Direct coupling of the HER4 intracellular domain (4ICD) and STAT5A signaling is required to induce mammary epithelial cell differentiation

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A R T I C L E   I N F O

Article history:
Received 24 February 2016
Received in revised form 17 June 2016
Accepted 18 July 2016
Available online 20 July 2016

Keywords:
Mammary epithelial differentiation
EGFR-family
STAT5A
HER4/ERBB4
Gene expression
Transactivation

A B S T R A C T

The HER4 receptor tyrosine kinase and STAT5A cooperate to promote mammary luminal progenitor cell maturation and mammary epithelial cell differentiation. Coupled HER4 and STAT5A signaling is mediated, in part, through association of the HER4 intracellular domain (4ICD) with STAT5A at STAT5A target gene promoters where 4ICD functions as a STAT5A transcriptional coactivator. Despite an essential role for coupled 4ICD and STAT5A signaling in mammary gland development, the mechanistic basis of 4ICD and STAT5A cooperative signaling remains unexplored. Here we show for the first time that 4ICD and STAT5A directly interact through STAT5A recruitment and binding to HER4/4ICD residue Y984. Accordingly, altering the 4ICD Y984 to phenylalanine results in a dramatic reduction of STAT5A and 4ICD-Y984F interacting complexes coimmunoprecipitated with HER4 or STAT5A specific antibodies. We further show that disrupting the 4ICD and STAT5A interaction has an important physiological impact on mammary epithelial cell differentiation. HC11 mammary epithelial cells with stable expression of 4ICD undergo differentiation with significantly increased expression of the STAT5A target genes and differentiation markers β-casein and WAP. In contrast, HC11 cells stably expressing 4ICD-Y984F failed to undergo differentiation with basal expression levels of β-casein and WAP. Differentiation in this cell system was induced in the absence of exogenous prolactin indicating that 4ICD activity is sufficient to induce mammary epithelial cell differentiation. Finally, we show that suppression of STAT5A expression abolishes the ability of 4ICD to induce HC11 differentiation and activate β-casein or WAP expression. Taken together our results demonstrate for the first time that direct coupling of 4ICD and STAT5A is both necessary and sufficient to drive mammary epithelial differentiation. In conclusion, our findings that 4ICD and STAT5A directly interact to form a physiologically important transcriptional activation complex, provide a mechanistic basis for the in vivo observations that HER4/4ICD and STAT5A cooperate to promote mammary gland progenitor cell maturation and initiate lactation at parturition.

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1. Introduction

Mammary epithelial differentiation requires the complex interplay between receptor tyrosine kinases (RTK), cytokine receptors, and downstream transcription factors. It is well established that signal transducer and activator of transcription 5A (STAT5A) represents the obligate transcription factor regulating mammary epithelial differentiation. For example, mammary specific deletion of STAT5A results in multiple defects including disengaged secretory epithelial differentiation, loss of milk-gene expression, and catastrophic lactational failure resulting in the death of offspring [1–4]. Significantly, recent experimental evidence indicates that STAT5A is required to establish the luminal progenitor cell population [4,5], providing a mechanistic basis for the loss of differentiated secretory epithelium and subsequent failure to lactate observed in STAT5A-null mice. Ultimately, activation of STAT5A in the differentiating mammary gland is regulated through cooperation between cytokine and the RTK receptor families. Although cytokine signaling through the prolactin receptor contributes to STAT5A activity during early pregnancy [2,8], the RTK HER4 (also referred to as ERBB4) appears to be the obligate regulator of STAT5A mediated luminal progenitor function and differentiation at late pregnancy and parturition [5,6].

HER4 is a unique member of the epidermal growth factor receptor (EGFR) family. Although, all four members of this RTK

Abbreviations: 4ICD, HER4 intracellular domain; ATCC, American type culture collection; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; EGFR, epidermal growth factor family; ERα, estrogen receptor alpha; FBS, fetal bovine serum; HEK, human embryonic kidney; HRGα, heregulin alpha; HRGα1, heregulin beta 1; NLS, nuclear localization signal; PCR, polymerase chain reaction; PI3K, phosphoinositide 3-kinase; RIP, regulated intramembrane cleavage; RT, reverse transcription; RTK, receptor tyrosine kinase; SH2, src homology 2; STAT5A, signal transducer and activator of transcription 5A; TACE, tumor necrosis factor-α converting enzyme; WAP, whey acidic protein; YAP, yes-associated protein

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family localize to the cell nucleus, under specific experimental conditions [7]. HER4 is the only EGFR-family member that undergoes regulated intramembrane cleavage (RIP) to release an independently signaling intracellular domain. Accordingly, ligand activation of HER4 results in tumor necrosis factor-α-converting enzyme (TACE)-mediated cleavage of the extracellular domain followed by γ-secretase catalyzed RIP to release the HER4 intracellular domain (4ICD) [8]. Interestingly, nuclear 4ICD functions as a potent transcriptional coactivator and is recruited to target gene promoters through association with the transcriptional coactivator yes-associated protein (YAP) [9] or the DNA-binding transcription factors estrogen receptor alpha (ERα) [10−12] or STAT5A [13].

Compelling developmental and experimental evidence indicates that HER4 employs multiple novel activities to regulate STAT5A induced luminal progenitor cell maturation and mammary epithelial cell differentiation. The HER4 ligand heregulin alpha (HRGα), HER4, and STAT5A all have overlapping mammary gland phenotypes [1,2,4−6,8,14,15]. Accordingly, HRGα stimulation of HER4 regulates STAT5A activation and subsequent luminal progenitor cell maturation during late pregnancy [5]. Moreover, HRGα promotes proteolytic processing of HER4 to generate 4ICD, which functions as a STAT5A nuclear chaperone and transcriptional coactivator by binding with STAT5A at target gene promoters [13]. STAT5A association with 4ICD and subsequent nuclear co-translocation is essential for STAT5A mediated gene expression and mammary epithelial differentiation [6,13,16]. Mechanistically the association between STAT5A and 4ICD requires an intact STAT5A Src homology 2 (SH2) domain [13] suggesting that STAT5A activity requires direct interaction with a specific phosphorylated tyrosine within 4ICD. However, the 4ICD residue targeted by this developmentally and functionally important phosphorylation event has remained elusive. Here, we demonstrate that phosphorylation of 4ICD Y984 is required for 4ICD interaction with STAT5A and activation of STAT5A target genes during mammary epithelial differentiation. Our results provide the first evidence for a direct interaction between 4ICD and STAT5A, further establishing 4ICD as a unique and obligate regulator of STAT5A activity during mammary progenitor cell maturation and mammary epithelial cell differentiation.

2. Materials and methods

2.1. Expression plasmids

To generate the p4ICD expression vector, the HER4 intracellular domain (4ICD) (residues 673−1308), was subcloned into pEFPN3 (Clontech) with the N-terminal EGFP removed. The Y984F mutation was produced by PCR-assisted site-directed mutagenesis of p4ICD (Clontech) with the N-terminal EGFP removed. The Y984F mutation of EBC lysate (Input) was probed by western blot using the indicated immunoblotting antibodies at 4°C overnight. Immune complexes were recovered by adding Protein A sepharose to each immunoprecipitation reaction and in- elut, 20 μg of lysate (Input) was probed by western blot using the indicated immunoblotting (IB) antibodies.

2.2. Cells

All cell lines were obtained from American Type Culture Collection (ATCC). The human embryonic kidney cell line HEK 293T was cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. HC11 mouse mammary epithelial cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FBS, 10 ng/ml epidermal growth factor (EGF) (Invitrogen), 5 μg/ml insulin (Sigma-Aldrich), and 1 μg/ml hydrocortisone (Sigma-Aldrich). For stable expression of 4ICD or 4ICD-Y984F, HC11 cells were transfected with p4ICD, p4ICD-Y984F or the empty vector using Fugene6 (Roche) and selected with G418 to generate the pooled cell lines HC11/4ICD, HC11/4ICD-Y984F, and HC11/ vector, respectively. Where indicated, differentiation of HC11 stable cell lines was induced by culturing confluent cells in FBS and EGF free culture media.

2.3. Immunoprecipitation and western blot analysis

Co-immunoprecipitation of STAT5A and 4ICD or 4ICD-Y984F was performed exactly as described previously [13,17] using HEK 293T cells were transfected with pEFPN-STAT5A, p4ICD or p4ICD-Y984F using Lipofectamine with Plus Reagent (Invitrogen). At 2 days post-transfection, cell lysates were prepared in EBC buffer (50 mM Tris, pH 7.5, 50 mM Tris, pH 7.5, 0.5% NP-40, with 1 mM phenylmethylsulfonyl fluoride) and 500 μg of lysate was immunoprecipitated overnight.
at 4 °C with antibodies directed against HER4 (Santa Cruz; sc-283) or STAT5 (Santa Cruz; sc-835). Immune complexes were collected by incubating with Protein A sepharose (Roche) at 4 °C for 3 h. and eluted by boiling in 60 μl of NuPAGE LDS Sample Buffer (4X) (Life Technologies) containing NuPAGE Sample Reducing Agent (10X) (Life Technologies). Twenty μl of each immunoprecipitate was loaded in each lane of a NuPAGE 4–12% Bis–Tris Gel (Life Technologies). Total cell lysates were collected using RIPA Buffer (10 mM NaPO4, pH 7.2, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Na-deoxycholate, 1% Nonidet P40) containing Complete EDTA-free Protease Inhibitor Cocktail (Roche) and PhosSTOP phosphatase inhibitor (Roche) and western blot analysis was performed exactly as described previously [18] using the primary antibodies directed against HER4 (Abcam; E200), Stat5 (Santa Cruz; sc-835), and α-tubulin (Upstate Biotechnology; 05829).

2.4. Quantitative RT-PCR

Triplicate total RNA samples were isolated from stable HC11 cell lines induced to undergo differentiation and quantitative RT-PCR was performed exactly as described elsewhere [10] using the following PCR primer pairs to amplify β-actin 5′-CGACTGTGCTATGACAATGAC and 5′-TGAAAGATTGCTTCTCTCGG, β-casein 5′-CGACTGTGCTATGACAATGAC and 5′-TGAAAGATTGCTTCTCTCGG or whey acidic protein (WAP) 5′-CATCCTGCGCTCGTGTGCGC and 5′-CCATGATTGCACACTTTGAAAG. The Ct analysis for each reaction was performed using the supplied 7500 Software v2.0.5 (Applied Biosystems) and gene expression levels were normalized to β-actin using the ΔΔCt method.

2.5. STAT5A knockdown

To suppress expression of endogenous STAT5, the HC11 stable cell lines HC11/vector and HC11/4ICD were grown to confluence and transfected with siRNA targeting STAT5 (Sigma; WD03944063) or scramble siRNA (Sigma; WD03148542) in FBS- and EGF-free RPMI 1640 using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer’s instructions.

3. Results and discussion

3.1. HER4 Y984 mediates the interaction between 4ICD and STAT5A

Our previously published data suggests that 4ICD directly interacts with STAT5A [8,13,17]. Furthermore, this interaction requires an intact STAT5A Src homology 2 (SH2) domain [13], which mediates STAT5A interaction with phosphorylated tyrosine residues.
Although HER4 harbors a total of 27 intracellular tyrosine residues one report indicates that only 19 undergo phosphorylation in response to the HER4 ligand HRGβ1. However, STAT5A was not included in the subsequent unbiased phospho-peptide screen designed to identify SH2-mediated HER4 interacting partners [19]. This limitation precluded identification of potential HER4 phosphotyrosines required to recruit STAT5A. A similar unbiased interactome of all 27 HER4 cytosolic tyrosines revealed STAT5A interaction with a phosphotyrosine representing Y984 positioned within the final carboxyl residues of the intrinsic tyrosine kinase domain [20] (Fig. 1). However, the synthetic peptide-peptide interaction was not confirmed experimentally and the physiological relevance of a HER4/STAT5A interaction remains to be established.

To confirm that HER4 Y984 mediates an interaction between 4ICD and STAT5A we used site-directed mutagenesis of 4ICD to replace Y984 with phenylalanine (Y984F). Both ectopically expressed 4ICD and 4ICD Y984F displayed relatively low but equivalent levels of autophosphorylation (Supplementary Fig. S1) indicating that the Y984F alteration failed to dramatically impact the 4ICD intrinsic tyrosine kinase activity. Using a communoprecipitation assay of HEK 293T transiently transfected with STAT5A and 4ICD or 4ICD-Y984F, we show that, consistent with previously published results [13,17], both the HER4 and STAT5A specific antibodies communoprecipitate the HER4 and STAT5A interacting complex (Fig. 2; lane 5). Significantly, altering the 4ICD Y984 to phenylalanine results in a dramatic reduction of STAT5A and 4ICD-Y984F interacting complexes communoprecipitated with HER4 or STAT5A specific antibodies (Fig. 2; lane 6). These results indicate that Y984 is the major HER4 phosphotyrosine required to mediate a direct interaction between 4ICD and STAT5A.

3.2. Mammalian epithelial cell differentiation requires an intact 4ICD Y984

Previous reports have shown that expression of 4ICD is sufficient to induce mammary epithelial cell differentiation in both in vivo and in vitro experimental systems [21,22]. The mouse mammary epithelial HC11 cell line is a commonly employed model of prolactin-induced mammary differentiation. These cells also undergo differentiation, in the absence of prolactin signaling, in response to 4ICD expression and serum starvation. To determine the physiological role for 4ICD Y984 during mammary epithelial cell differentiation, we induced differentiation of HC11 cells stably transfected with 4ICD or 4ICD-Y984F. The two pooled HC11/4ICD and HC11/4ICD-Y984F stable cell lines displayed equivalent levels of ectopic 4ICD expression (Fig. 3A). Consistent with a role for 4ICD in mammary epithelial cell differentiation, ectopic expression of 4ICD resulted in a significant increase in HC11 expression of the differentiation markers and STAT5A target genes β-casein (Fig. 3B) and WAP (Fig. 3C). In contrast, ectopic expression of 4ICD-Y984F failed to significantly increase HC11 expression of β-casein (Fig. 3B) or WAP (Fig. 3C). These results indicate that an intact 4ICD Y984 is required to induce mammary epithelial differentiation and suggests that phosphorylated Y984 recruits STAT5A to drive mammary differentiation.

3.3. 4ICD-induced mammary epithelial cell differentiation requires STAT5A expression

Activation of STAT5A during 4ICD-induced HC11 cell differentiation suggests that STAT5A is the downstream effector of 4ICD activity in this experimental system [21]. Our findings that ablating 4ICD Y984, which is required for 4ICD to recruit STAT5A, abolishes the ability of 4ICD to induce HC11 differentiation, further substantiates an obligate role for STAT5A in 4ICD-induced mammary epithelial cell differentiation. To confirm a role for STAT5A in 4ICD-induced HC11 differentiation, we determined the impact of siRNA-mediated suppression of STAT5A on the differentiation of HC11/4ICD cells. Consistent with previous results (Fig. 3), differentiation of HC11/4ICD cells transfected with non-specific siRNA (Fig. 4A) resulted in a significant increase in expression the β-casein (Fig. 4B) and WAP (Fig. 4C) differentiation genes when compared to control HC11/vector cells. We successfully used siRNA to suppress STAT5A expression in the HC11/4ICD cells stimulated to undergo differentiation (Fig. 4A). However, in the absence of STAT5A the HC11/4ICD cells failed to induce significant levels of β-casein (Fig. 4B) or WAP (Fig. 4C) expression demonstrating for the first time that STAT5A is required for 4ICD induced differentiation of HC11 cells. Prolactin stimulation of β-casein and WAP was also suppressed, further confirming functional knockdown of STAT5A in the HC11/4ICD cells (Supplementary Fig. S2).

In conclusion, compelling in vivo and in vitro evidence suggests that 4ICD and STAT5A cooperate to drive mammary epithelial cell differentiation; however, experiments demonstrating direct coupling of these two signaling pathways have remained elusive. Here we show for the first time that STAT5A directly interacts with 4ICD and this interaction occurs through STAT5A binding to Y984 of 4ICD. We further demonstrate that the 4ICD and STAT5A interaction is physiologically relevant and required to drive mammary epithelial cell differentiation. Taken together our findings are significant because they indicate that 4ICD and STAT5A can induce mammary epithelial cell differentiation in the absence of prolactin signaling. Furthermore, our findings that 4ICD and STAT5A directly interact to form a physiologically important functional complex provide a mechanistic basis for the observations that HER4/4ICD and STAT5A cooperate to promote mammary gland progenitor cell maturation and initiate lactation at parturition.

Acknowledgments

We thank Elenore Semmes and Alicia Meyer for assistance with experiments and other members of the Jones lab for helpful discussions. This work was supported by NIH/NCI Grants R01CA089783 (FEJ) and R01CA096717 (FEJ), Tulane University Office of Research Bridge Funding (FEJ), and the Gerald and Flora Jo Mansfield Piltz Professorship in Cancer Research (FEJ).

Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.07.015.

Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.07.015.

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