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Phosphorylation of glucocorticoid receptor tau1c transactivation domain enhances binding to CREB binding protein (CBP) TAZ2

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ABSTRACT

The glucocorticoid receptor (GR) N-terminal domain (NTD) contains a transactivation domain (activation function 1; AF-1). GR AF-1 is phosphorylated, but effects of this modification upon AF-1 activity and cofactor recruitment are not completely clear. GR AF-1 activity is mostly confined to a short unstructured domain called tau1c (amino acids 187–244) that contains three phosphorylation sites and binds a short cysteine rich fragment (CH3) of the coactivator CREB binding protein (CBP). Since the CH3 domain overlaps the CBP transcriptional adaptor zinc binding (TAZ) 2 domain, implicated in phosphorylation dependent binding to other unstructured transcription factor domains, we set out to investigate whether GR interacts with TAZ2 and whether this binding event is modulated by phosphorylation. We find that GR tau1c is absolutely required for enhancement of GR function and GR/CBP association in cultured cells. Tau1c interacts with TAZ2 *in vitro* and peptide mapping reveals CBP binding determinants throughout tau1c. Phosphorylation at GR Ser203, not involved in transactivation, does not affect tau1c/TAZ2 interactions. However, phosphorylation at Ser211 and Ser226, markers of GR transcriptional activity, greatly enhances TAZ2 binding in a synergistic fashion. We propose that GR tau1c phosphorylation could promote CBP recruitment and enhance AF-1 activity.

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1. Introduction

Glucocorticoid actions are mediated by the glucocorticoid receptor (GR), a nuclear hormone receptor (NR) [1–4]. Like other NRs, GR comprises a C-terminal ligand binding domain (LBD), central DNA binding domain and a long N-terminal domain (NTD) with a poorly defined transactivation function (AF-1). Glucocorticoids trigger GR nuclear translocation, allowing liganded GR to bind glucocorticoid response elements (GREs) or engage in protein–protein contacts with heterologous transcription factors (TFs) near target genes. From these locations, GR modulates gene expression by nucleating large coregulator complexes which influence transcription. For GR, AF-1 can display strong activity in certain contexts [5]. While the structure and mechanism of action of the hormone-dependent LBD activation function (AF-2) is understood in atomic detail, current models suggest that NR NTDs are intrinsically disordered and little is known of NR and GR AF-1 organization [6].

GR is subject to multiple phosphorylation events, many of which affect the NTD [1–3]. Serine (S) 203 and 211 (human GR α numbering) phosphorylation is mediated by cyclin/cdk2 complexes or mitogen activated protein kinases (MAPK) and is enhanced by agonist binding. Ser226 phosphorylation is mediated by c-Jun N-terminal kinase (JNK) and is unaffected by hormone. Relationships between GR phosphorylation and transactivation are complex; changes in GR NTD phosphorylation lead to changes in the spectrum of GR target genes and phosphorylation is thought to influence both transcriptional activity and nuclear trafficking [1,2]. However, of the three phosphorylation events, Ser211 phosphorylation correlates well with GR nuclear localization, DNA binding and optimal transcriptional response at a GR-regulated minimal promoter driven reporter in mammalian cells and GR activity in yeast and is therefore considered to be a marker for transcriptional activity [1,7]. Likewise, Ser226 phosphorylation is also associated with transcriptionally active GRs, although it can also blunt GR activity via enhanced GR nuclear export. By contrast, phosphorylation at Ser203 promotes GR cytoplasmic localization and is associated with transcriptional inactivity.

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TF phosphorylation often leads to changes in coregulator binding [1–3] and, accordingly, GR phosphorylation enhances NTD interactions with mediator complex component med14 (DRIP150/TRAP170) and inhibits interactions with TSG101, a coregulator with roles in cell growth [8]. AF-1 deletion mutagenesis revealed a minimal GR NTD transactivation unit 1 core peptide (tau1c, amino acids 187–244) that retains more than 60% of activity of intact AF-1 in mammalian cells and nearly all GR activity in yeast [9]. Importantly, tau1c overlaps all three major GR NTD phosphorylation sites raising the possibility that this type of modification could play a role in tau1c activity. Tau1c also contains a high percentage of acidic amino acids, similar to transactivation domains (TAD) from p53 and others [10]. Tau1c is disordered in standard conditions, but displays α-helical content in the organic solvent trifluoroethanol, raising the possibility that it acquires structure on binding to a partner [11]. Mutational analysis revealed important roles for tau1c hydrophobic residues that map to predicted α-helices and revealed that some mutations that increase helical hydrophobic content also enhance tau1c activity [10].

CREB binding protein (CBP) and its homolog p300 are histone acetyl transferases that function as GR cofactors [12,13]. CBP/p300 is comprised of multiple domains, including two transcriptional adaptor zinc binding (TAZ) domains that are important for coactivator function and interact with TADs from several TFs including p53 [14,15], STATs [16], adenovirus E1A [17], nuclear receptors [18] and erythroid Kruppel-like factor (EKLF/KLF1) [19]. Phosphorylation of p53 TAD1 enhances binding to p300 TAZ2 [14,20], pointing to an important role for phosphorylation in modulation of TAD/TAZ2 interactions. Interestingly, GR tau1c binds to a cysteine-histidine rich CBP fragment (CH3 domain; amino acids 1678–1868) that overlaps TAZ2, part of the CBP HAT domain and a nearby independently folded ZZ zinc binding domain [10] (PMID). Moreover, effects of tau1c mutations are known to correlate with their effects on binding to CBP [10]. Since TAZ2 binds intrinsically disordered TADs and contacts with p53 are regulated by phosphorylation [14,20], we set out to investigate whether GR also interacts with TAZ2 and whether this binding event is modulated by phosphorylation.

2. Materials and methods

2.1. Transactivation assays

U2-OS cells were grown to ~80% confluency in 24 well plates using no phenol DMEM supplemented with 5% charcoal stripped FBS at 37 °C, 5% CO_2. Cells were transiently transfected with Fu-Gene HD system using 300 ng of promoter only or 300 ng promoter and 100 ng GR (or GR Δtau1c mutant). 1 ng Renilla construct was added to all wells as an internal transfection control. After 6 h, cells were treated with either 100% ethanol (vehicle) or 100 nM dexamethasone. After 18 h cells were lysed and luciferase activities measured according to the Promega Dual-Luciferase Reporter Assay System manual. Values reported are the average of three independent experiments.

2.2. Western analysis and immunoprecipitation

Immunoprecipitations were performed from extracts of HeLa cells transfected with 1 μg HA tagged CBP and GR wild type or GR Δtau1c expression plasmids. Cells were treated ±10 nM dexamethasone for 6 h following transfection, and harvested in RIPA cell lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2.5 mM EGTA, 1% NP-40, protease inhibitor cocktail (Roche)). Whole-cell lysate (400 μg) was incubated with 2 μg of anti-GR (Santa Cruz Biotechnology sc-1002) antibody conjugated agarose bead slurry (Sigma–Aldrich) for 12 h at 4 °C. Antibody conjugated agarose beads were washed three times with RIPA buffer at 4 °C, and bound proteins were separated by SDS–PAGE. Proteins were transferred to a PVDF membrane (Bio-Rad), subjected to western blot analysis with anti-HA (Cell Signaling #2999) and anti-GR (Santa Cruz Biotechnology sc-1002), and then detected with an ECL kit (Amersham Pharmacia).

2.3. Purification of GR tau1c, CBP TAZ2 and CBP ZZ

We ligated cDNAs for human GR tau1c (amino acids 187–244), CBP ZZ (amino acids 1701–1741) and TAZ2 (amino acids 1741–1870) into pET 41a(+). All three constructs have a polyhistidine tag and TEV consensus site (ENLYFQ^G) inserted N-terminally to the protein sequence. GR tau1c cys223 was mutated to ala and an extra cys residue introduced downstream of the TEV site to facilitate fluorophore labeling. GR tau1c was labeled with Alexa Fluor 488 C_5 Maleimide (Molecular Probes, Life Technologies, Grand Island, NY). TAZ2 non-zinc binding cys 1775, 1826, 1827 and 1883 were mutated to ala to facilitate expression and prevent aggregation, as previously described. Proteins were expressed in *Escherichia coli* BL21 DE3, purified according to previously published methods and treated with TEV protease overnight at 4 °C. Cleaved product was passed over a 5 mL nickel charged IMAC column and GSTrap HT column in tandem and flow-through collected, concentrated to 5 mL and purified over a HiLoad Superdex 75 prep grade column. Peak purity was verified by SDS–PAGE and peaks were pooled, concentrated, snap frozen with liquid nitrogen and stored at −80 °C til use.

2.4. Fluorescence anisotropy

GR tau1c was expressed and labeled as above or synthetic peptides (described in each figure) and p53 peptide (amino acids 1–39) were purchased from Genscript (Piscataway, NJ) as 98% pure and N-terminally labeled with FITC, C-terminally amidated and phosphorylated if noted. CBP domains were titrated into 120 μL of 10 nM peptide solution in 25 mM Tris pH 7.4, 150 mM NaCl, 1 mM DTT. Anisotropy was measured at 25 °C on an ISS Inc. (Champaign, IL) PC-1 Spectrophotometer while excited at 490 nm and emission read at 520 nm with a slit width of 2 nm and 1 nm respectively. Anisotropy (r) was converted to fraction bound using the equation $f_{FM\ bound} = (r_{observed} - r_{free})(r_{bound} - r_{free})$ with $r_{free} = r_{observed}$ with no protein titrated and $r_{bound} = r_{observed}$ at saturation. Values were plotted in Prism and fit to the "One site-Total binding" model.

3. Results and discussion

3.1. GR tau1c mediates glucocorticoid response and CBP interactions

We verified that GR tau1c is important for GR activity in cultured cells. Transfection of a GR expression vector into U2-OS osteosarcoma cells, which contain low levels of endogenous GRs, enhanced dexamethasone response at a minimal reporter driven by three copies of the tyrosine aminotransferase (TAT) GRE and the GRE-dependent mouse mammary tumor virus (MMTV) long terminal repeat (Fig. 1A and B) [21]. No increase in dexamethasone response was observed with a GR mutant that lacked tau1c (GR Δtau1c). Instead, this GR mutant exerted weak dominant negative activity upon dexamethasone response observed with endogenous GR.

We also determined whether GR tau1c played a role in CBP binding (Fig. 1C). We introduced an expression vector for full length GR or GR Δtau1c with an expression vector for epitope

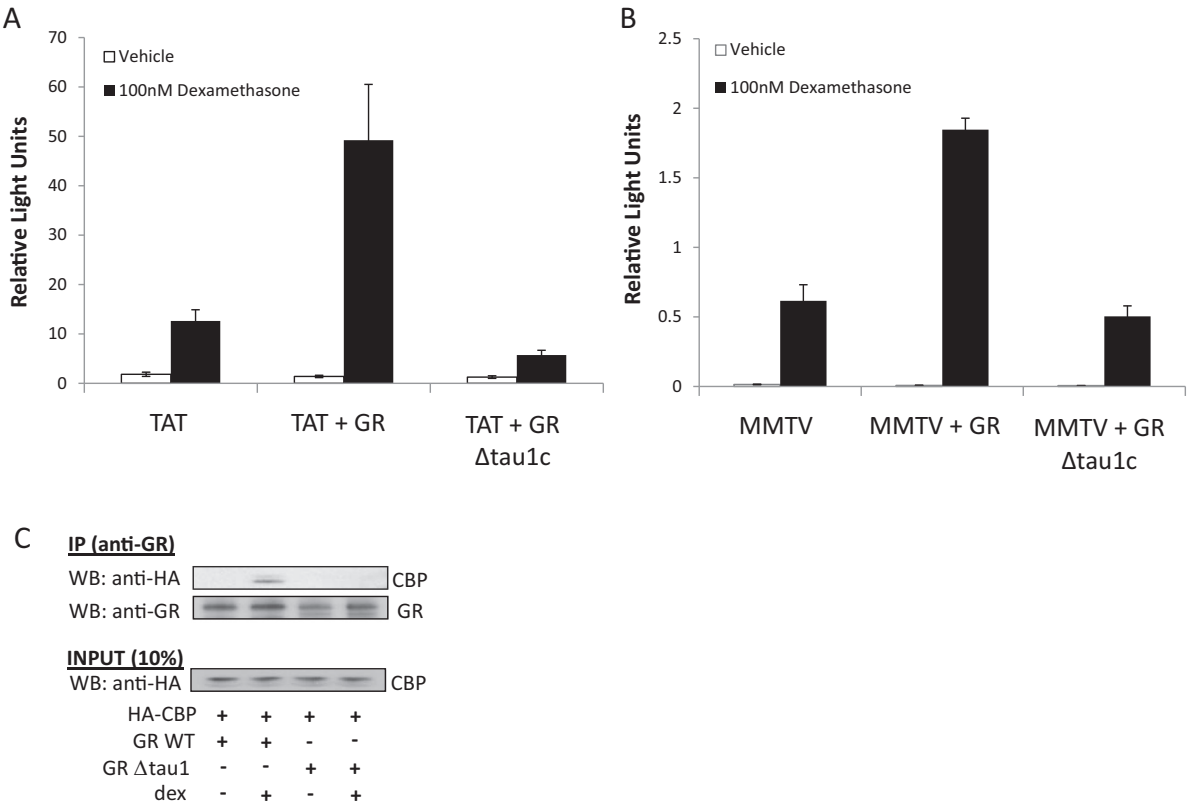

Fig. 1. Tau1c is needed for optimal GR activity and CBP association. (A) The panel represents luciferase assays performed on extracts of U2-OS cells transfected with TAT3-Luc reporter ± control vector, GR expression vector or GR ΔTau1c. (B) As Fig. 1A, except that MMTC-Luc was used. (C) Panels represent western blots of co-immunoprecipitations from HeLa transfected with expression vectors for HA tagged CBP and GR and treated ±10 nM dexamethasone for 6 h. Antibody used for immunoprecipitation is indicated at top and antibody used for western analysis is indicated at the side. Panel below represents western blots of input CBP.

(HA) tagged CBP into HeLa. Immunoprecipitation of GR followed by assessment of CBP pull-down (upper panels) revealed that CBP binds to full length GR in a hormone-dependent fashion. However, GR Δtau1c does not bind to CBP at all. Control CBP westerns confirmed that equivalent amounts of CBP were present in each input sample (lower panel). Thus, tau1c is necessary for GR/CBP association, as well as potentiation of GR activity.

3.2. Tau1c binds CBP TAZ2 and not ZZ

We next used fluorescence anisotropy (FA) based assays to recapitulate GR/CBP interactions *in vitro* and to define whether GR bound to the TAZ2 or ZZ domain. We incubated labeled bacterially expressed GR tau1c peptide (amino acids 187–244) with increasing amounts of CBP TAZ2 and ZZ domains. While we did not detect specific interactions between GR tau1c and the ZZ domain, the GR tau1c peptide displayed saturation binding to the TAZ2 domain with calculated affinity of 9.3 ± 1.8 μM (Fig. 2A). As mentioned, previous studies revealed that the CBP TAZ2 domain binds to the p53 acidic TAD and we verified that a p53 TAD peptide bound to CBP TAZ2 with an affinity of 4.6 ± 2.4 μM in this system (Fig. 2B), comparable to previous results [14,15]. Thus, GR tau1c binds to the CBP TAZ2 domain with slightly weaker affinity than unphosphorylated p53.

3.3. Multiple GR tau1c peptides bind to CBP TAZ2

To define GR tau1c contact points for CBP TAZ2, we synthesized a series of short labeled peptides corresponding to different regions of tau1c that overlap predicted helical and non-helical regions [10] (Fig. 3). We then measured affinity of each peptide for TAZ2 using FA. Interestingly, several GR peptides displayed significant affinity for TAZ2. Peptide T1, which overlapped tau1c H1, bound to TAZ2 with K_d approximately 2 μM affinity whereas an peptide (T2) that overlapped the neighboring non-helical region bound to TAZ2 with much lower affinity (K_d = 24.5 μM). Peptides T3–4, which overlapped H2, also bound TAZ2 with reasonable affinity (K_d = 2.3 and 1.7 μM, respectively). Finally, peptides T5 and T6, which overlap predicted short helix H3, bound TAZ2 with K_d values of 3 and 3.6 μM. The fact that several GR tau1c peptides display significant affinity for TAZ2 suggests that CBP interaction determinants may be spread throughout tau1c. Further, it is noteworthy that tau1c peptides display improved affinity for CBP TAZ2 relative to intact tau1c (Fig. 2). This may imply that TAZ2 binding determinants are partially masked in the context of a full length tau1c peptide. Alternatively, mutation of cys223, needed for fluorophore labeling of the GR tau1c fragment (Methods), could reduce affinity for TAZ2. Finally, GR tau1c peptides bind bacterially expressed TAZ2 with K_d values in the low micromolar range, comparable to the VP16 TAD peptide [14]. This suggests that tau1c peptides interact with TAZ2 with similar affinity to a bona fide TAZ2 target, consistent with the idea that GR/TAZ2 interactions could be relevant for function.

Structural studies have revealed that TAZ2 forms a four α-helix core structure linked by three short loops that comprise zinc binding motifs [14,22] and those of TAZ2 in complex with p53, Stat1 and E1a reveal that α-helical TAD segments dock against different surfaces of the TAZ2 α-helical core with different binding modes [14–17]. Since our studies raise the possibility that GR helical segments could be responsible for binding to the TAZ2 (Fig. 3), it is intriguing to suggest that tau1c may adopt binding modes similar to subsets of other α-helical TADs.

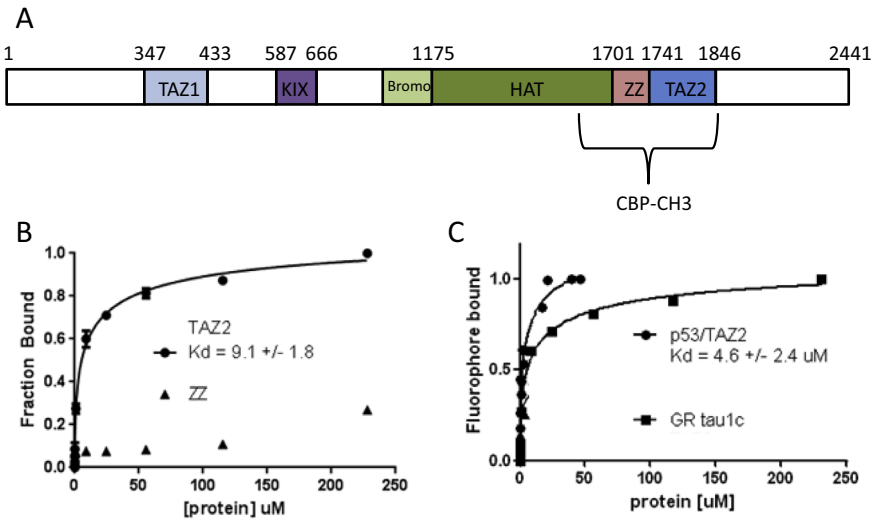

Fig. 2. GR binds CBP TAZ2. (A) Schematic of CBP structure showing positions of the CH3 domain, HAT, TAZ2 and ZZ. (B) Fluorescence anisotropy shows GR tau1c binding CBP TAZ2 and not CBP ZZ. Binding of either CBP TAZ2 (amino acids 1741–1846, circles) or CBP ZZ (amino acids 1701–1741, triangles) to N-terminally fluorescently labeled GR tau1c (amino acids 187–244) as measured using fluorescence anisotropy. CBP TAZ2 showed saturation binding, with a calculated K_d of 9.1 ± 1.8 μM, while CBP ZZ showed no measurable binding. (C) As in Fig. 2B, with p53 peptide [14].

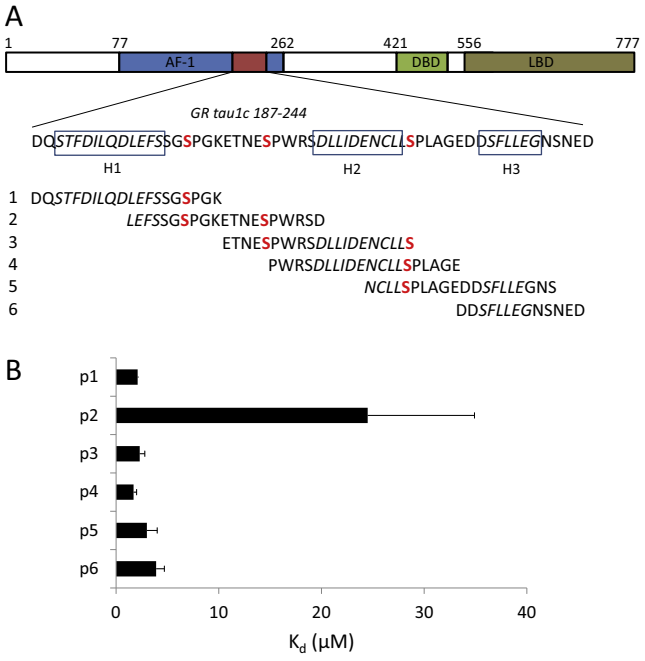

Fig. 3. GR TAZ2 binding determinants are spread throughout tau1c. (A) GR schematic showing position of tau1c and sequence. Individual test peptide (p1–p6) sequence and positions are outlined below (B). The panel represents average K_d values determined for binding of each GR peptide for CBP TAZ2.

3.4. Phosphorylation at Ser211 and Ser226 enhances GR tau1c peptide binding to CBP TAZ2

Since tau1c contains three GR phosphorylation sites [1], we assessed effects of this modification upon affinity of individual tau1c peptides for TAZ2. To do this, we compared binding of phosphorylated and non-phosphorylated versions of GR tau1c peptides to TAZ2. Ser203 phosphorylation, not associated with GR transactivation capacity or other markers of GR activity, did not increase the capacity for TAZ2 binding (K_d unphosphorylated 2.1 μM, phosphorylated 1.8 μM) (Fig. 4). By contrast, phosphorylation at Ser211 and Ser226 both enhanced CBP TAZ2 binding. Unphosphorylated peptide bound TAZ2 with a K_d of approximately 2.3 μM, whereas peptides that were phosphorylated at Ser211 or Ser226 bound TAZ2 with K_d values of approximately 0.8 and 0.7 μM respectively. This represents a threefold increase in affinity versus the unphosphorylated form, similar to that seen with a monophosphorylated form of the p53 TAD (Ser15P) for the p300 TAZ2 domain but less than an elevenfold increase in affinity of another monophosphorylated form of p53 TAD (T18P) [14]. The fact that both GR phosphorylation events enhance CBP TAZ2 binding is consistent with suggestions that they are markers of transcriptional activity [1].

Strikingly, diphosphorylated GR p2 (Ser211P, Ser226P) bound TAZ2 with a K_d value of approximately 0.2 μM, four times higher than either monophosphorylated form and around 11 times higher than unphosphorylated peptide (Fig. 4). This is different from effects of double phosphorylation of the p53 TAD, which yields an affinity for TAZ2 that is intermediate between both forms of monophosphorylated p53 [14]. However, p53 phosphorylation sites are closely juxtaposed, whereas GR Ser211 and Ser226 are separated by fifteen amino acids and bracket a predicted α-helix. Our findings suggest that dual phosphorylation at Ser211 and Ser226 could result in synergistic enhancement of CBP recruitment (Fig. 4) and also suggest that effects of multiple phosphorylation events upon TAZ2 binding will depend upon the particular TAD.

Previous analysis of a p53 TAD/TAZ2 complex structure allowed interpretation of effects of p53 T18 phosphorylation upon binding to TAZ2 in terms of enhanced electrostatic interactions with TAZ2 arginine residues [14]. While it is not yet possible to explain why GR phosphorylation enhances TAZ2 binding, we propose two (non-mutually exclusive) explanations for enhanced affinity of phosphorylated GR peptides. Perhaps phosphorylation promotes local helical organization of the unstructured GR TAD, enhancing binding of GR tau1c helical segments to TAZ2. Alternatively, phosphorylation could enhance electrostatic interactions with charged or polar TAZ2 residues, as seen with p53 T18P [14]. While it may be possible to dissect contributions of phosphorylation events upon hydrophobic and electrostatic interactions with isothermal titration calorimetry, a detailed description of the roles of GR phosphorylation in CBP binding awaits structures of the complex.

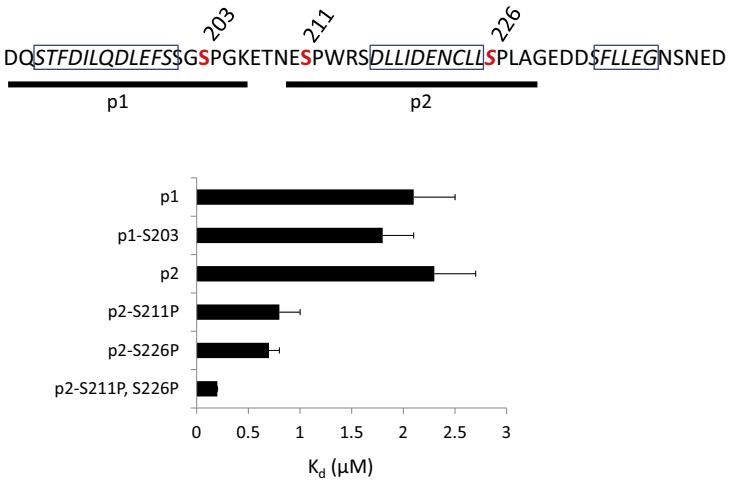

Fig. 4. Phosphorylation at Ser211 and Ser226 enhances GR tau1c/CBP TAZ2 interactions. Peptide sequence of tau1c with ser phosphorylation sites marked and peptides p1 (DQSTFDILQDLEFSS) and p2 (ESPWRSDLLIDENCLL) marked. Panel represents average K_d values determined for binding of unphosphorylated GR peptides (p1 and p2) for CBP TAZ2 versus phosphorylated forms.

3.5. Summary

GR tau1c, which contains three GR phosphorylation sites, is needed for optimal GR activity and absolutely required for enhancement of GR function and GR/CBP association in cultured cells. We localized the GR tau1c binding site to CBP TAZ2 and found that CBP binding determinants are spread throughout GR tau1c and may coincide with putative helical regions. While phosphorylation at Ser203 does not affect CBP binding, phosphorylation at Ser211 and Ser226 enhances CBP TAZ2 binding in a synergistic fashion. This suggests that Ser phosphorylation could promote CBP recruitment and enhance AF-1 activity.

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