

**Membrane tethered bursicon constructs as heterodimeric modulators of the
Drosophila GPCR rickets**

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Drosophila Leucine-rich repeat receptors 1, 2 and 3.

Abstract

The study of complex heterodimeric peptide ligands has been hampered by a paucity of pharmacological tools. To facilitate such investigations we have explored the utility of membrane tethered ligands (MTL). Feasibility of this recombinant approach was explored with a focus on *Drosophila* bursicon, a heterodimeric cystine-knot protein that activates the G protein-coupled receptor rickets (rk). Rk/bursicon signaling is an evolutionarily conserved pathway in insects required for wing expansion, cuticle hardening, and melanization during development. We initially engineered two distinct MTL constructs each comprised of a type II transmembrane domain, a peptide linker, and a C-terminal extracellular ligand that corresponded to either the α or β bursicon subunit. Co-expression of the two complementary bursicon MTLs triggered rk mediated signaling *in vitro*. We were then able to generate functionally active bursicon MTLs in which the two subunits were fused into a single heterodimeric peptide, oriented as either α - β or β - α . Carboxy-terminal deletion of 32 amino acids in the β - α MTL construct resulted in loss of agonist activity. Co-expression of this construct with rk inhibited receptor-mediated signaling by soluble bursicon. We have thus generated membrane-anchored bursicon constructs that can activate or inhibit rk signaling. These probes can be used in future studies to explore the tissue and/or developmental stage-dependent effects of bursicon in the genetically tractable *Drosophila* model organism. In addition, our success in generating functionally diverse bursicon MTLs offers promise that such technology can be broadly applied to other complex ligands including the family of mammalian cystine-knot proteins.

Introduction

The *Drosophila* receptor rickets (rk, dLGR2) is a member of the leucine-rich repeat subfamily of G protein-coupled receptors (GPCRs) (Van Loy et al., 2008). Rk activation is required for wing expansion, cuticle sclerotization, and melanization. The endogenous rk agonist, bursicon, is a heterodimeric cystine-knot protein. Bursicon has been known as the insect tanning hormone for more than four decades, however it was only in 2005 that it was discovered that the active ligand is comprised of two unique subunits, BURS (Bur α) and Partner of bursicon (Bur β) (Luo et al., 2005; Mendive et al., 2005).

Rk/bursicon signaling is highly conserved among insects and has been shown to play an important role in development (Bai and Palli, 2010; Loveall and Deitcher, 2010; Van Loy et al., 2007). In *Drosophila*, bursicon is sequentially secreted from two distinct clusters of neuroendocrine cells shortly following eclosion. An initial wave of hormone is released from neurons in the subesophageal ganglion, which in turn induces secondary release of bursicon from another subset of neurons in the abdominal ganglion. This sequence ultimately triggers wing expansion, cuticle hardening and pigmentation (Luan et al., 2006; Peabody et al., 2008). RNAi studies in *Drosophila* revealed that down regulation of rk during development compromises insect survival (Dietzl et al., 2007; Loveall and Deitcher, 2010). Although bursicon and rk signaling have been most extensively investigated in *Drosophila*, other studies have shown that this pathway is essential for viability of other insect species including *T. castaneum* (Bai and Palli, 2010). Rk and bursicon like sequences have been identified in a wide variety of insects,

suggesting that the physiological significance of this signaling cascade has been highly conserved (An et al., 2009; Honegger et al., 2008; Honegger et al., 2011; Van Loy et al., 2007). The vast majority of research on rk/bursicon has focused on the functional role of this regulatory system during development. One limitation of these efforts stems from the paucity of pharmacologic modulators of this GPCR which can be used as experimental tools.

Both bursicon subunits (Bur α and Bur β) are members of the eight membered ring cystine-knot proteins. This family also includes the TGF β bone morphogenetic protein antagonists known to be required for development and organogenesis (Avsian-Kretchmer and Hsueh, 2004). Bursicon is also structurally related to the family of glycohormone cystine-knot proteins that activate leucine-rich repeat GPCRs. Corresponding mammalian GPCRs include the luteinizing hormone (LHR), follicular stimulating hormone (FSHR), and thyroid stimulating hormone receptors (TSHR). The glycohormone ligands share a common α subunit and each have a unique β subunit that confers receptor specificity (Hearn and Gomme, 2000). Comparison of bursicon/rk, with LH, FSH, and TSH ligand/receptor pairs suggest that these structurally related hormones and GPCRs arose from common ancestors (Van Loy et al., 2008).

Generating pharmacological tools to probe the physiology of rk/bursicon *in vivo* presents considerable practical hurdles. Like mammalian glycohormones bursicon is comprised of two large and complex molecules. As a result, conventional peptide synthesis is impractical for making functionally active ligand. In addition, introduction of mutations into corresponding recombinant DNA constructs aimed at expressing variant

cystine-knot proteins in heterologous cell lines may be hampered by impaired processing and/or secretion of the peptide (Darling et al., 2000; Galet et al., 2009) .

To circumvent these challenges we have extended a strategy that our lab has previously utilized to study relatively short GPCR peptide ligands. Membrane tethered ligand (MTL) technology uses recombinant DNA to encode an extracellular peptide hormone fused to a linker sequence and a transmembrane domain. To date, a variety of short peptide MTLs have been generated that selectively activate cognate class B GPCRs (Choi et al., 2009; Fortin et al., 2011; Fortin et al., 2009). Previous investigations also demonstrated the utility of membrane tethered toxins as ion channel blockers (Auer and Ibanez-Tallon, 2010).

In the current report we demonstrate that large, complex cystine-knot proteins which require a heterodimeric partner can be generated as functionally active MTLs. Furthermore, we utilize this extended MTL technology to identify a ligand domain that is required for rk receptor activation and to generate an inhibitor of rk signaling. In addition to providing insights into the structure function relationships underlying bursicon activity, respective constructs provide novel tools for further analysis of associated physiology *in vivo*. Extending from our current investigation, the approach developed for bursicon can be utilized to study related cystine-knot proteins (e.g. glycohormones, bone morphogenetic protein antagonists) as well as other complex peptide ligands.

Materials and Methods

Cell Culture

Human embryonic kidney cells (HEK293) were cultured in Dulbecco's modified eagle medium (DMEM, Life Technologies, Grand Island, NY) with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals, Lawrenceville GA), 100U/mL penicillin, and 100 μ g/mL streptomycin (Life Technologies, Grand Island, NY). Cells were maintained at 37 °C in a humidified 5% CO₂ atmosphere.

Plasmids

DLGR2 (rk), GenBank: AF142343.1 was generously provided by Dr. Cornelis Grimmelikhuijzen and subcloned into pcDNA1.1 using the restriction enzymes HindIII and XbaI (Eriksen et al., 2000). Bursicon α and β cDNAs in pcDNA3.1 were generously provided by Dr. J. Vanden Broeck (Mendive et al., 2005). The type II MTL backbone was generated by PCR amplification of the transmembrane domain (amino acids 10-56) of Tumor Necrosis Factor alpha (TNF α) from a cDNA template (NCBI accession # BC028148) (Marmenout et al., 1985). The nucleotide and corresponding amino acid sequence of the Type II construct is presented in Supplemental Figure 1. The bursicon α and β subunits were subcloned by PCR into the Type II MTL backbone. The bursicon MTL constructs included nucleotide sequences corresponding to amino acids 30-143 for Bur α and 21-141 for Bur β (Mendive et al., 2005). For the CHE-tBur- β - α construct, a

cDNA encoding cherry fluorescent protein was ligated in frame 5' of the TNF α transmembrane domain coding sequence to create an intracellular fluorescent tag. The negative control MTL, CHE-tCCL2 contains the same fluorescent tag and backbone as CHE-tBur- β - α with the alpha and beta subunits replaced by amino acids 25-99 of human chemokine ligand 2 (NCBI accession # NP_002973.1). All cAMP response element (6X-CRE) reporter genes and β -galactosidase plasmids were as previously described (Choi et al., 2009; Fortin et al., 2009).

Transfections

Polyethylenimine (PEI) transfection reagent was prepared as previously described (Zaric et al., 2004). All transfections were done using a final PEI concentration of 2 μ g/mL. Transfections were performed in serum free DMEM with antibiotics at 37 °C. Cells were incubated with transfection mix for 20-48 hours as indicated in the figure legends prior to initiating functional or MTL expression assays.

Bursicon Conditioned Media

HEK293 cells were seeded in 75cm² flasks at 1.2 million cells/flask. Twenty four hours later cells were co-transfected with 4 μ g each of bursicon α and bursicon β cDNAs (or 4 μ g of a chimeric α - β cDNA construct as indicated) with PEI as previously described. Following a 24 hour incubation, the transfection media was aspirated and 12ml serum free DMEM with antibiotics was added. Medium was conditioned for 48 hours, then collected, centrifuged at 1600 g for 5 minutes to remove cellular debris, aliquoted, and stored at -80 °C.

Luciferase Assays

Luciferase assays were done as previously described (Al-Fulaij et al., 2007; Fortin et al., 2009; Hearn et al., 2002) with minor modifications. HEK293 cells at ~80% confluence in 96-well plates were transiently transfected using PEI. To assess rk activity, each well was co-transfected with cDNAs encoding; rk (0.25ng), a luciferase reporter gene under the control of a cAMP response element (6X-CRE-Luc, 5ng), bursicon constructs as indicated in the figure legends, and β -galactosidase as a transfection control (5ng). To assess the function of tethered ligands, luciferase levels were quantified 24 hours after transfection using Steady-Light (PerkinElmer, Waltham, MA) and normalized relative to β -galactosidase as previously described (Fortin et al., 2009). To assess the function of soluble ligands, bursicon conditioned media was added twenty hours after transfection for an additional 4 hour incubation. Luciferase and β -galactosidase levels were then measured as indicated above.

Confocal Microscopy

HEK293 cells were transfected in 35mm glass bottom dishes (Mattek, Ashland, MA). Cells were transfected with cDNAs encoding rk, a cherry fluorescent protein tagged tBur- β - α construct, and a GFP reporter gene under the control of a cAMP response element (6X-CRE-GFP) reporter gene (Fortin et al., 2011). After 48 hours, the cells were fixed for 10 minutes using 4% paraformaldehyde in PBS. The cells were subsequently washed with PBS containing 100mM glycine and then kept covered with PBS to prevent drying. Microscopy was performed on a Leica TCS SP2 confocal microscope with an inverted

40x oil objective. Two channels were used to simultaneously monitor MTL expression (mCherry fluorescent protein) and rk activation (GFP).

ELISA

ELISA was performed to quantify the expression of membrane tethered ligands. MTL encoding cDNAs were transfected into HEK293 cells grown in 96-well plates. Twenty four hours after transfection, the media was replaced with 50 μ L of DMEM +10% FBS with antibiotics; the cells were then grown for an additional 24 hours. Following this period, ELISA was performed as previously described (Doyle et al., 2012; Fortin et al., 2009) using a rabbit polyclonal c-myc conjugated HRP antibody at 1:2500 dilution (Abcam, Cambridge, MA).

Programs and Statistics

All luciferase and expression data were graphed and analyzed using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA). All cDNA sequences were designed and analyzed using Vector NTI Advanced 9 software (Life Technologies, Grand Island, NY).

Results

In this study we explored the utility of MTLs as pharmacological tools for studying complex heterodimeric protein ligands. We applied this technology to bursicon and rickets as a prototypical ligand-receptor pair. As a first step we generated two independent tethered constructs; one encoding the alpha subunit and the other encoding the beta subunit of bursicon (Figure 1).

The design of these membrane tethered subunits included a type II transmembrane domain (TMD) with the intent of expressing the peptide ligand at the extracellular C-terminus. Type II transmembrane domains specifically orient within the plasma membrane such that the N-terminus is intracellular and the C terminus is extracellular. To verify the predicted orientation of the MTL, an ELISA directed at the extracellular c-myc epitope included in the construct was performed. In unpermeabilized HEK293 cells, both individually and co-expressed α and β subunit constructs were readily detected at the cell surface (Figure 2A and Supplemental Figure 2).

We next examined ligand induced signaling. Co-expression of cDNAs encoding both bursicon subunit MTLs together with rk and a 6X-CRE-Luc reporter gene led to concentration dependent receptor activation (Figure 2B). Co-expression of rk with each MTL alone (α or β) and a 6X-CRE-Luc reporter gene did not trigger signaling.

To quantify the magnitude of the signal obtained with co-expression of both bursicon MTLs, comparison was made relative to the soluble bursicon (sBur). To enable these studies, we generated bursicon conditioned media by co-expressing both α (sBur- α) and β (sBur- β) subunit cDNAs in HEK293 cells and collecting the supernatant as detailed in methods (Luo et al., 2005; Mendive et al., 2005). When conditioned media was applied

to rk expressing cells, the resulting heterodimeric bursicon ligand triggered concentration dependent luciferase reporter gene activity (Figure 2C).

In parallel studies we demonstrated that co-expression of freely soluble bursicon subunits in HEK293 cells together with rk and a 6X-CRE-luc reporter gene, also led to receptor mediated signaling (Figure 3A). Consistent with the known requirement of bursicon to form a heterodimer, expression of either subunit alone did not trigger receptor mediated signaling.

The above studies set the stage to examine whether co-expression of one soluble (α or β) and one tethered ligand (β or α) would trigger receptor mediated signaling. As shown in Figure 3B, the soluble and tether combinations were active. In contrast, when conditioned media was generated from a single subunit cDNA (α or β) and added to cells expressing the complementary tethered subunit (β or α), no rk activation resulted (data not shown).

To further simplify a system for studying complex heterodimeric ligands, we explored the potential of membrane tethered fusion proteins as functional ligands (Figure 4A). The initial MTL β - α fusion protein that was generated positioned the α subunit at the construct's free extracellular C-terminus. When co-expressed with rk and the luciferase reporter gene, this MTL triggered GPCR mediated signaling. A parallel construct with the opposite order of subunits (α - β , where the carboxy-terminus of the β subunit was at the free extracellular end of the protein) also activated rk. Activity of each bursicon fusion protein MTL was similar regardless of orientation of the subunits. In addition, signaling by these MTLs was comparable to co-expression of tBur- α and tBur- β (Figure 4B). Notably, expression levels of the fusion MTLs as assessed by

ELISA were also comparable to the levels observed with single subunit constructs (Figure 4C). These latter experiments confirmed, as observed with MTLs including a single bursicon subunit, that the ligand domains of tethered β - α and α - β are localized in the extracellular space. As an additional control we demonstrated that conditioned media cannot be made from cells expressing either a heterodimeric fusion MTL or co-expressing individual subunit MTLs (Supplemental Figure 3). This observation indicates that bursicon MTLs are not secreted.

As a complementary index of tBur- β - α function (in addition to luciferase activity) we visually monitored ligand expression as well as MTL induced signaling using a 6X-CRE-GFP reporter gene. To enable these studies, a tBur- β - α construct was generated that included a cherry fluorescent protein at the intracellular amino-terminus, (CHE-tBur- β - α). After co-transfection of cDNAs encoding: CHE-tBur- β - α , rk and a 6X-CRE-GFP reporter gene, MTL expression and receptor mediated signaling could be simultaneously observed by confocal imaging. As shown in Figure 5, CHE-tBur- β - α expression results in rk activation, triggering GFP production. In contrast, a non-specific MTL CHE-tCCL2 (designed to activate the CCR2 receptor) can be visualized at the cell surface, but does not trigger rk mediated signaling (no 6X-CRE-GFP expression is induced).

In summary, our results with recombinant bursicon demonstrate that co-expression of both α and β subunits, either as two soluble peptides, or as two independent membrane tethered constructs is sufficient to generate active hormone. In addition, a single heterodimeric MTL with the peptide ligand in either the β - α or α - β configuration results in active bursicon. All bursicon MTLs appear to specifically activate rk (dLGR2).

When tested on related *Drosophila* LGR receptors (dLGR1, dLGR3), no activation could be detected (Supplemental Figure 4).

The ability to express recombinant functionally active bursicon heterodimers as a single MTL fusion protein enabled an expedited strategy for structure-function studies. As a first step, we examined the effect of serial deletions at the C-terminus of the tBur- β - α construct (Figure 6A). Deletion of 10, 21, 32, or 35 amino acids from the C-terminus of tBur- β - α led to a progressive loss of MTL activity. The Δ 10 and Δ 21 constructs were partial agonists compared to full length tBur- β - α . In contrast, little if any activation of rk was detected with expression of Δ 32 and Δ 35 MTLs (Figure 6B). Deletion of the C-terminus had little effect on cell surface expression levels (Figure 6C). In contrast to the tBur- β - α constructs, corresponding deletions of the C-terminus of tBur- α - β (up to or including the final cysteine residue) did not result in loss of agonist activity (Supplemental Figure 5).

Further analysis of tBur- β - α Δ 32 and Δ 35 MTLs revealed these tethered constructs markedly inhibited receptor stimulation by soluble bursicon (conditioned media). Functional antagonism of tBur- β - α Δ 35 was suggested by a significant rightward shift of the conditioned media concentration response curve when this construct was expressed (Figure 6D). With the tBur- β - α Δ 32 an even more pronounced inhibition resulted, essentially eliminating agonist induced signaling.

Discussion

We have developed novel recombinant constructs that enable membrane-anchored expression of bioactive bursicon. Our study demonstrates that MTL technology can be applied to larger and considerably more complex GPCR ligands than those described in prior reports. Previously, only MTLs that included short peptide ligands (up to 39 amino acids) have been described (Auer and Ibanez-Tallon, 2010; Choi et al., 2009; Fortin et al., 2011; Fortin et al., 2009; Ibanez-Tallon and Nitabach, 2012). In contrast, the mature bursicon subunits, α and β , are 141 and 121 amino acids, respectively. Furthermore, each of these subunits is a cystine-knot protein that includes a series of intramolecular disulfide bridges which confer tertiary structure. As an additional prerequisite of agonist activity, the α and β subunits must interact to form a structurally integrated heterodimer (Mendive et al., 2005).

Given the stringent requirements underlying the formation of active soluble bursicon, including cellular co-expression, co-processing and co-secretion, the success in generating corresponding functional membrane tethered ligands could not have been anticipated. Initially, we demonstrated that expression of both single tethered bursicon subunits (alpha and beta) in the same cell was sufficient to generate an active ligand. Follow-up studies revealed that co-expression of soluble and tethered complementary subunits also enabled the formation of active ligand. In contrast when a single soluble subunit was added as conditioned media to cells expressing a tethered complementary subunit, no agonist activity was detectable (data not shown). This finding suggests that intracellular assembly of the alpha-beta heterodimer is a critical step in the formation of active hormone. These observations are consistent with reports on the heterodimerization requirements of soluble bursicon and other cystine-knot proteins that are known to undergo

intracellular assembly prior to secretion as an active ligand (Xing et al., 2004). Remarkably, both membrane tethered and soluble bursicon subunits, despite the complexity of processing, appear to be fully compatible with each other in forming active heterodimers.

In an attempt to further understand the structural requirements underlying tethered bursicon function, we generated constructs in which both the α and β subunits were included in a single MTL. Since an active tethered ligand can be generated as either a β - α or α - β fusion construct, neither a free N nor a free C-terminus is a requirement for agonist activity (Figure 4B). It is of note that conditioned media containing a soluble form of the bursicon fusion protein tested in the β - α arrangement also shows agonist activity (Supplemental Figure 6). Whether tethered or soluble, the bursicon fusions are active ligands. Our observations with bursicon reveal another parallel with mammalian heterodimeric cystine-knot proteins. Fusion of the α and β subunits of mammalian glycohormones including TSH, LH, and FSH as single soluble peptides, also results in ligands that can activate their corresponding mammalian GPCR (Fares et al., 1998; Park et al., 2005; Sen Gupta and Dighe, 2000; Setlur and Dighe, 2007; Sugahara et al., 1996).

The generation of tethered bursicon fusion proteins provided a simplified model system to define domains of the dimer that are important for agonist activity (Figure 6B). These experiments were guided by prior observations that the β subunit of mammalian glycohormones provides specificity and affinity for cognate receptors while the α subunit is required for receptor activation (Park et al., 2005). Furthermore, the literature suggests that the C-terminal domain of the glycohormone alpha subunit is an important determinant for ligand activity (Butnev et al., 2002; Sato et al., 1997; Sen Gupta and Dighe, 2000). Based on this knowledge, we generated a series of deletions in the C-terminus of Burs α in the context of the tBur- β - α heterodimer. These experiments demonstrated that the C-terminal domain in

tethered bursicon was essential for rk activation. One of the deletion mutants in which 32 C-terminal residues were truncated (designated as $\Delta 32$) not only led to loss of agonist activity, but also markedly inhibited the function of soluble bursicon (Figure 6D). This observation suggests that once a domain essential for agonist activity was removed in the corresponding MTL, the remaining truncated peptide can inhibit soluble agonist induced signaling. An MTL with a larger C-terminal deletion ($\Delta 35$), while also lacking agonist activity, was much less effective (vs. $\Delta 32$) in blocking soluble bursicon induced signaling. The difference between $\Delta 32$ and $\Delta 35$ is that three additional highly conserved residues including a critical cysteine are truncated in $\Delta 35$. The loss of these 3 residues may have compromised the tertiary structure of the tethered ligand, in turn explaining the functional difference in constructs. Soluble versions of the $\Delta 32$ and $\Delta 35$ constructs did not confer the same ability to block ligand induced signaling (Supplemental Figure 7). Thus it is possible that membrane anchoring is required to generate a functional antagonist.

It is of note that the GPCR targeted MTLs that had been reported prior to this study all shared a common orientation, in which the peptide ligand was expressed with a free extracellular N-terminus. In contrast, the bursicon MTLs were engineered with the opposite orientation (i.e., with a free extracellular carboxy-terminus). This was achieved by incorporating a different transmembrane domain anchor (a type II TMD) into the construct. The ability to generate membrane tethered ligands in either orientation markedly enhances the potential utility of MTL technology. For many peptides orientation may be a critical factor in generating an active MTL. It is well established that for peptide hormones recognizing class B GPCRs (e.g. secretin, PTH, CRF, GLP-1, GIP), the critical determinants of ligand efficacy reside in the N terminal domain of the hormone (Hoare, 2005). We have previously shown that each of these peptides remains

active when incorporated into an MTL that includes a type I TMD, i.e. the extracellular free end of the peptide is the N terminus (Fortin et al., 2009). In contrast, peptide ligands recognizing class A GPCRs are more diverse. As examples, the amino termini of chemokines are generally considered critical for ligand activity whereas for neuropeptides, functional determinants are often localized at the carboxyl terminus (Eipper and Mains, 1988; Mayer and Stone, 2001). In the latter case, it is anticipated that MTLs including a type II TMD will preserve biological activity when corresponding peptides are anchored to the cell membrane.

In summary, we have developed a strategy that can be widely applied to the study of peptide ligands. More specifically, we have identified bursicon MTLs that either activate or block rk mediated signaling. These findings set the stage for future *in vivo* studies. In the investigations to follow, we intend to selectively express tethered constructs in targeted tissues of *Drosophila* thus exploring the utility of the approach for defining corresponding rk mediated pathways/physiologies. Precedent with these bursicon MTLs will set the stage for parallel studies using other tethered cystine-knot proteins as tissue selective molecular probes. Candidate MTLs include mammalian glycohormones as well as non-GPCR regulators such as bone morphogenetic protein antagonists. The efficiency and flexibility of recombinant MTL technology will enable generation of a wide range of unique tools to complement the use of soluble ligands in understanding corresponding receptor mediated physiologies.

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Authorship Contributions

Participated in research design: Harwood, Fortin, and Kopin

Conducted experiments: Harwood, Gao, and Chen

Performed data analysis: Harwood, Fortin, Beinborn, and Kopin

Wrote or contributed to the writing of the manuscript: Harwood, Fortin, Beinborn, and Kopin

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Footnotes

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Figure Legends

Figure 1. Schematic representation of heterodimeric membrane tethered bursicon subunits coexpressed with rk. Abbreviations: *=c-myc epitope tag.

Figure 2. Bursicon membrane tethered α and β subunits together activate the *Drosophila* rickets receptor.

A) Quantification of cell surface expression of bursicon MTLs. Forty eight hours after transfection ELISA was performed using an antibody directed against a c-myc epitope. The x-axis denotes the total amount of cDNA transfected. B) Tethered ligand-induced activation of rk mediated signaling. HEK293 cells were transiently co-transfected with cDNAs encoding: rk, a 6X-CRE-Luc reporter gene, one or both bursicon MTL subunit(s), and a β -galactosidase gene to control for transfection variability. For tethered ligand activity, twenty four hours after transfection luciferase activity was quantified and normalized relative to a four hour maximal stimulation of rk with bursicon conditioned media. The x-axis denotes the amount transfected for each cDNA subunit. C) Concentration dependent activation of rk with bursicon conditioned media. A series of ten fold dilutions of conditioned media (1=undiluted conditioned media) was added to cells 20 hours after transfection; the duration of ligand stimulation was 4 hours. Data represent the mean \pm SEM from 3 independent experiments, each performed in triplicate. Abbreviations: tBur= bursicon MTL subunit cDNA.

Figure 3. Rk is activated by co-expression of either two complementary soluble bursicon subunits or complementary combinations of soluble and tethered bursicon subunits.

HEK293 cells were transiently co-transfected with cDNAs encoding: rk, a 6X-CRE-Luc reporter gene, and either soluble bursicon subunits (A) or combinations of soluble and tethered bursicon subunits (B). The x-axes denote the amount transfected for each cDNA subunit. Twenty four hours following transfection, luciferase activity was quantified and activity values were normalized relative to maximal stimulation of rk with the addition of independently prepared bursicon conditioned media. Data represent the mean \pm SEM from 3 independent experiments, each performed in triplicate. Abbreviations. sBur = soluble Bursicon subunit cDNA, tBur=bursicon MTL subunit cDNA.

Figure 4. Bursicon MTLs are active fusion proteins independent of C-terminal subunit positioning

A) Cartoon illustrating the protein structure of membrane tethered constructs that include the two complementary bursicon subunits. B) Tethered ligand induced activation of rk mediated signaling. HEK293 cells were transiently co-transfected with cDNAs encoding: rk, a 6X-CRE-Luc reporter gene, the indicated bursicon MTL encoding construct(s), and a β -galactosidase control gene. Twenty four hours after transfection, luciferase activity was quantified and normalized relative to maximal stimulation of rk with addition of bursicon conditioned media. The x-axis denotes the amount transfected for each cDNA subunit. C) Quantification of cell surface expression of bursicon MTLs. Forty eight hours after transfection ELISA was performed using an antibody directed against a c-myc epitope. The x-axis denotes the total amount of cDNA transfected. Data represent the mean \pm SEM from 3 independent experiments, each performed in triplicate. Abbreviations: tBur= bursicon MTL subunit cDNA, TNF α =Tumor necrosis factor α .

Figure 5. Rk activation by a bursicon MTL fusion protein can be visually monitored by confocal microscopy.

Representative images showing bursicon mCherry-fluorescent protein (CHE) MTL triggering rk mediated GFP expression. HEK293 cells were transiently co-transfected with cDNAs encoding: CHE-tBur β - α or CHE-tCCL2 (negative control), rk, and a 6X-CRE-GFP reporter gene. Confocal images were obtained 48 hours after transfection. Data represent 3 independent experiments. Abbreviations: CRE-GFP=cAMP response element green fluorescent protein reporter gene.

Figure 6. Development of a membrane tethered inhibitor of rk signaling.

A) Cartoon illustrating the secondary structure of the bursicon α subunit, a cystine-knot protein, highlighting the relative positions of deleted domains (dotted lines) (adapted from Honegger et al., 2008, Figure 2). B) Activity screen of bursicon MTL serial deletions. HEK293 cells were transiently co-transfected with cDNAs encoding: rk, a 6X-CRE-Luc reporter gene, the indicated bursicon MTL, and a β -galactosidase control gene. Twenty four hours after transfection, luciferase activity was quantified and normalized relative to maximal stimulation of rk after addition of bursicon conditioned media. C) Quantification of cell surface expression of full length vs. C-terminally truncated bursicon MTLs. Forty eight hours after transfection ELISA was performed using an antibody directed against a c-myc epitope. D) Expression of rk bursicon MTL C-terminal deletion constructs disrupts receptor activation by soluble bursicon. HEK293 cells were transiently transfected with 2ng of the indicated MTL construct, rk, a 6X-CRE-Luc

reporter gene, and a β -galactosidase control gene. Twenty hours following transfection bursicon conditioned media was added at a series of ten fold dilution (1=undiluted conditioned media). Following a four hour incubation with bursicon conditioned media, luciferase activity was quantified and normalized relative to maximal stimulation of rk by bursicon conditioned media in the absence of a tethered inhibitor. Data represent the mean \pm SEM from 3 independent experiments, each performed in triplicate.

Abbreviations: tBur= bursicon MTL subunit cDNA.

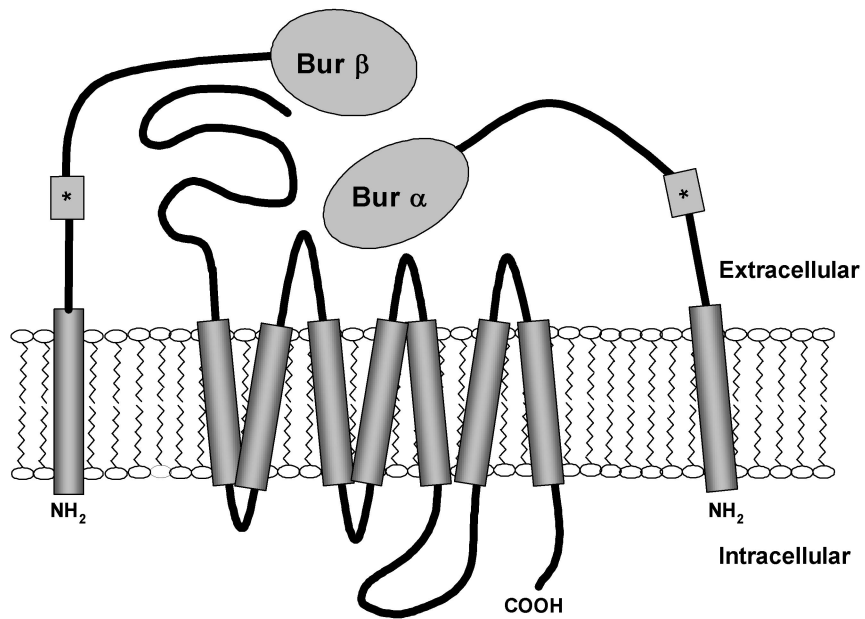


Figure 1

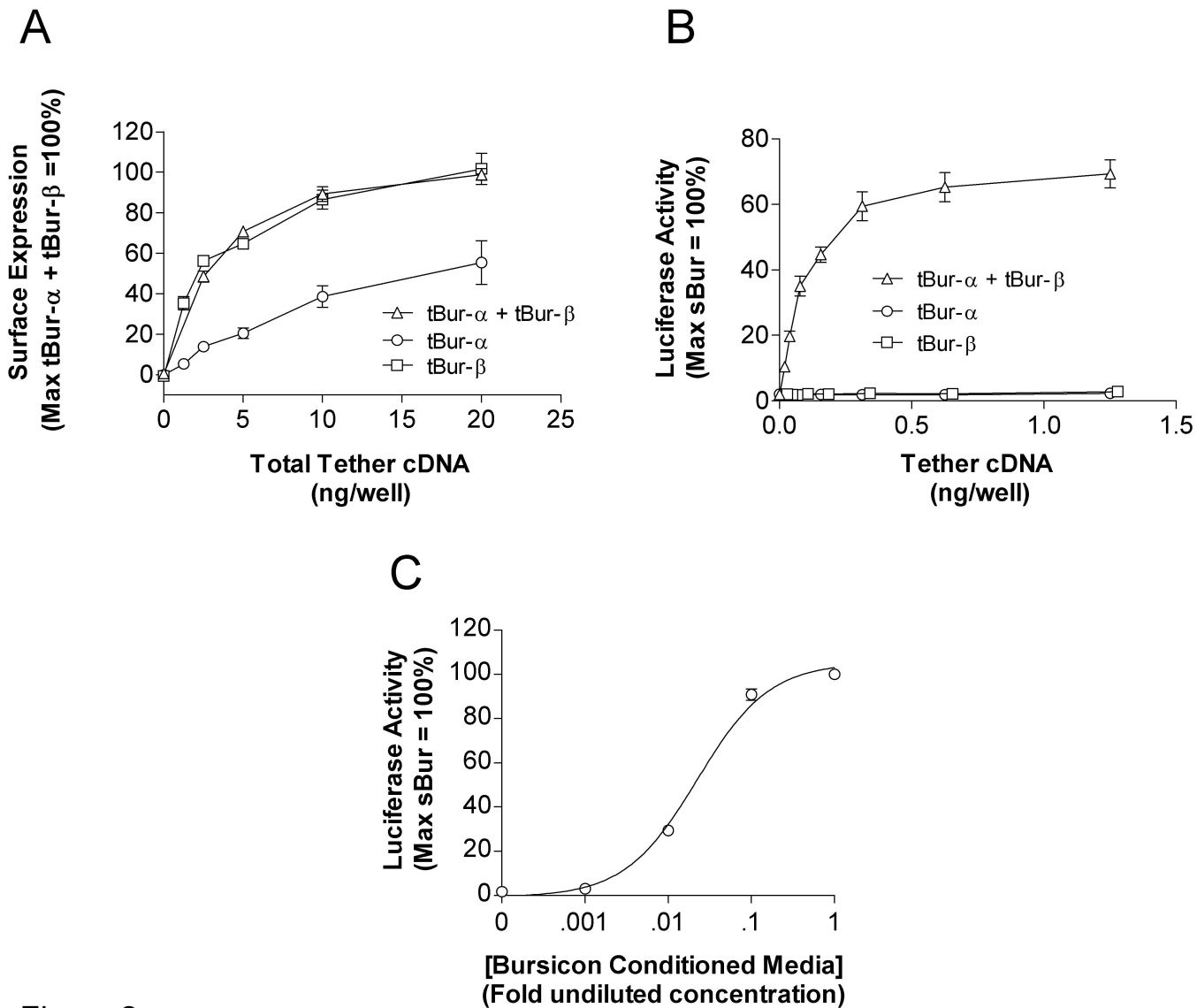


Figure 2

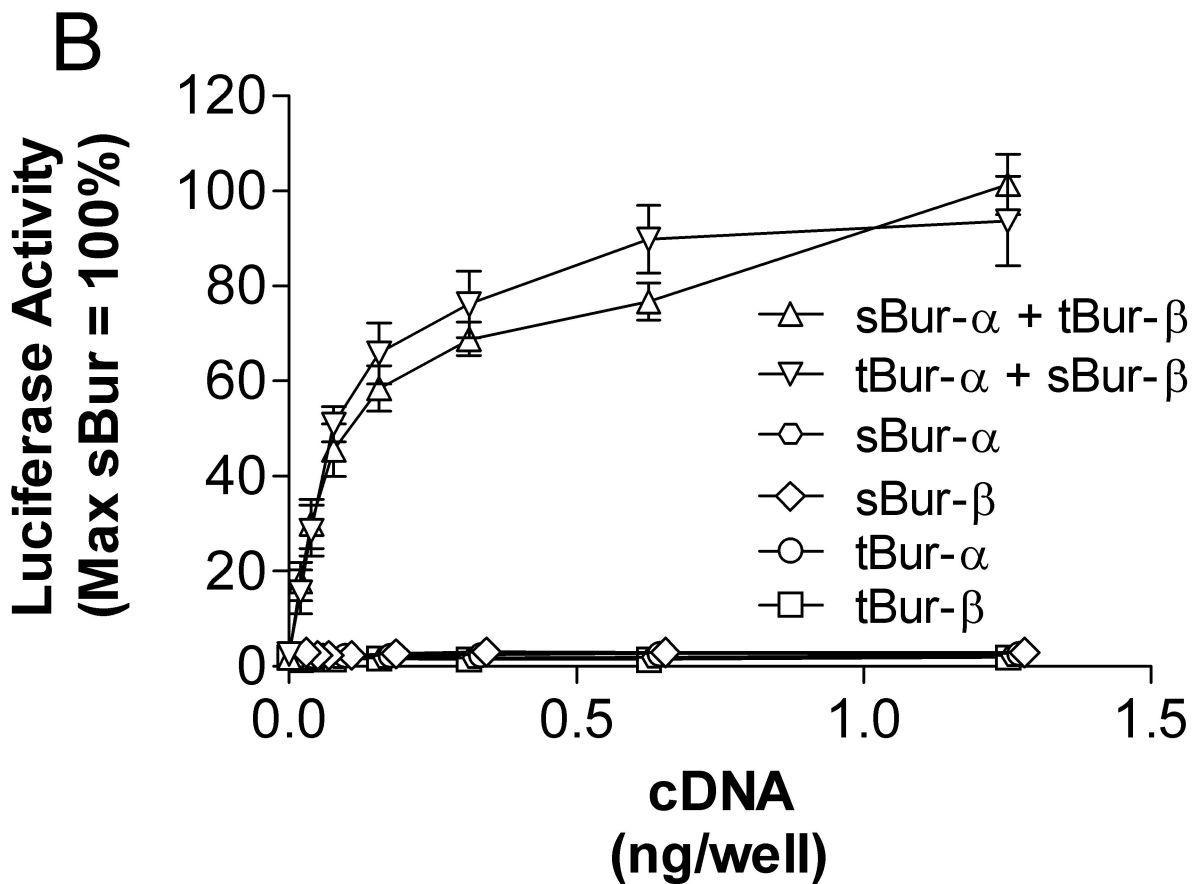
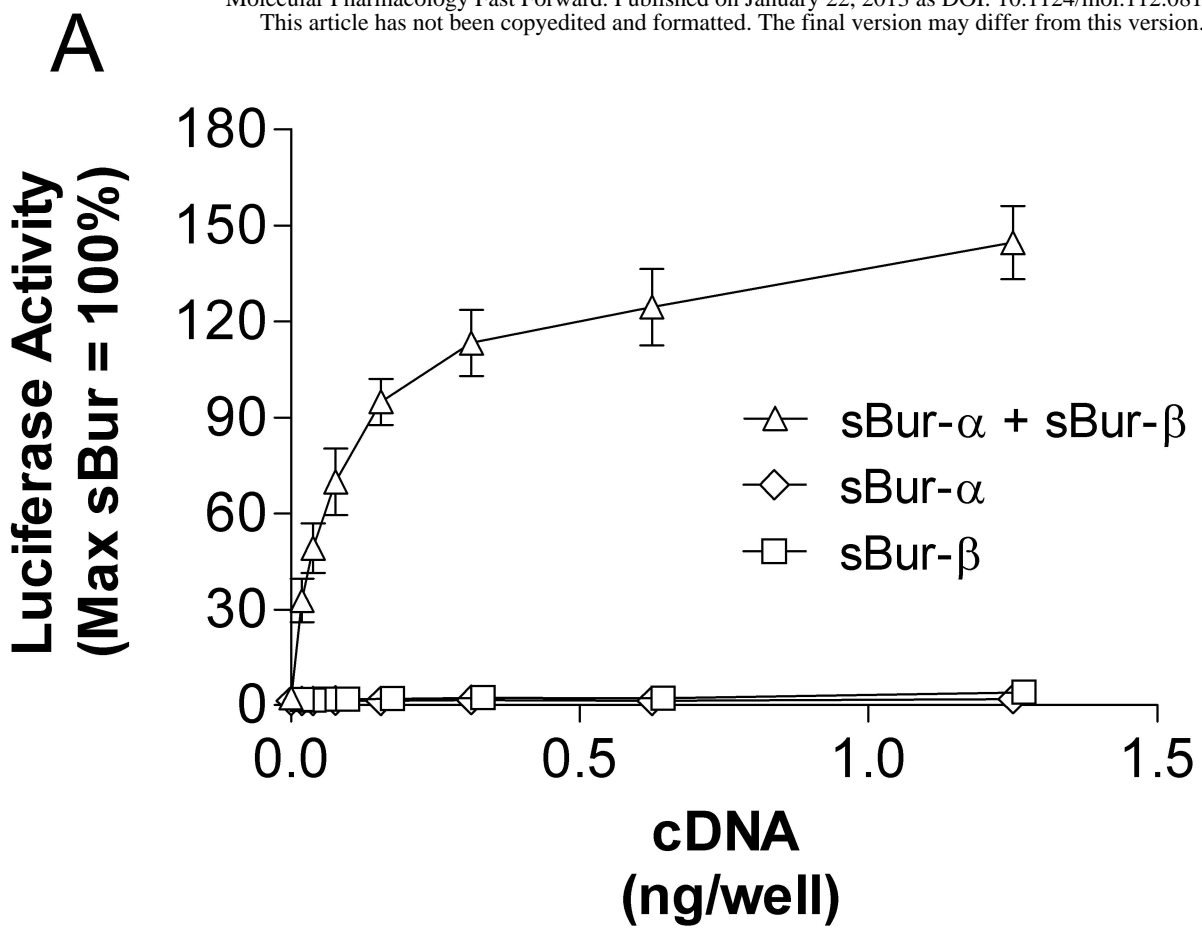


Figure 3

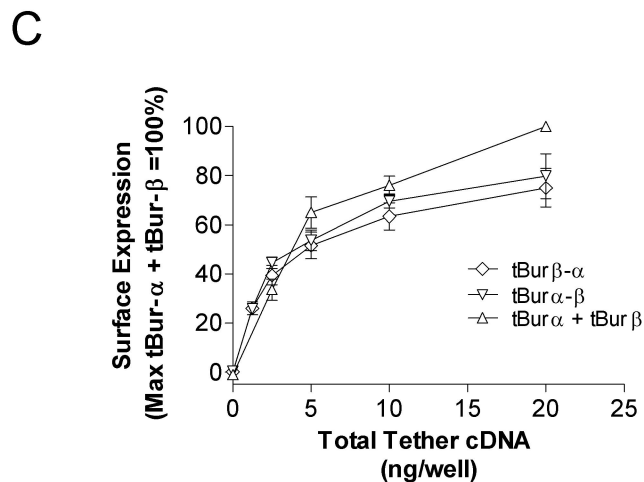
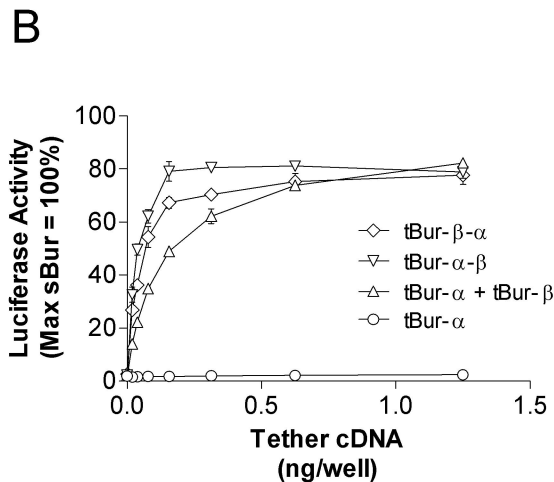
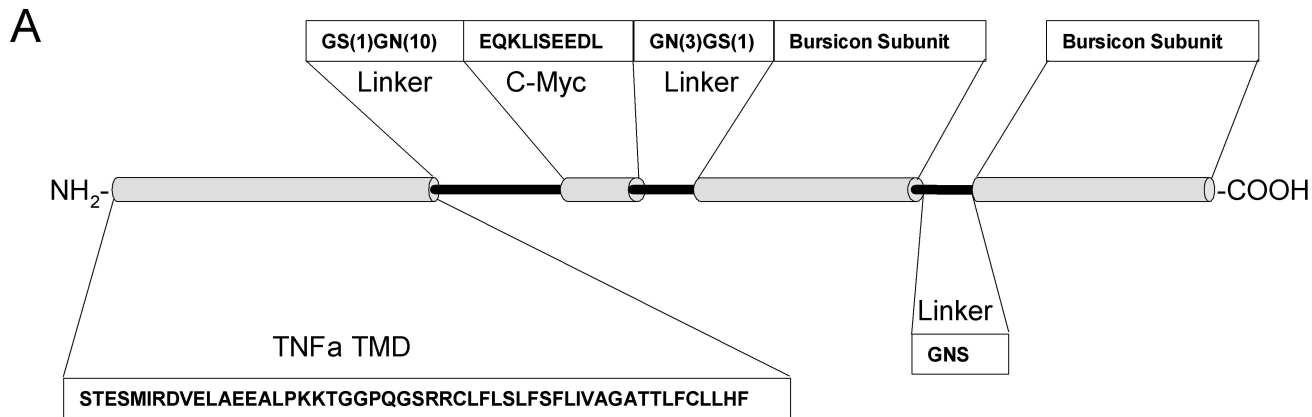


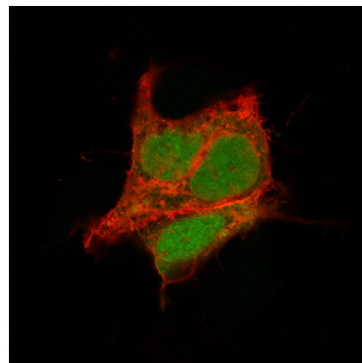
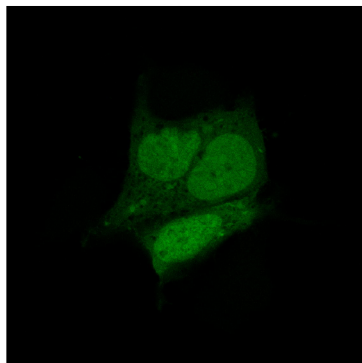
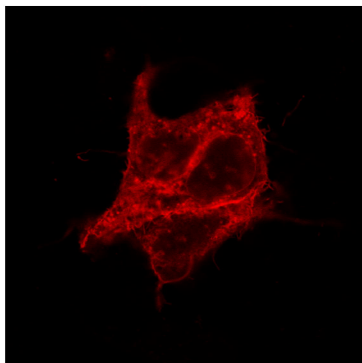
Figure 4

mCherry

GFP

Overlay

- CHE-tBur β - α
- 6X-CRE-GFP
- Rk-pcDNA1.1



- CHE-tCCL2
- 6X-CRE-GFP
- Rk-pcDNA1.1

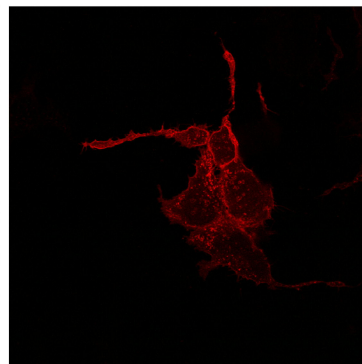
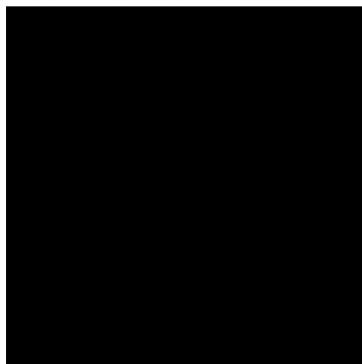
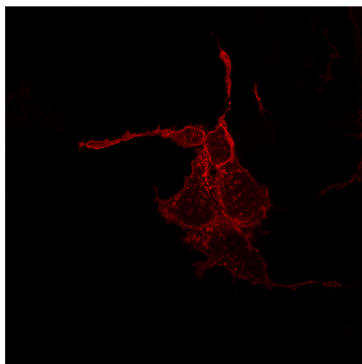


Figure 5

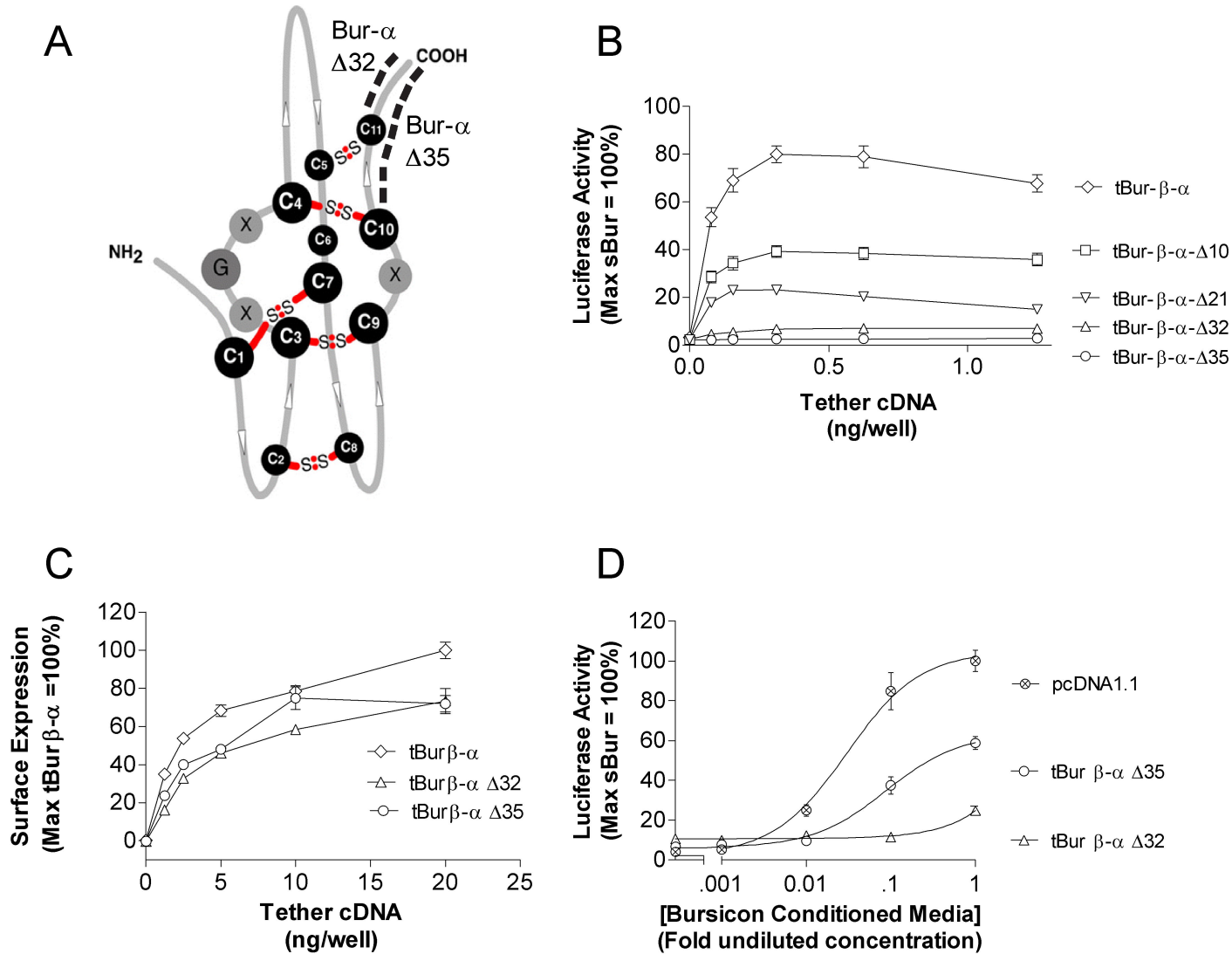


Figure 6