the same conditioned media (Fig. 1E, middle panel) and lysates (Fig. 1F) from S2R\textsuperscript{\textgamma}m1-knockdown cells, suggesting that decreased MMP9 activity in media is not due to changes in MMP9 levels.

The shRNA targeting S2R\textsuperscript{\textgamma}m1 binds a sequence in the 3′-untranslated region of the S2R\textsuperscript{\textgamma}m1 transcript, and we expressed an exogenous S2R\textsuperscript{\textgamma}m1 transcript lacking this sequence. Exogenous S2R\textsuperscript{\textgamma}m1 restored NGAL levels in conditioned media (Fig. 2A, lane 4) to levels approximating the control cells (Fig. 2A, lanes 1 and 2). NGAL expression also decreased after treatment with AG-205, an S2R\textsuperscript{\textgamma}m1 small molecule inhibitor (26, 28) in a dose-dependent manner (Fig. 2B). MMP9 was unchanged (Fig. 2B, third panel). While we
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NGAL Requirement for Tumor Formation—NGAL biological activity has not been reported previously for lung cancer. We inhibited NGAL expression (Fig. 4A) by introducing an NGAL-targeting shRNA into A549 NSCLC cells. Zymography of conditioned media from control and NGAL-knockdown cells indicated that both NGAL-MMP9 and MMP9 activity were decreased (26-fold and 52-fold, respectively, Fig. 4B). A 65 kDa band, which could be a cleaved form of MMP9 or uncleaved MMP2, also decreased 11-fold but was detectable in the NGAL-knockdown cells (Fig. 4B, MMP9/2/9). Tumor xenograft formation was sharply impaired in the mice infused with NGAL-knockdown cells (p = 0.002, t test, Fig. 4C), indicating an essential role for NGAL in lung tumor growth. NGAL also promoted motility in A549 cells in a modified Boyden chamber assay (p = 0.0002, t test, Fig. 1D), consistent with its function in regulating metalloproteinases.

NGAL Expression Driven by an EGFR-NFκB Pathway in Lung Cancer—S2R<sup>pgrm1</sup> associates with EGFR, increasing pools of EGFR at the plasma membrane and elevating erlotinib sensitivity (28). In A549 cells, the EGFR inhibitors erlotinib and AG1478/tyrphostin inhibited NGAL expression (Fig. 4, A–C) and RNA levels (Fig. 5D), while NGAL was absent in S2R<sup>pgrm1</sup>-knockdown cells (Fig. 5, A and D, lanes 3–4). Complete NGAL inhibition was detected at higher doses of erlotinib (Fig. 5B) and targeting shRNA into A549 NSCLC cells. Zymography of conditioned media from control and S2R<sup>pgrm1</sup>-knockdown cells showed decreased levels of activated cathepsin D (top panel) but no change in Timp-2 (second panel) in S2R<sup>pgrm1</sup>-knockdown cells. D. Western blot analysis of control and S2R<sup>pgrm1</sup>-knockdown cells revealed no change in levels of the stress response proteins p53, calnexin, and hsp90.

focused this study primarily on Ngal, other proteins were altered in the conditioned media, including activated cathepsin D (Fig. 2C), although Timp2 was unchanged (Fig. 2C).

FIGURE 2. Restoration of S2R<sup>pgrm1</sup> elevates NGAL levels. A, A549 control (lanes 1 and 2) and S2R<sup>pgrm1</sup>-knockdown (lanes 3 and 4) cells were transfected with a control plasmid (lanes 1 and 3) or a plasmid encoding S2R<sup>pgrm1</sup> (lanes 2 and 4). Conditioned media were analyzed by Western blot for NGAL (top) and by SDS-PAGE and Coomassie Brilliant Blue staining (CBB, bottom). Lane 4 shows elevated NGAL levels when S2R<sup>pgrm1</sup> was re-expressed. B, NGAL levels in conditioned media after treatment with increasing doses of the S2R<sup>pgrm1</sup> inhibitor AG-205 for 72 h. While NGAL levels decreased (upper panel), MMP9 levels were unchanged (lower panel). Ku70 served as a loading control. C, Western blot of conditioned media from control and S2R<sup>pgrm1</sup>-knockdown cells showed decreased levels of activated cathepsin D (top panel) but no change in Timp-2 (second panel) in S2R<sup>pgrm1</sup>-knockdown cells. D. Western blot analysis of control and S2R<sup>pgrm1</sup>-knockdown cells revealed no change in levels of the stress response proteins p53, calnexin, and hsp90.

One potential mechanism governing NGAL levels in S2R<sup>pgrm1</sup>-knockdown cells is the activation of a general stress response. However, levels of the stress response genes p53, calnexin and hsp90 (39–41) were unchanged in S2R<sup>pgrm1</sup>-knockdown cells (Fig. 2D). Instead, the decline in NGAL protein levels was reflected in decreased NGAL RNA levels in S2R<sup>pgrm1</sup>-knockdown cells by RT-PCR (Fig. 3A) or real-time quantitative PCR (Fig. 3B). Decreased NGAL RNA levels were also detected after expression of a heme-binding-deficient (S2R-hbd) S2R<sup>pgrm1</sup> mutant (Fig. 3C).

NGAL protein levels are dependent on S2R<sup>pgrm1</sup>. A, proteome array containing antibodies to secreted proteins was probed with conditioned media from control A549 NSCLC cells (top, con) or S2R<sup>pgrm1</sup>-knockdown cells (bottom, shPGR). NGAL is indicated. B, quantitation of NGAL spots in duplicate from a pair of arrays probed with control (top) or S2R<sup>pgrm1</sup>-knockdown (bottom) conditioned media. C and D, Western blots of control (c, odd lanes) or S2R<sup>pgrm1</sup>-knockdown (kd, even lanes) cells that were serum-starved for the indicated times. C, Western blot of NGAL in conditioned media with Coomassie brilliant blue-stained media (CBB, as a loading control. D, Western blot of NGAL in cellular lysates (upper panel) with a Western blot of TIMP2 (lower panel). E, zymography of control and S2R<sup>pgrm1</sup>-knockdown conditioned media showing decreased gelatinase activity in S2R<sup>pgrm1</sup>-knockdown cells. MMP9 expression (Western blot) in the same media samples is shown in the middle panel. Loading controls are analogous to those in panel C. F, Western blot of MMP9 (upper panel) in lysates from control and S2R<sup>pgrm1</sup>-knockdown cells. Ku70 (lower panel) is shown as a loading control.
AG1478 (Fig. 5C). EGFR is upstream of Akt and ERK, two key effector kinases, and the Akt and ERK inhibitors LY294002 and PD98059, respectively, decreased NGAL levels (Fig. 5E). We then took a genetic approach and overexpressed EGFR in control and S2RPgrmc1-knockdown cells (Fig. 5F). EGFR overexpression partially restored NGAL levels in S2RPgrmc1-knockdown cells (Fig. 5F, lane 4). We conclude that EGFR signaling contributes to NGAL transcription. NFκB has been previously implicated in NGAL transcription, and p65-RelA is activated by acetylation and phosphorylation and inhibited by histone decetylases or HDACs (11, 42–44). Acetylated p65/RelA (Ac-RelA) decreased 8-fold in the nuclear fraction of S2RPgrmc1-knockdown cells (Fig. 6A, top panel), while p65-phosphoS536 levels diminished by 5.7-fold (Fig. 6A, second panel) and total nuclear p65 by 3.9-fold (Fig. 6A, third panel). In contrast, the nuclear marker E2F5 was unchanged (Fig. 6A, lower panel). The nuclear and cytoplasmic protein separation was validated with markers for each fraction (supplemental Fig. S4). The results are consistent with a model in which p65 phosphorylation, acetylation, and nuclear transport are partially dependent on S2RPgrmc1. We detected a similar decrease in acetylated p65 in NCI-H226 cells following infection with the Ad-S2R-hbd mutant (supplemental Fig. S3C).

Because HDACs decrease NFκB acetylation, we posited that the HDAC inhibitor SAHA would have the opposite effect. Indeed, control cells treated with SAHA exhibited increased secreted NGAL (Fig. 6B, lane 2), and SAHA restored NGAL to near basal levels in S2RPgrmc1-knockdown cells (Fig. 6B, lane 4). Furthermore, NGAL transcription increased following treatment with the HDAC inhibitor sodium butyrate in control and shPGR cells by 2.2- and 8-fold, respectively (Fig. 6C, lanes 2 and 5). SAHA treatment slightly repressed NGAL transcription in control cells (Fig. 6C, lane 3) but induced NGAL transcription by 4-fold in S2RPgrmc1-knockdown cells. As expected, SAHA treatment had little effect on p65 acetylation in control cells (Fig. 6D, lane 2) but increased p65 acetylation by 2.8-fold in S2RPgrmc1-knockdown cells (Fig. 6D, lane 4). In contrast, E2F5 levels decreased slightly after SAHA treatment and were

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**FIGURE 3.** 
S2RPgrmc1 increases NGAL RNA levels. A, RT-PCR for NGAL (top) and actin (bottom) in A549 control (odd lanes) and S2RPgrmc1-knockdown cells (even lanes) at increasing times after serum starvation. The figure is an agarose gel. B, real-time PCR of NGAL cDNA. The sample pattern is the same as in panel A. C, real-time PCR of NGAL in A549 cells infected with a control adenovirus (LacZ) or increasing doses of the Ad-S2R-hbd adenovirus (see Fig. 2A).

**FIGURE 4.** 
NGAL is required for A549 xenograft tumor growth. A, Western blot of NGAL (top panel) in conditioned media, concentrated 10-fold, from A549 cells infected with a control lentivirus (con, lane 1) or a lentivirus directing expression of an NGAL-targeting shRNA (shNGAL, lane 2). The second panel shows the same samples stained with Coomassie. The lower panels are Western blots of NGAL or ku70 (loading control) in lysates from control and NGAL-knockdown cells. In each case, cells were grown in the absence of serum for 72 h. B, top panel shows zymography of conditioned media from control (lane 1) or NGAL-knockdown cells (lane 2). MMP9 expression is shown in the middle panel, and Coomassie staining is shown as a loading control. MMP-9 and ku70 expression in cell lysates is shown in the lower two panels. C, box plot of tumor weight in control and NGAL-knockdown A549 tumor xenografts, showing decreased tumor growth in NGAL-knockdown cells. D, diagram of invasion assays of control and NGAL-knockdown A549 cells, showing a significant decrease in migration in NGAL-knockdown cells.
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S2RPgrmc1 is elevated in lung cancers and contributes to tumor growth, metastasis and invasion in lung cancer cells. In the present study, we demonstrate that S2RPgrmc1 elevates the transcription and protein levels of NGAL, a secreted glycoprotein that complexes with the MMP9 metalloproteinase, stabilizing the NGAL-MMP9 complex. NGAL also binds to iron (2), and the yeast S2RPgrmc1 homologue is regulated by iron (45) and is essential for iron storage (24). Our results suggest a model in which S2RPgrmc1 activates NGAL transcription. The role of NGAL in promoting or suppressing tumor growth is highly dependent on the tissue of origin, and our results support a role for NGAL in cancer cell invasion and tumor formation in lung cancer.

In addition to increasing NGAL expression, S2RPgrmc1 elevated NGAL-MMP9 activity. Surprisingly, uncomplexed MMP9 activity was also increased by S2RPgrmc1 (Fig. 1), even though MMP9 levels were largely unchanged in S2RPgrmc1-knockdown cells. One potential mechanism is the induction of Timp1, -2, and -3 expression in S2RPgrmc1-knockdown cells (supplemental Fig. S1), which can inhibit MMP9 activation (46). However, Timp2 was not induced in conditioned media by Western blot (Fig. 2), suggesting that the proteome array results may be tightly dependent on specific epitopes recognized by the antibodies embedded in the arrays. Alternately, MMP9 can be activated by multiple proteases (46), and any of them could be altered by loss of S2RPgrmc1. Loss of S2RPgrmc1 reduced activated cathepsin D (Fig. 2), which in turn suggests alterations in cathepsins L and B (47). Interestingly, cathepsin G is important in MMP9 processing (46), and one potential model is that multiple cathepsins are activated by S2RPgrmc1. This model, which is purely speculative, is currently under investigation. The results are important because MMP9 plays a key role in tumor growth and progression (48), and NGAL is a potential mediator of tumor invasion by stabilizing MMP9.

One enigmatic finding in the present study is that S2RPgrmc1 has little effect on MMP9 levels (Fig. 1), while NGAL expression increased MMP9 levels (Fig. 4). In the simplest sense, the expected result is that both S2RPgrmc1 and NGAL inhibition should have the same phenotype, decreased MMP9, reflecting a common pathway. The results suggest that S2RPgrmc1 may have NGAL-independent functions, increasing MMP9, that are uncharacterized.

Based on previously published studies, we propose that the S2RPgrmc1-EGFR complex (28) drives NGAL expression. According to this model, NFκB p65/RelA, an essential protein in K-ras-driven lung cancer (49), is subsequently phosphorylated, acetylated and transported to the nucleus, driving NGAL transcription (Fig. 7). One caveat to this model is that the S2RPgrmc1-knockdown affected NGAL levels more potently than EGFR inhibitors (Fig. 5), suggesting that S2RPgrmc1 may utilize pathways other than EGFR in activating NGAL. Exogenous EGFR expression restored NGAL levels (Fig. 5), but only partially, likely because of the important role of S2RPgrmc1 in stabilizing EGFR pools at the plasma membrane (28). The
The significance of the findings is that tumor invasion is a hallmark of aggressive cancers and is driven, at least in part, by MMPs. Therapeutic targeting of MMPs has had mixed results (48), and the current findings suggest a new approach for targeting MMP-NGAL complexes. Indeed, a small molecule S2R\textsuperscript{Pgrmc1} inhibitor suppressed NGAL expression, and other S2R ligands have activity against cancer cells (29, 52) and may indirectly inhibit MMPs. Our results suggest that strategies inhibiting S2R\textsuperscript{Pgrmc1} could potentially target MMP9 activity in lung cancer and perhaps other cancer types.

The mechanism linking S2R\textsuperscript{Pgrmc1} and EGFR to p65/RelA includes Akt (for maintaining nuclear, acetylated p65/RelA and for activation) and ERK activation (for nuclear, acetylated p65/RelA, Figs. 5 and 6), but does not include altered HDAC1 expression (Fig. 6D). An alternate model is that HDAC1 can be activated by non-EGFR metabolic pathways. For example, S2R\textsuperscript{Pgrmc1} ligands have been implicated in oxidative stress induction (50), suggesting that S2R\textsuperscript{Pgrmc1} normally increases oxidative stress. In turn, oxidative stress inhibits HDAC activity (51), which is predicted to elevate p65/RelA acetylation. The role of S2R\textsuperscript{Pgrmc1} in oxidative stress is a subject of ongoing investigation.
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