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14. ABSTRACT Prolactin receptor (PRLR) is a single transmembrane receptor that normally requires ligand-binding to trigger intracellular signaling. Several isoforms of the human PRLR have been identified, including a long form (LF) and two short forms (SF1a and SF1b). These isoforms share identical amino acid sequence in the extracellular domain, but different cytoplasmic domain as a consequence of alternative splicing. The extracellular domain consists of two fibronectin-like subdomains, S1 and S2. Recently we have identified the existence of naturally-occurring S2 deleted (delta S2) variants in several human cancer cell lines. We also showed that these human delta S2 isoforms were constitutively dimerized in the absence of added PRL. When overexpressed in human breast cancer cells (T-47D) driven by a Tet-responsive promoter, the short isoform delta S2 SF1b produced prolonged activation of ERK and up-regulated both the cell cycle inhibitor, p21, and the milk protein, beta-casein. In this report, a soluble receptor lacking the S2 subdomain is also described. This isoform, termed SS1, is down-regulated in human breast cancer and is capable of modulating PRL-stimulated signaling in T-47D cells.					
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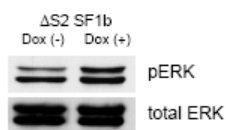
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INTRODUCTION

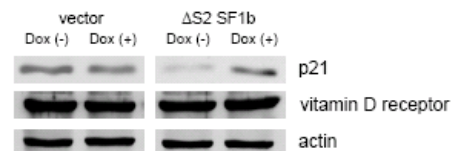
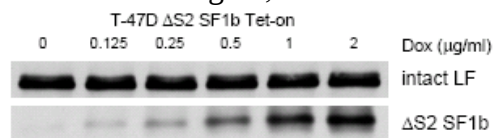
Prolactin (PRL) has been shown to play an important role in controlling the proliferation and differentiation in mammary gland development (1-3). Due to its proliferative property in normal breast tissue, PRL has also been proposed to contribute to the pathogenesis of breast cancer (4). PRL binds its cognate single-membrane receptor, the PRL receptor (PRLR) and triggers multiple signaling pathways involved in a large number of cellular processes (5, 6). Several PRLR isoforms have been identified. Besides the extensively studied long form (LF), the shorter isoforms (including two short forms SF1a and SF1b) are generated by alternative splicing of the same gene, producing transcripts with different lengths in their cytoplasmic signaling domains (7, 8). The ligand-binding extracellular domain, which is identical in primary sequence in all isoforms, consists of two fibronectin-like subdomains, S1 and S2. We have previously shown that deletion of the S2 domain results in constitutive dimerization in the absence of PRL. We also demonstrated these deleted variants (Δ S2 LF, SF1a and SF1b) to be naturally occurring receptors (9). The Δ S2 form of PRLR LF has a growth-promoting effect and both Δ S2 LF and Δ S2 SF1a increase beta-casein expression in transiently transfected human breast cancer cells (T-47D) without added PRL. In an effort to evaluate the individual roles of the different Δ S2 PRLRs in mammary cells, stable T-47D breast cancer cell lines under the control of a tetracycline responsive promoter were established. Analysis showed that overexpression of one short receptor, Δ S2 SF1b, induced prolonged ERK activation and up-regulated the cell cycle inhibitor p21 and the milk protein beta-casein in the absence of added PRL. This suggests both an anti-proliferative and pro-differentiative role for this particular receptor. In this report, a soluble receptor lacking the S2 subdomain was also described. This isoform, termed SS1, was down-regulated in human breast cancer and was capable of modulating PRL-stimulated signaling in T-47D cells.

BODY

We have demonstrated in previous year's work that the Δ S2 variant of the PRL short receptor, Δ S2 SF1b, inhibits growth and migration of human cancer cells. In order to further investigate the biological functions of this apparently beneficial isoform in breast cancer, we established human breast cancer cells (T-47D) stably expressing the Δ S2 SF1b construct. This construct was driven by a Tet-responsive transcriptional activator so that the expression of Δ S2 SF1b could be turned on by tetracycline when needed. As shown in the figure, cells treated with doxycycline showed a marked, dose-dependent induction of Δ S2 SF1b. After treatment of T-47D Δ S2 SF1b cells with doxycycline and subsequent starvation in low serum medium, Western blotting analysis showed increased activation of ERK in the absence of added PRL (left). An increased level of the cell cycle inhibitor, p21, was also observed, whereas expression of another cell cycle regulator, vitamin D receptor, remained unchanged (right).

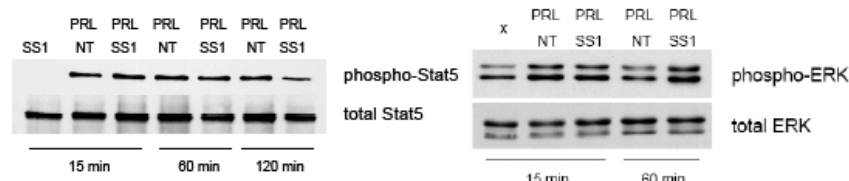
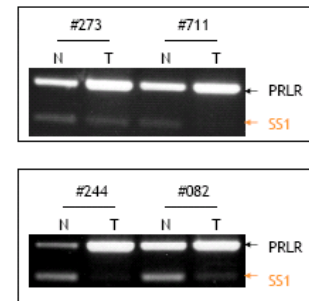


The effect of Δ S2 SF1b on the expression of a milk protein, beta-casein, was also examined. Detection of both the promoter activity and the



mRNA level showed a moderate but significant increase in a 24-hour time frame of the experiment (not shown).

In the course of the study, we also identified another isoform in which the S2 domain was missing. Sequence analysis revealed an exon 6 deleted transcript. This deletion creates a frameshift in the open reading frame, resulting in a shortened soluble receptor essentially composed of half of the extracellular domain. The isoform, designated SS1 (for soluble S1) was first described at the mRNA level by Laud et al (10). They showed that the SS1 transcript was highly expressed in one sample of normal mammary tissue and tissue from fibrocystic disease and was expressed at lower levels in mammary tumors. In our study, we extended this observation by examining tumor samples from patients with invasive ductal carcinomas versus histologically normal contiguous parts from the same patients. Our results from four sample pairs showed higher expression of the intact PRLR in tumor samples and a larger complement of SS1 in adjacent normal regions (right). Immunoprecipitation of T-47D conditioned medium with an antibody recognizing the extracellular domain of the PRLR confirmed the expression at the protein level (not shown). Given that SS1 is secreted soluble receptor, we next examined whether this protein was capable of modulating PRL stimulated Jak2-Stat5 and ERK signaling pathways. Concomitant treatment of T-47D cells with



PRL (at 100 ng/ml) and SS1 conditioned medium showed an earlier decrease in Stat5 activation after 120 minutes, as determined by Western blotting analysis. However, while ERK

activation declined rapidly after 30 minutes in the control group, ERK remained activated in the presence of SS1.

KEY RESEARCH ACCOMPLISHMENTS

- established T-47D Tet-on cell lines expressing Δ S2 PRLR
- characterized a soluble PRLR (SS1) in breast cancer cell lines and human primary tissue
- determined the role of Δ S2 PRLR SF1b and SS1 in receptor signal transduction

REPORTABLE OUTCOMES

Data obtained in this training period was in part presented in a poster session in the Prolactin and Growth Hormone Gordon Research Conference 2008. One paper has been published and one manuscript was prepared for submission.

1. Tan D, Huang KT, Ueda E and Walker AM. (2008) S2 deletion variants of human PRL receptors demonstrate that extracellular domain conformation can alter conformation of the intracellular signaling domain. *Biochemistry*, 47(1), 479-89.
2. Huang KT and Walker AM. (2008) A naturally occurring, inducible, constitutively

- active isoform of the human prolactin receptor, Δ S2 SF1b, reduces proliferation and migration in human prostate cancer cells. Manuscript in preparation.
3. Ueda E, Huang KT, Nguyen V and Walker AM. (2008) Evidence suggesting that prolactin functions in the mammary duct. The 2008 Gordon Research Conference: Prolactin and Growth Hormone Family.

CONCLUSION

Given that the Δ S2 PRLRs are constitutively activated, these receptors are a great tool for studying biological functions of each individual isoform. The experiments can be conducted in the absence of added PRL. Therefore the problem that multiple ligand binding varieties of the PRLR are turned on by one single ligand can be avoided. Based on our preliminary results described above, we conclude that the short receptor Δ S2 SF1b, is both anti-proliferative and pro-differentiative. This is at least in part through prolonged activation of ERK. SS1, a secreted isoform, is a PRL-binding protein that has the capacity to modulate PRL signaling. There is a correlation between loss of SS1 and development of invasive ductal carcinoma, a result which suggests beneficial aspects to increased expression of SS1. Future directions will focus on the molecular mechanisms of Δ S2 PRLRs in mediating cell proliferation, migration, and differentiation. Also examined will be the effect of SS1 expression on the phenotypic changes of breast cancer cells. The Tet-on system will be utilized.

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