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Altered phosphorylation of [Beta]-catenin in glucocorticoid treated 235-1 rat pituitary tumor cells

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ALTERED PHOSPHORYLATION OF b-CATENIN IN GLUCOCORTICOID TREATED 235-1 RAT PITUITARY TUMOR CELLS

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Dedications

To my family, Bill, Sandie, Betsy, Amy and Billy Saunders (oh yeah, and of course Kurt Geveke), who gave never ending support and encouragement for my interest in research and medicine

To my second family, Andy, Jim, Garrett, Tricia, Lucy, Pat, Bob and Barry, who were always there for me when I needed them. I wouldn't be who I am today without them

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ABSTRACT:

Beta-catenin is an essential cell adhesion and signaling protein, associated with high prolactin levels in rat pituitary tumor cells. It has been shown that phosphorylation affects the location and activity of β-catenin. Glycogen synthase kinase (GSK3-β) is a serine-threonine kinase that phosphorylates β-catenin on N-terminal residues, targeting it for proteasomal degradation. Studies have shown that C-terminal tyrosine phosphorylation decreases the association of β-catenin with cadherin. In 235-1 rat pituitary tumor cells, our lab has shown that the glucocorticoid analog dexamethasone (Dex) decreases the half-life of β-catenin while increasing the activity of GSK3-β. The current study was undertaken to examine whether active GSK3-β is necessary for the regulation of β-catenin in Dex-treated cells. Duplicate cultures of 235-1 cells were grown for four days in the presence of 100 nM Dex. To inhibit the activity of GSK3-β, control and Dex-treated cells were treated with 20 mM LiCl, a known inhibitor of GSK3-β activity. Cell lysates were collected and analyzed by Western blotting. Phosphorylation-specific antibodies were used to detect levels of phosphorylated β-catenin (Ser 33/37/Thr 41) and (Ser 45/Thr41), active GSK3-β (Tyr 216) and inactive GSK3-β (Ser 9). Additional experiments examined tyrosine phosphorylated β-catenin through immunoprecipitation using a phosphotyrosine specific antibody, and by using a LAR tyrosine phosphatase which removes phosphates from ß-catenin. Using λ-phosphatase, phosphates were removed nonspecifically from Ser, Thr, and Tyr residues in β-catenin to evaluate total phosphorylation. After four days of treatment with Dex, β-catenin levels were reduced by approximately 50%. Levels of active GSK3-β (Tyr 216) were increased in Dex-treated cells and levels of inhibited GSK3-ß (Ser 9) were decreased in Dex-treated cells. This shows that Dex treatment alters activation of GSK3-ß and likely functions in the regulation of β-catenin in 235-1 cells. Tyrosine C-terminal phosphorylation of ß-catenin leads to a loss of cell-to-cell adhesion, which would lead to morphological changes. Microscopic examination of Dex-treated cells showed the presence of more rounded cells than control cells. Therefore, phosphorylation studies were conducted to examine changes in tyrosine C-terminal phosphorylation. These studies demonstrated no detectable alterations in tyrosine C-terminal phosphorylation; however, the changes in cellular morphology suggest a loss of the cadherin-catenin adhesion complex in Dex-treated cells. In Dex-treated cells λ-Phosphatase removed phosphates in one major step. However, in control cells an additional step was needed to remove phosphates from β-catenin, indicating that there is an alteration in phosphorylated β-catenin in Dex-treated cells. Dex-treated cells showed an increase in the amount of phosphorylated (Ser 45/Thr41 and Ser33/37/Thr41) ß-catenin. After a 24 hour treatment with LiCl, there was a decrease in the amount of Ser33/37/Thr41 phosphorylated ß-catenin. Phosphorylation on Ser 45 was still present after treatment with LiCl indicating that two separate kinases phosphorylate ß-catenin. Furthermore, ß-catenin levels were restored, even though there was no apparent change in the level of active GSK3-β. These data suggest that the presence of Dex caused an increase in the degradation rate of β-catenin by stimulating GSK3-β activity. Supported by NSF grant #IBN-9810327 to BCD.

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List of Abbreviations

ACTH	**adrenocorticotropic hormone**
ADH	**antidiuretic hormone**
APC	**adenomatous polyposis coli**
BCA	**bicinchoninic acid**
BSA	**bovine serum albumin**
CHX	**cyclohexamide**
CK1	**casein kinase**
Dex	**dexamethasone**
DMEM	**Dulbecco's Modified Eagle Medium**
ECL	**enhanced chemiluminescence**
EDTA	**ethylenediaminetetracetic acid**
FSH	**follicle stimulating hormone**
GH	**growth hormone**
GSK3-ß	**glycogen synthase kinase**
HEPES	**N-[2-hydroxyethyl] poperazine-N' -[2-ethane sulfonic acid]**
IgG	**immunoglobulin G**
LH	**luteinizing hormone**
LiCl	**lithium chloride**
NaCl	**sodium chloride**
NP-40	**nonidet P-40**
PBS	**phosphate buffered saline**
PMSF	**phenylmethyl-sulfonyl fluoride**
PRL	**prolactin**
SDS	**sodium dodecyl sulfate**
SDS-PAGE	**SDS polyacrylamide gel electrophoresis**
TBST	**tris buffer saline with tween**
TCF-1	**T-cell factor**
TEMED	**N,N,N',N'-tetramethylethylenediamine**
Tris	**tris[hydroxymethyl]aminomethane**
TSH	**thyroid stimulating hormone**
Tween-20	**polyethylene-20-sorbitan monolaurate**

INTRODUCTION:

Animals are composed of cells organized specifically to perform certain functions. In order for an animal to function properly, their cells rely on constant, carefully orchestrated communication with each other, allowing homeostasis. Cellular regulation is accomplished through both hormonal or electrical mechanisms and direct cell-to-cell contact. Together, these processes allow animals to balance the needs of several body organ systems at one time.

The Pituitary

The pituitary is an endocrine gland, which secretes hormones directly into the blood stream. Located at the base of the brain, the pituitary is fully enclosed within the skull (Figure 1A). The pituitary rests on the sella turcica, which is a depression in the cranial sphenoid bone, and it is divided into two lobes: the anterior pituitary and the posterior pituitary (Netter and Hansen, 2003) (Figure 1B). The posterior pituitary serves a neurocrine function and releases two hormones: antidiuretic hormone (ADH) and oxytocin. ADH helps the body regulate water, while oxytocin plays a role in uterine contraction during childbirth and allows contraction of milk glands for lactation.

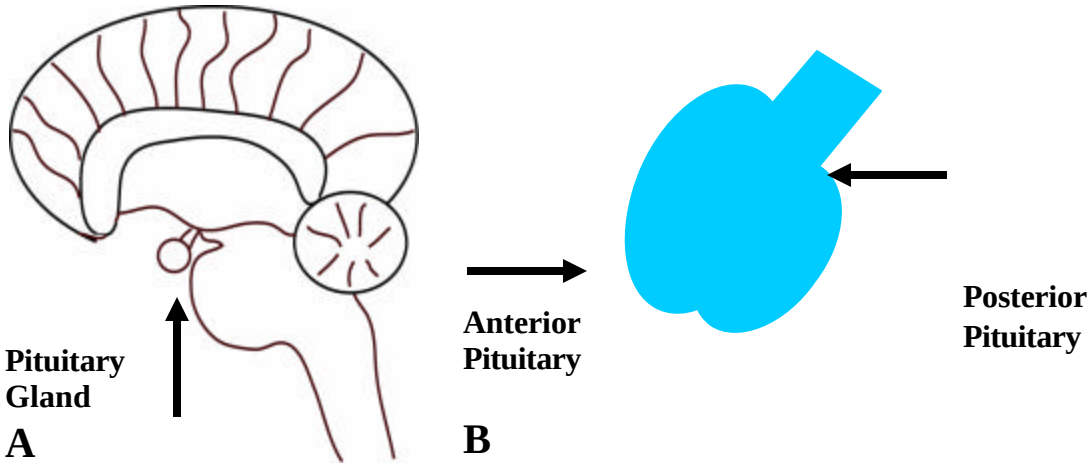


Figure 1: (A) Location of the pituitary gland: below the cerebrum and resting on bone. (B) The division of the pituitary into the two different lobes: anterior pituitary and posterior pituitary.

The anterior pituitary is an endocrine gland containing three different types of cells, which are classified according to the staining characteristics of their granules: basophils, chromophobes and acidophils (Cotran et al., 1999) (Figure 2). The basophils take up basic stain; therefore, their granules stain blue. The basophils comprise two types of endocrine cells: the gonadotrophs and the thyrotrophs. The gonadotrophs secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH), which act upon reproductive systems in male and females. The thyrotrophs secrete thyroid stimulating hormone (TSH), which stimulates the thyroid to produce thyroid hormone. The chromophobes do not take up any dye and they represent one type of hormone-secreting cells, the corticotrophs. These endocrine cells secrete adrenocorticotropic hormone (ACTH), which stimulates the adrenal cortex to secrete cortisol, important in helping the body respond to stress. The acidophils are the most common cell type found in the anterior pituitary. These cells take up an acidic stain causing their granules to appear pink. The acidophils consist of three types of hormone-producing cells: the lactrotrophs

somatotrophs and somatolactotrophs. The lactrotrophs produce prolactin (PRL), which stimulates milk production in the mammary glands. The somatotrophs secrete growth hormone (GH), which promotes growth in long bones (Cotran et al., 1999). The somatolactotrophs can produce both GH and PRL. One characteristic of the acidophils is that they can undergo phenotypic switching, depending on the physiological condition of the animal (Kineman et al., 1992) (Figure 3).

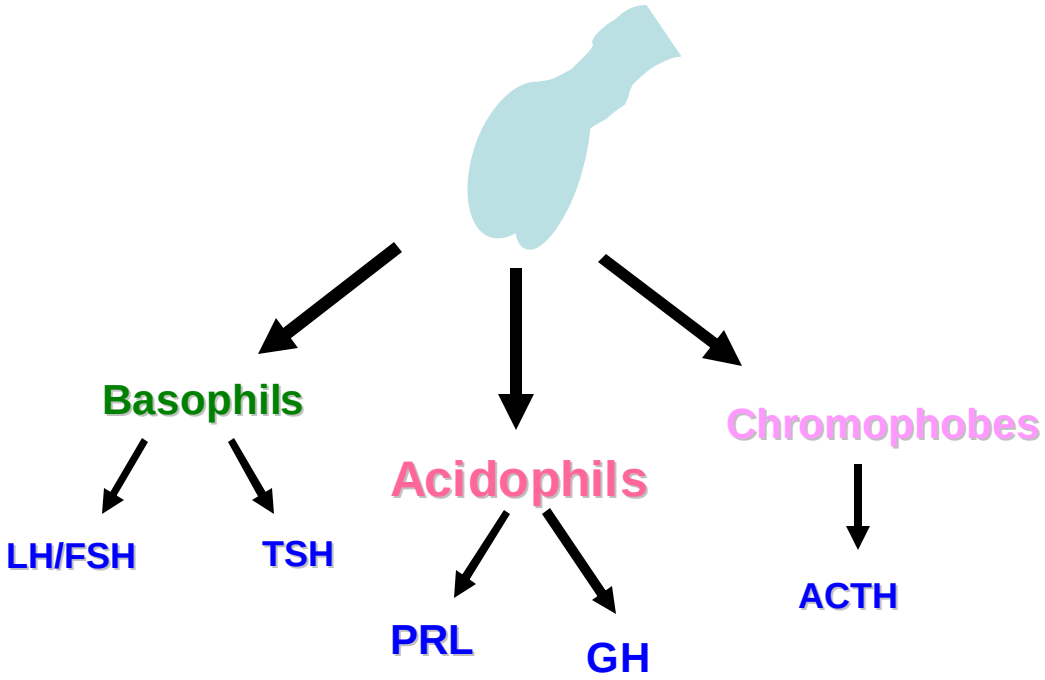


Figure 2: Three classes of anterior pituitary cells and the hormones produced by these cells.

In mature female animals, the main stimulus to induce an increase in the lactrotrophic acidophil population is from the hormone estrogen (Wilson, 1998; Johnson et al., 1998). This situation applies in pregnant and post-partum females who need prolactin to produce milk for lactation. Furthermore, the balance is shifted toward the somatotrophs in young animals, which do not require high levels of prolactin, but must

have growth hormone for development. Cortisol, a glucocorticoid produced by the adrenal cortex, is known to stimulate GH secretion while decreasing PRL levels. As these cells switch phenotypes from the lactrotrophs and somatotrophs, they undergo a transition stage in which the cells have characteristics of both groups. These cells are called the somatolactotrophs and they can produce both prolactin and growth hormone.

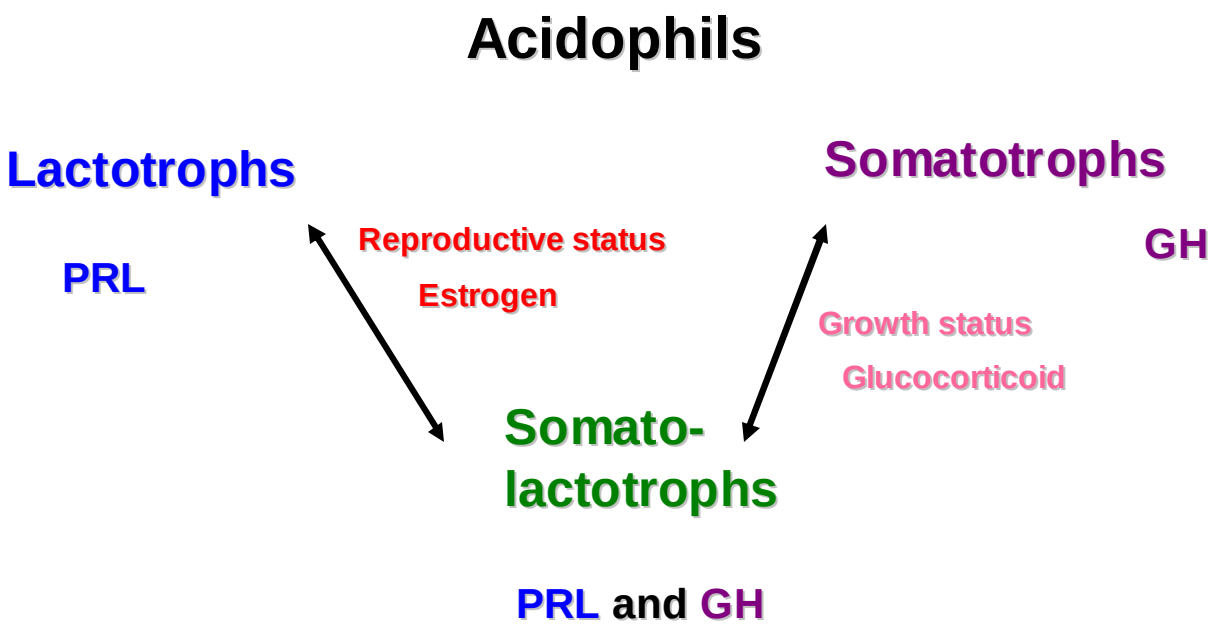


Figure 3: Phenotypoic switching of acidophil populations. Estrogen stimulates prolactin production in mature female animals, while glucocorticoid stimulates growth hormone in young animals.

Pituitary Regulation

A characteristic unique to the acidophils is that the lactotrophs are the only pituitary cell type under primary inhibitory control. The lactotrophs are under tonic dopaminergic inhibition from the hypothalamus (Johnson et al., 1998; Porter et al., 1994). Dopamine is a catecholamine neurotransmitter found in the central nervous system.

When it is released into the hypophysial portal blood system which connects the hypothalamus to the pituitary gland, dopamine will inhibit prolactin secretion. Conversely, some normal physiological conditions will stimulate prolactin secretion. Prolactin-releasing stimuli, such as nursing, will inhibit dopamine release from the hypothalamus, allowing plasma levels of prolactin to rise, permitting milk production in the mammary glands.

The adrenal glands, located at the superior pole of each kidney, also function in the regulation of the pituitary. The adrenals produce the three classes of corticoidsteroids: the mineralocorticoids, gonadocorticoids and glucocorticoids. Glucocorticoids are essential for life, as they prepare an individual for stress by suppressing inflammation, breaking down muscle to amino acids, converting amino acids and glycogen to glucose and promoting fat metabolism. In the immune system they likewise serve as an immunosuppressant (Cotran et al., 1999; Andreoli et al., 1997).

The most biologically active glucocorticoid is cortisol, which is a natural inhibitor of prolactin secretion (Figure 4). Cortisol is secreted by the adrenals in response to ACTH, which is produced by the corticotrophs. The presence of high levels of cortisol can inhibit the corticotrophs, thus decreasing the amount of ACTH secreted through an inhibitory feedback loop. Without the ACTH stimulation, the adrenals over time will stop producing cortisol.

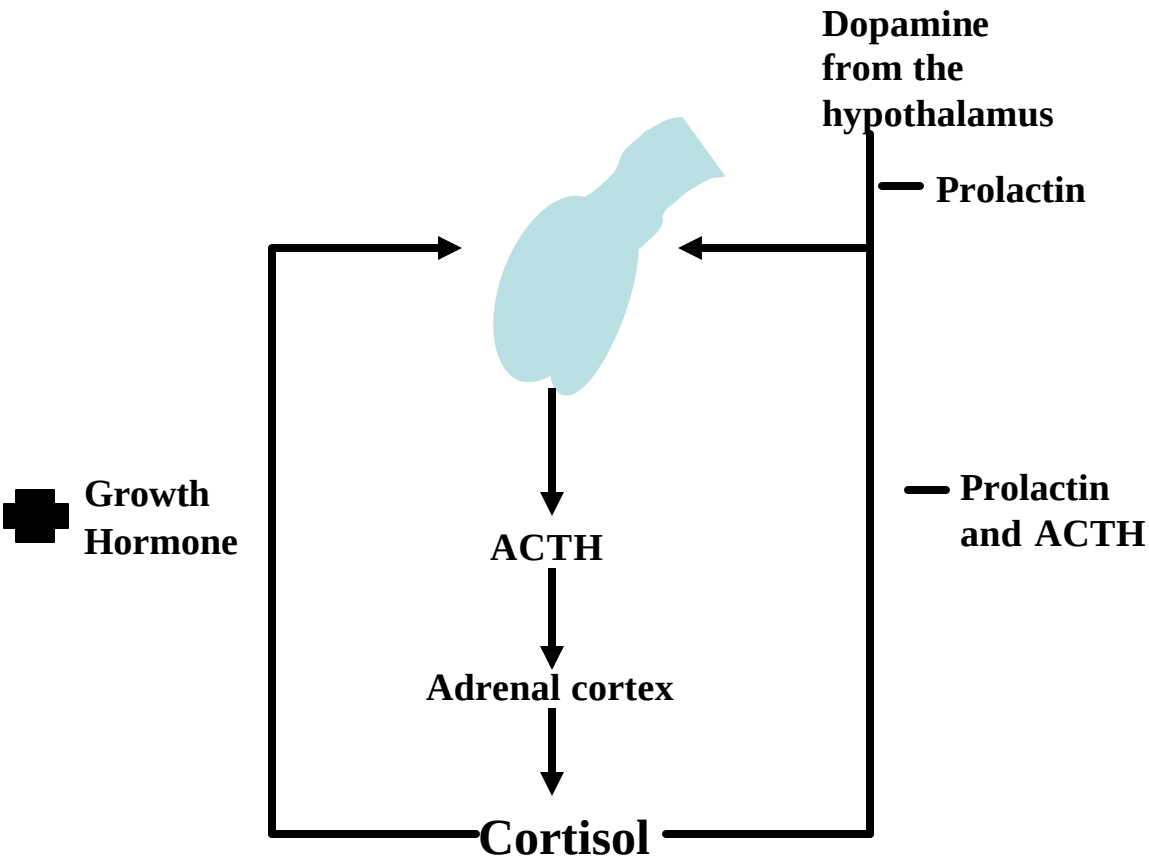


Figure 4: Normal inhibition and control of PRL and GH secretion from the anterior pituitary.

Pituitary Adenomas

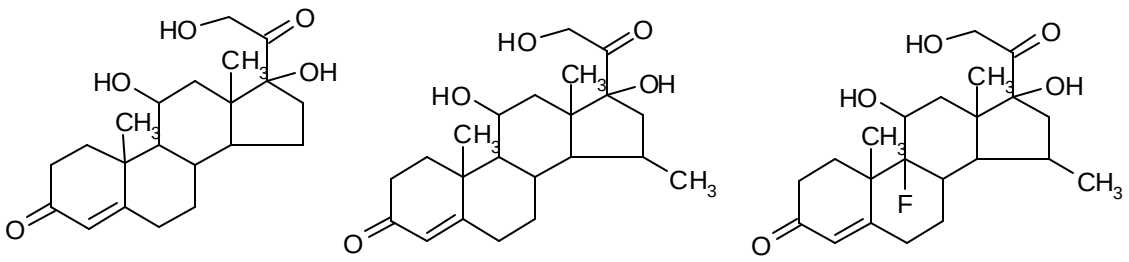
Prolactin secreting pituitary adenomas are the most common type of pituitary tumor, which produce and secrete excess levels of prolactin (Hardman et al., 2003). Controlling the growth of these tumors has been of clinical interest for some time due to the mass effect of the tumor itself and the problems associated with high prolactin levels in the bloodstream. The space inside the skull is limited and an increase in the size of the pituitary can compress structures such as the optic nerve or cerebral blood vessels which result in loss of vision or ischemia to parts of the brain. High prolactin levels in the

bloodstream can result in infertility and changes in menstruation in women and cause impotence and gynecomastia in men.

The treatment options for prolactinomas are very limited. The primary regulation of prolactin secretion is from dopamine secreted from the hypothalamus (Porter et al., 1994). Therefore, the first line of treatment for pituitary tumors is through dopaminergic drugs, such as bromocriptine, which decrease prolactin levels and lowers the growth rate (Hardman et al., 2003). However, some pituitary tumors are not responsive to dopamine and, therefore, more invasive procedures including radiation or surgery must be implemented to remove the tumors. In some cases, these are still ineffective in reducing hyperprolactinemia. Thus, finding additional means to treat dopamine resistant tumors is of interest.

Dexamethasone

Our laboratory and others have found that the synthetic glucocorticoid analog dexamethasone (Dex) (Figure 5) inhibits prolactin levels (Spangler and Delidow 1998; Sakai et al., 1988). This effect is not unexpected because it is part of the normal pituitary inhibition, where by cortisol hinders the lactotrophs from secreting PRL while stimulating the somatotrophs to secrete GH (Figure 4). Naturally-occurring cortisone is produced from methylation of the fourth ring on cortisol. However, the body must convert cortisone back to active cortisol, which is used by body systems to perform particular functions such as stimulating gluconeogenesis, or inhibiting prolactin levels and stimulating growth hormone. Dexamethasone is a fluorinated analogue of cortisone and in our laboratory it was used instead of cortisol because Dex has a biological half-life of 36-54 hours, while cortisol has a half-life of 8-12 hours.



Cortisol **Cortisone** **Dexamethasone**

Figure 5: Structure for cortisol the most biological active glucocorticoid. Cortisone is formed from methylating the fourth ring and then dexamethasone is a synthetic cortisone analogue.

235-1 Rat Pituitary Tumor Cells

The 235-1 cell line are rat pituitary acidophilic tumor cells, producing prolactin only. These cells are a pure clone of lactotrophs and contain no somatotrophs: therefore, these cells are unable to produce growth hormone (Delidow et al., 1992; Billis et al., 1992). Previous work in our laboratory has shown that Dex inhibits cellular division in the 235-1 cells by blocking late G1 stage of the cell cycle, while simultaneously decreasing prolactin levels and decreasing cellular adhesion (Delidow et al., 2002).

Cellular Adhesion

In the 235-1 cells, prolactin levels are directly related to cellular adhesion. Cell-to-cell adhesion is established by the catenin-cadherin complex, which is involved in signaling during embryonic development and in the maintenance and function of epithelial cell layers (Kemler, 1993; Papkoff, 1996; Hirano et al., 1992; Oyama et al., 1994) (Figure 6).

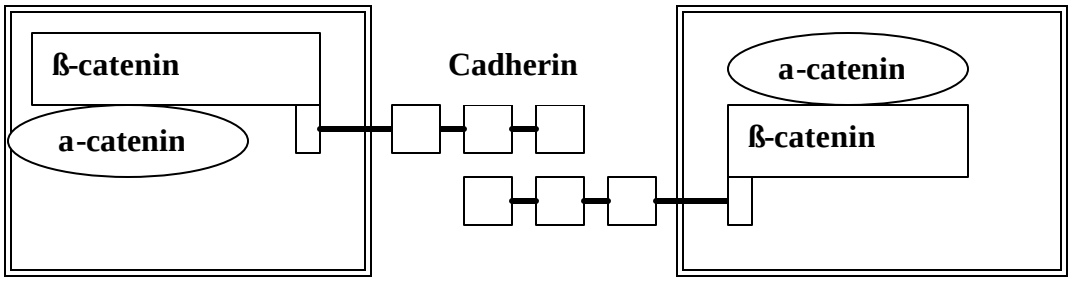


Figure 6: Diagram of the cadherin-catenin adhesion complex. This is a homophilic interaction that occurs between two cells. ß-catenin provides linkage of a-catenin to cadherin.

The cadherin-catenin adhesion complex mediates the homophilic interaction occurring between cells at the adherens junction. Cadherin is a calcium dependent adhesion glycoprotein, containing three domains: the extracellular cadherin repeats, a transmembrane region, and a cytoplasmic tail. The extracellular cadherin repeats allow communication with other cells. The cytoplasmic domain interacts with linker proteins including α-, ß- and γ-catenin (Aberle et al., 1996). The interactions between linker proteins are necessary for establishing a connection between the adhesion complex and the actin cytoskeleton. The linker proteins may undergo phosphorylation, which results in the reduction or loss of cell-to-cell adhesion (Ohsugi et al., 1999). Research in our laboratory has implicated dexamethasone (Dex) in reducing prolactin gene expression while simultaneously altering cadherin function through the regulation of β-catenin (Spangler and Delidow, 1998).

Wnt Signaling

Beta-catenin is a multifunctional protein involved in both the cadherin adhesion complex and the Wnt signaling pathway (Figure 8). ß-catenin's structure contains three main domains: the N-terminus, armadillo repeats, and the C-terminus (Figure 7). The N-terminus can become phosphorylated to regulate the degradation of ß-catenin, while the C-terminus is involved in association with cadherin. There are eleven positively charged armadillo repeats found in ß-catenin's structure. These repeats aid in the binding of ligands such as a-catenin and cadherin, and they are used in recognition by the tumor suppressor protein adenomatous polyposis coli (APC) which aids in the degradation of ß-catenin.

Figure 7: Structure of ß-catenin showing the N and C terminus and the armadillo repeats which are found in the center of the primary structure. The colored boxes indicate the eleven positively charges armadillo repeat units.

In the adhesion complex, ß-catenin provides the linkage of cadherin to a-catenin and the actin cytoskeleton. Cell-to-cell adhesion is dependent on the C-terminal phosphorylation state of β-catenin. In the absence of phosphorylation, the armadillo repeats of ß-catenin can interact with its C-terminal tail or with E-cadherin. E-cadherin and the β-catenin armadillo repeats interact electrostatically,

due to the acidic residues in cadherin and the positively charged groove in the armadillo repeats. When β-catenin is phosphorylated at Tyr 654, the resulting steric hindrance forces the loss of binding to E-cadherin (Piedra et al., 2001; Takeda et al., 1995; Hoschuetzky et al., 1994) (Figure 10). In this case, ß-catenin dissociates from the adhesion complex and can undergo either translocation to the nucleus or rapid degradation. In the presence of Wnt signaling, secreted Wnt glycoproteins bind to the Frizzled receptor. The signal is transduced through a cytoplasmic protein cascade. First, the binding to the Frizzled receptor activates Dishevelled, which inhibits glycogen synthase kinase (GSK3-ß) from phosphorylating ß-catenin (Miller et al., 1999). Absence of this phosphorylation inhibits the formation of the degradation complex of ß-catenin with axin, APC and GSK3-ß (Figure 8). Ultimately activation of Wnt signaling, allows ß-catenin to be translocated to the nucleus where it binds to T-cell factor (TCF-1), which then stimulates gene transcription and cell proliferation (Amit, et al., 2002; Chunming, et al., 2002)

Glycogen Synthase Kinase

Glycogen Synthase Kinase (GSK3-ß) is a ubiquitously expressed serine/threonine protein kinase. GSK3-ß participates in energy metabolism, regulating glycogen synthesis in response to insulin (Welsh et al., 1996). GSK3-ß is involved in neuronal cell development and plays a role in Alzheimer's disease through the hyper-phosphorylation of Tau protein, which leads to formation of amyloid plaques (Phiel et al., 2003). Lastly, GSK3-ß is involved in the Wnt signaling pathway. In this pathway GSK3-ß plays an important role in regulating ß-catenin. In the absence of Wnt signaling, GSK phosphorylates ß-catenin. Once phosphorylated, ß-catenin forms a complex with

adenomatous polyposis coli (APC) and axin, which targets the protein for proteasomal degradation (Figure 8).

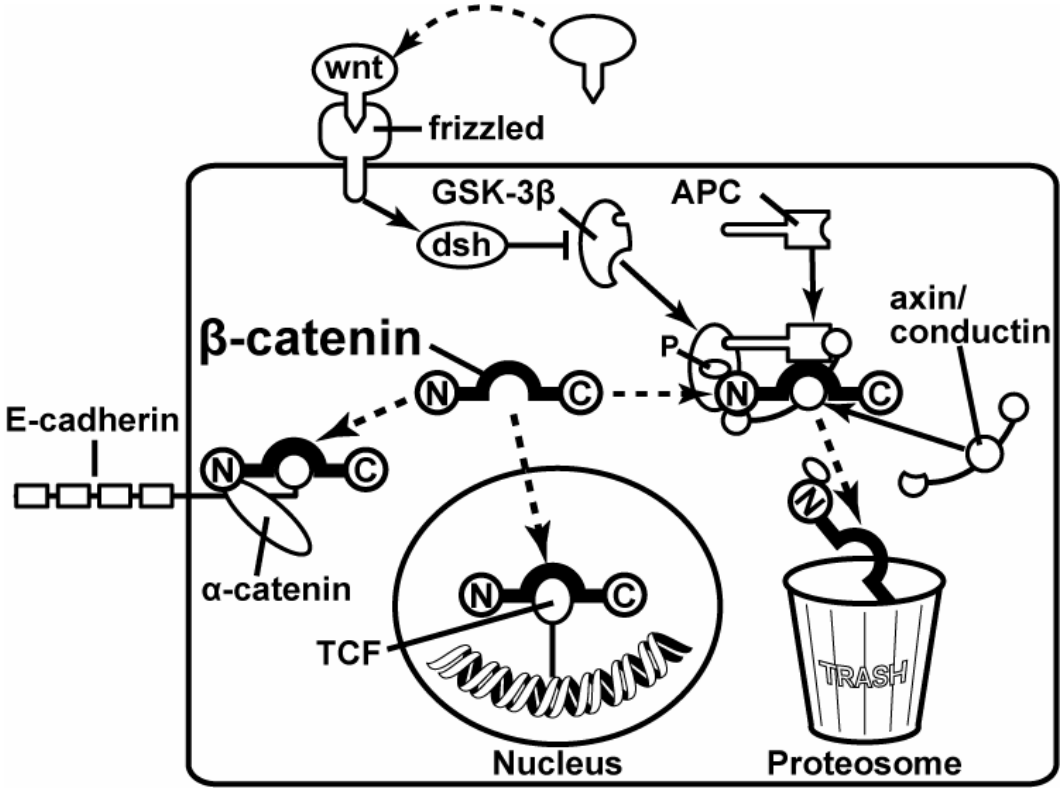


Figure 8: Diagram of Wnt signaling. The figure illustrates the pathway ß-catenin undergoes in the presence or absence of Wnt signaling. In the presence of Wnt signaling ß-catenin is translocated to the nucleus where it binds to TCF to initiate gene transcription. In the absence of Wnt ß-catenin is phosphorylated by GSK3-ß and targeted for proteasomal degradation. Illustration provided by S. Fish Department of Anatomy, Cell and Neurobiology, Marshall University School of Medicine.

ß-catenin Phosphorylation Sites

N-terminal phosphorylation is important for β-catenin regulation by the Wnt signaling pathway, which takes place through the following mechanism: Casein kinase (CK1) phosphorylates β-catenin on Ser 45, by a non-Wnt related signaling pathway, thus priming β-catenin for further phosphorylation (Provost et al., 2003). In the absence of

Wnt signaling, GSK3-ß targets ß-catenin for proteasomal degradation by phosphorylating additional N-terminal (Ser 33/37 and Thr 41) sites (Figure 9). The CK1 phosphorylation site (Ser 45) and the GSK3-ß targeted sites are conserved from *Drosophila* to humans. Mutations in the N-terminal phosphorylation sites of β-catenin results in protein stabilization, which can lead to tumorigenesis. Mutating Ser 45 results in a loss of the priming phosphorylation on ß-catenin. This results in a reduction of the ability of GSK3-β to phosphorylate ß-catenin, which then leads to a decrease in the degradation of β-catenin (Provost et al., 2003).

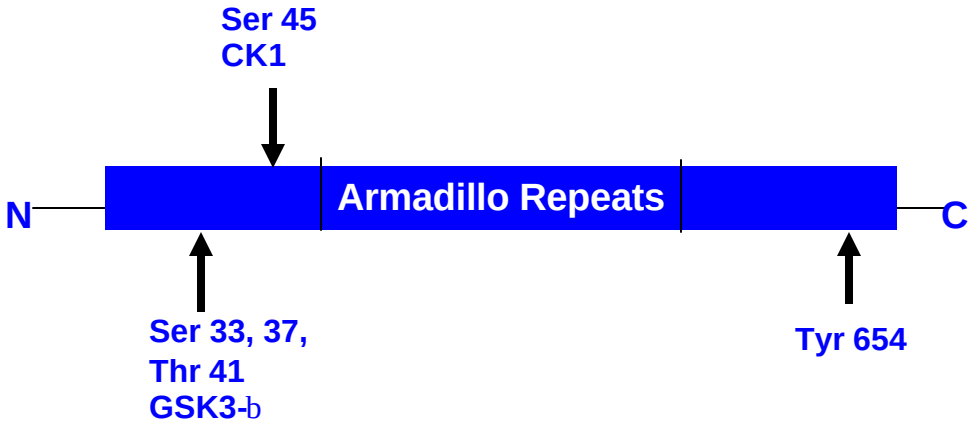


Figure 9: Diagram of phosphorylation sites on ß-catenin. C-terminal Tyr 654 phosphorylation releases ß-catenin from the adhesion complex. The N-terminal phosphorylation sites are involved in degradation of the protein. Ser 45 is a priming site and allows ß-catenin to be further phosphorylated on Ser 33/37/Thr41 which targets the protein for proteasomal degradation.

Our lab has previously shown that treatment of 235-1 rat pituitary cells with 100 nM Dex causes a 50% reduction in the levels of ß-catenin by altering its half-life. Current research has shown global reduction in nuclear, cytoplasmic and membrane-bound ß-catenin. There is no change in the level of total GSK3-ß, which suggests that there is an alteration in the amount of active GSK3-ß enzyme in Dex-treated cells. In the

current study, phosphorylation-specific antibodies were used to detect the amount of active GSK3-β (Tyr 216) enzyme and changes in the inhibitory phosphorylation of GSK3-ß (Ser 9). Lithium, a known inhibitor of the GSK3 family of protein kinases (Stambolic, et al., 1996) was used to determine the effect of blocking GSK3-β enzyme activity on ß-catenin protein levels. It is believed that glucocorticoid treated 235-1 cells regulate ß-catenin levels by increasing the activity of GSK3-ß. Because phosphorylation leads to the degradation of ß-catenin, phosphospecific antibodies (Ser 33/37/Thr41 and Ser 45/Thr41) were used to examine the effects of Dex on the phosphorylation state of ß-catenin. If active GSK3-ß is increased in glucocorticoid- treated cells, it is also expected that there will be an increase in the amount of phosphorylated forms of ß-catenin, since these sites regulate the degradation of the protein. Additional experiments examined total phosphorylation sites on ß-catenin in order to determine the effect of Dex on the Ser, Thr and Tyr phosphorylation.

Methods:

Cell Culture

The 235-1 rat pituitary cells were maintained in tissue culture flasks in DMEM-high glucose media (Life Technologies), containing 10% iron-supplemented bovine calf serum (Hyclone), 10 mM HEPES (pH 7.4), 100 U/mL of penicillin, 100 µg/mL of streptomycin and 5 µg/mL of plasmocin (InvivoGen). Cells were split 1:20 once weekly in phosphate buffered saline (PBS) containing 0.5 mM EDTA (ethylenediaminetetraacetic acid, PBS, pH 7.4) then seeded onto 100 mm culture dishes (Falcon). Cells were grown for a 24 hour period before the beginning of each experiment to allow for attachment and growth.

A 1 mM stock solution of Dex (Sigma) was prepared in ethanol. A 100 µM working solution of Dex was made by dilution in sterile PBS. Cells were treated with 100 nM Dex or vehicle (10 µL of 10% ethanol in PBS) for a period of four days. The cells were fed and treated every two days for the duration of the experiment. In certain experiments, duplicate cultures of control and Dex cells were treated with 20 mM LiCl for 24 hours prior to cell collection. Cells were collected on day five by scraping; washed once in 1 mL of PBS; then lysed and analyzed as described.

Sample Preparation

Cell pellets were lysed by sonication using the Tekmar Sonic Distruptor. Cells were suspended in two pellet volumes (up to 180 µL) HEPES/NP-40 lysis buffer (10 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM EDTA and 1% NP-40) containing protease

inhibitor cocktail (Sigma: AEBSF, Aprotinin, Leupeptin, Bestatin, Pepstatin A, E-64). The sonicator was set to an output of 45 Hz and the samples were sonicated 3X for five-second bursts. The phosphospecific experiments included phosphatase inhibitor cocktails 1 (Sigma: cantharidin, bromotetramisole, microcystin LR) and cocktail 2 (Sigma: sodium orthovanadate, sodium molybdate, sodium tartrate, imidazole) in the lysis buffer. Lysates were cleared by centrifugation. The bicinchoninic acid (BCA) assay (Pierce) was then employed to determine protein concentration. In this assay proteins interact with Cu^{2+}, forming Cu^{1+}, and this in turn interacts with the BCA compound forming a purple color that can be measured at an absorbance of 562 nm.

Electrophoresis and Transfer

Aliquots of 30-50 µg of protein were added to 2X SDS sample buffer (0.046% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.0125 M Tris, pH 6.8, 0.01% bromophenol blue) and samples were separated by 10% SDS-PAGE in running buffer at 125 V for one hour. Samples were then electroblotted onto nitrocellulose (Osmonics) in transfer buffer at 100 V for one hour.

Western Blotting

All experiments analyzed by Western blotting were performed according to the manufacturers's protocols with antibodies against the protein of interest (β-catenin, Sigma; GSK3-β, Biosource International; Phospho-GSK3-β (Ser 9), Cell Signaling; Phospho-β-catenin (Ser 45/Thr 41) and (Ser33/35 Thr41), Cell Signaling; Phospho-tyrosine (PY-20), Transduction Laboratories). The non-phosphospecific blots were

blocked with 5% nonfat dried milk (Carnation) in TBST (10 mM Tris, pH 8.0; 150 mM NaCl; 0.1% Tween-20) for one hour at room temperature with gentle agitation. Phosphospecific antibodies were used at a 1:1000 dilution according to manufacturer recommendations, in 5% Bovine Serum Albumin (BSA) in TBST at 4°C overnight. Blots were washed three times (5 minutes each) in TBST and then incubated for an hour at room temperature with the appropriate peroxidase-labeled secondary antibody (1:3000 in 5% milk with TBST) (Vector Labs). All additional antibodies (β-catenin and GSK3-β) were blocked and incubated with a primary antibody dilution ranging from 1:4000 to 1:5000 in 3-5% milk in TBST; the horseradish peroxidase (HRP)-conjugated secondary antibody was diluted 1:3000 in the same buffer. Signals were detected using enhanced chemiluminescence (SuperSignal reagent, Pierce) and exposed on to x-ray film. Blots were analyzed by the Personal Densitometer, using Image Quant software (Molecular Dynamics).

Phosphatase Treatment of Cellular Extracts

Aliquots of 100 µg of protein collected from control and Dex-treated cultures were incubated with λ phosphatase buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.1 mM $MnCl_2$, 0.1 mM EGTA, 2 mM dithiothreitol, 0.01 Brij 35 and 50% glycerol) and 400 U of λ phosphatase (New England Biolabs) in a reaction volume of 20 µL. The samples were incubated at 30°C for 0-30 minutes. Samples then were mixed with an equal volume of SDS sample buffer, denatured at 100°C and resolved on a 7.5% polyacrylamide gel. Samples were transferred to nitrocellulose and analyzed for β-catenin by Western blotting.

LAR Protein Tyrosine Phosphatase

Aliquots of 50 µg of protein collected from control and Dex-treated cultures were incubated with LAR Buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.0, 2 mM Na_2EDTA, 5 mM dithiothreitol, 0.01 Brij 35, 50% glycerol and purified 10 mg/ml of BSA) and 5 U of LAR Protein Tyrosine Phosphatase (New England Biolabs) in reaction volume of 20 µL. The samples were incubated at 30°C for 0-30 minutes. Samples then were mixed with an equal volume of SDS sample buffer, denatured at 100°C and resolved on a 7.5% polyacrylamide gel. Samples were transferred to nitrocellulose and analyzed for β-catenin by Western blotting.

Subcellular Fractionation

Control and Dex-treated samples were collected and washed with PBS to remove any extra media from the cells. Five pellet volumes (up to 500 µL) of hypotonic lysis buffer (20 mmol/L HEPES pH 7.5, 10 mM potassium acetate, 1.5 mM magnesium acetate) was added to the cellular pellet. The cells were dounce homogenized (rubbed against ground glass) for a total of 10 strokes. Samples were then subjected to low speed centrifugation (approximately 2,000 rpms) for five minutes. After reserving the supernatant, the pellet was resuspended in two pellet volumes (100 µL) hypotonic lysis buffer and again subjected to low speed centrifugation two more times, with reserving the supernatant each time. The nuclear pellet was resuspended in 1% NP-40 in PBS also containing protease inhibitor cocktail. The pooled supernatant was centrifuged at 4 °C for 1 hour at 13,000 rpms. The pellet containing the membrane fraction was resuspended

in 1% NP-40 in PBS containing protease inhibitor cocktail. The supernatant represented the cytoplasmic fraction.

Immunoprecipitation and Detection of Tyrosine Phosphorylation

Aliquots of 250 μg of protein were precleared by incubation with 0.5 μL of normal mouse IgG (Sigma) and 20 μL protein G-agarose (Oncogene Research Products) for 30 minutes at 30°C. The samples were subjected to centrifugation at 2,500 rpms for 5 minutes at 4°C. The supernatant was transferred to a new tube with 2 μg of β-catenin antibody (Tranduction Laboratories) and incubated for one hour at 4°C. Protein G agarose beads (20 μL) were added to the tubes and incubated overnight at 4°C. The samples then were cleared by centrifugation (2,500 rpms) for five minutes at 4°C and the pellets were washed four times with 100 μL of lysis buffer containing 100 nM vanadate (pH 7.4). The pellet then was resuspended in 20 μL 2X SDS-PAGE sample buffer. The samples were denatured and 10 μL of the samples were resolved on a 10% polyacrylamide gel, transferred to nitrocellulose and analyzed for tyrosine phosphorylation by immunoblotting.

RESULTS:

To determine the mechanism by which glucocorticoid acts to alter the levels of ß-catenin in 235-1 cells, replicate cultures of 235-1 cells were treated with 100 nM Dex for a period of either two or four days. Cells were collected, lysed and BCA analysis was employed to determine protein concentration. Previous work demonstrated there is no change in the mRNA levels of ß-catenin; therefore, we analyzed post-translational regulation by measuring the protein level of ß-catenin by Western blotting (Fig. 10). In this and other repeated experiments, we found that Dex causes a 50% decrease in the expression of ß-catenin. Because previous work showed no alternation in ß-catenin mRNA levels in Dex-treated cells, half-life studies were conducted to determine the degradation rate of ß-catenin (Jones, R. unpublished results). Cells were treated with Dex for two days followed by cyclohexamide (CHX) for 120 minutes. The samples were resolved on a polyacrylamide gel, transferred to nitrocellulose and then analyzed by Western Blotting. This revealed a decrease in the half-life of ß-catenin from twenty minutes in control cells compared to nine minutes in Dex-treated cells.

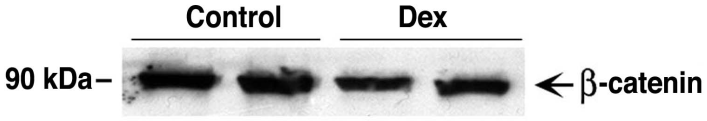


Figure 10: Expression of ß-catenin in 235-1 cells, determined by Western blotting. Duplicate cultures were isolated after four days from control or 100 nM Dex-treated cells. Aliquots of protein (40 µg) were probed with antiserum against ß-catenin. A representative blot is shown. [Lanes 1 and 2 control; lanes 3 and 4 Dex]. There is a 40% decrease in ß-catenin levels in Dex-treated cells.

In glucocorticoid-treated cells, cellular morphology is altered such that the cells appear more rounded and have shorter processes than controls (Fig. 11A). These

alterations suggested a loss of cellular adhesion. Dex causes a decline in the growth rate which is evident in Figure 11A, since we can see a decrease in cell numbers (Delidow et al., 2002). Previous work in the lab also showed that treatment with Dex results in a 50% reduction in the re-aggregation of cells (Spangler and Delidow, 1998). Phosphorylation of Tyr 654 decreases ß-catenin binding to the cadherin-catenin adhesion complex; therefore we examined changes in Tyr phosphorylation in both control and Dex-treated cells. Duplicate cultures were treated for four days with Dex. Figure 11B is a representative blot showing Tyr phosphorylated ß-catenin analyzed by immunopreciptating ß-catenin from 250 µg of cellular protein. The blot was probed with an anti-Tyr antibody (PY-20). This experiment revealed no changes in Tyr phosphorylated ß-catenin, even though changes in cellular morphology were evident (Fig 11A). Figure 11C is a representative blot showing the removal of phosphates from tyrosine in ß-catenin. Aliquots of protein (50 µg) were treated for 30 min with LAR tyrosine phosphatase, which served to remove phosphates from Tyr. After gel electrophoresis (7.5%) and transfer, the blots were probed with an antibody against ß-catenin. By running this experiment on a low percentage gel, as the phosphates are removed there should be shifts in the protein bands due to loss of the phosphates since the molecular weight of ß-catenin is decreasing by approximately 80 Da each time a phosphate is removed. As shown in Figure 11C, there are no shifts in the bands due to the loss of phosphates therefore there are no apparent alteration in Tyr phosphorylated ß-catenin. It is possible that the resolving power was not sufficient to detect the loss of phosphates. This is because ß-catenin is not a highly Tyr phosphorylated protein. There

are only three Tyr phosphorylated sites in the protein, therefore it may be hard to detect the shifts in molecular weight.

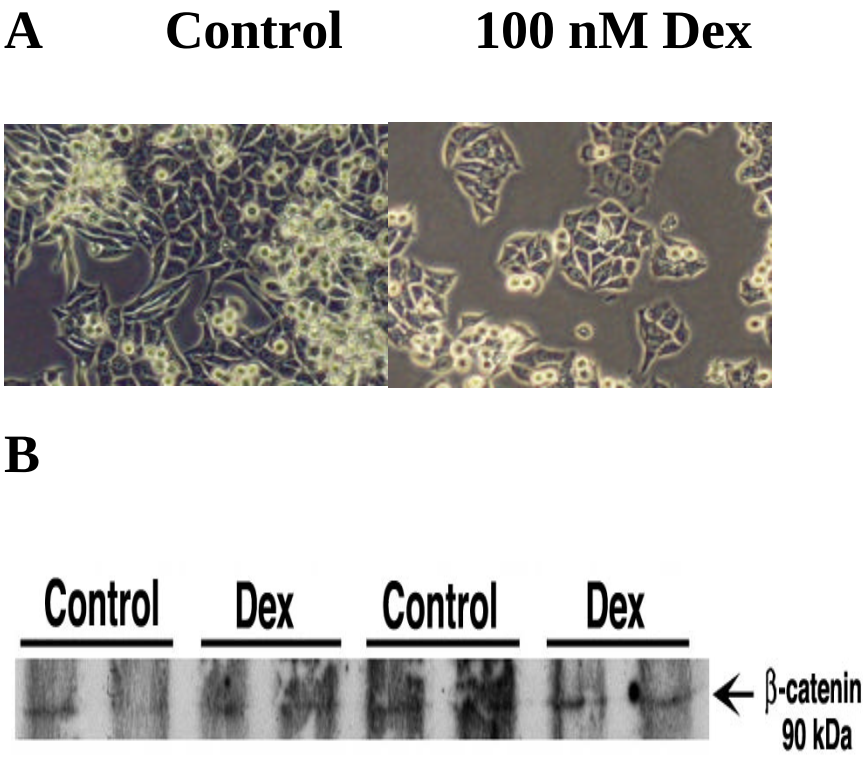


Figure 11: (A) Morphological changes induced by addition of glucocorticoid Cells were treated with Vehicle or Dex for four days and images were taken at 100X magnification. Dex-treated cells are more rounded and have shorter appendages. (B) Analysis of Tyr 654 phosphorylated ß-catenin. Aliquots of protein lysates (250 mg) were immunoprecipitated with antiserum against b-catenin. Samples were then analyzed by Western blotting using a tyrosine specific antibody (PY-20). A representative blot is shown. [Lanes 1, 2, 5 and 6 control; lanes 3, 4, 7 and 8 Dex]. There are no observable changes in tyrosine phosphorylated b-catenin between the control and Dex-treated cells. (C) Removal of phosphates from tyrosine phosphorylated ß-catenin. Duplicate cultures were analyzed by Western blots of protein isolated from control and 100 nM Dex-treated cells. Aliquots of protein (50 mg) were treated for 30 min with LAR Tyrosine phosphatase, which served to remove all Tyr phosphate groups. Samples were probed with antiserum against b-catenin and a representative

blot is shown. [Lanes 1, 2 and 3 control samples; lanes 5, 6 and 7 represent Dex-treated cells at indicated time intervals]. There does not appear to be any difference in Tyr phosphorylated b-catenin with glucocorticoid treatment.

Beta-catenin is localized in three separate places within the cell. We attempted to determine by subcellular fractionaltion whether glucocorticoid treatment causes any alterations in ß-catenin's localization. It is expected that Dex-treated cells will show a decrease in cytoplasmic ß-catenin due to the increased degradation rate of the protein. Using a cell fractionation extraction protocol, protein was isolated from the nucleus, cytoplasm and membrane. The results in Figure 12 indicate that glucocorticoid treatment results in a decrease in cytoplasmic, nuclear and membrane-bound ß-catenin. The greatest percentage decrease is shown in the nucleus and membrane. The loss of any of the linker proteins (a- or ß-catenin) from the adhesion complex results in a decrease in cellular adhesion (Ohsugi et al., 1999). In our cell line, glucocorticoid treatment reduces the amount of membrane bound ß-catenin. This process alone can cause a reduction in cell-to-cell adhesion and this is evident in morphological changes that occur with glucocorticoid treatment (Spangler and Delidow, 1998) (Figure 11A).

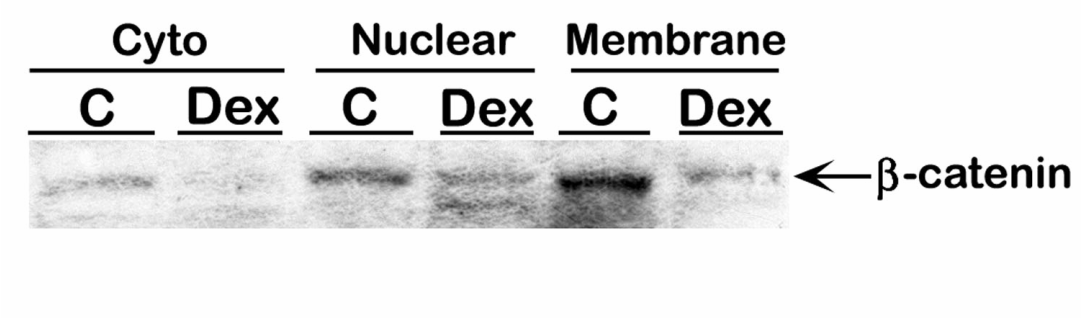


Figure 12: Expression of total ß-catenin localization in 235-1 cells determined by Western blots of protein isolated from replicate cultures of control or 100 nM Dex-treated cells isolated from the cytoplasm, nucleus and membrane. Cells were treated for two or four days and then collected. Aliquots of protein (40 µg) were examined using a specific antibody against ß-catenin. A representative blot is shown. ß-catenin levels were decreased in the cytoplasm, nucleus and membrane.

Cytoplasmic β-catenin is regulated by Wnt signaling and phosphorylation at key N-terminal sites. To study the mechanism by which 235-1 cells regulate ß-catenin levels we examined GSK3-ß, a kinase that phosphorylates ß-catenin, leading to proteasomal degradation. Since ß-catenin levels are reduced by 50% when 235-1 cells are treated with glucocorticoid, it might be expected that there is an increase in the amount of active kinase essential to the phosphorylation of ß-catenin. We examined the levels of total GSK3-ß in both control and Dex-treated cells. Duplicate cultures were treated with Dex for periods of two and four days. As shown in Figure 13A, the levels of total GSK-ß remain unchanged after four days of treatment with Dex.

In order to ascertain whether Dex treatment causes any alterations in the amount of active GSK3-ß we examined the protein levels of Tyr 216 phosphorylated GSK3-ß. This site is phosphorylated in active GSK3-ß (Figure 13B). After a four day treatment with Dex, the amount of active GSK3-ß was increased two-fold. When GSK3-ß is phosphorylated on Ser 9, the kinase activity is inhibited; therefore we wanted to note if there is any alteration in the inhibition of GSK3-ß in the presence of Dex (Fig 13C). When 235-1 cells are treated for a period of four days in the presence of Dex, we see a 25% decrease in the amount of the Ser 9 phosphorylated inactive GSK3-ß. Thus, treatment with glucocorticoid caused an increase in the amount of active Tyr 216 phosphorylated GSK3-ß, and a decrease in the amount of inhibitory Ser 9 phosphorylated GSK3-ß. Both suggest that the 235-1 cells regulate ß-catenin levels in the presence of Dex by increasing the activity of GSK3-ß.

The levels of ß-catenin and GSK3-ß were then measured in the presence of LiCl, a known inhibitor for GSK3-ß enzymes. When GSK3-ß activity is inhibited, ß-catenin will not be targeted for proteasomal degradation. As shown in Figure 13D, glucocorticoid treatment results in a decrease in the amount of ß-catenin with a simultaneous increase in the amount of active GSK3-ß. However, when LiCl is added to duplicate cultures of control and Dex-treated cells, active GSK3-ß is still present, but ß-catenin levels are restored. This suggests that while GSK3-ß can be activated in the presence of LiCl, it is unable to phosphorylate ß-catenin.

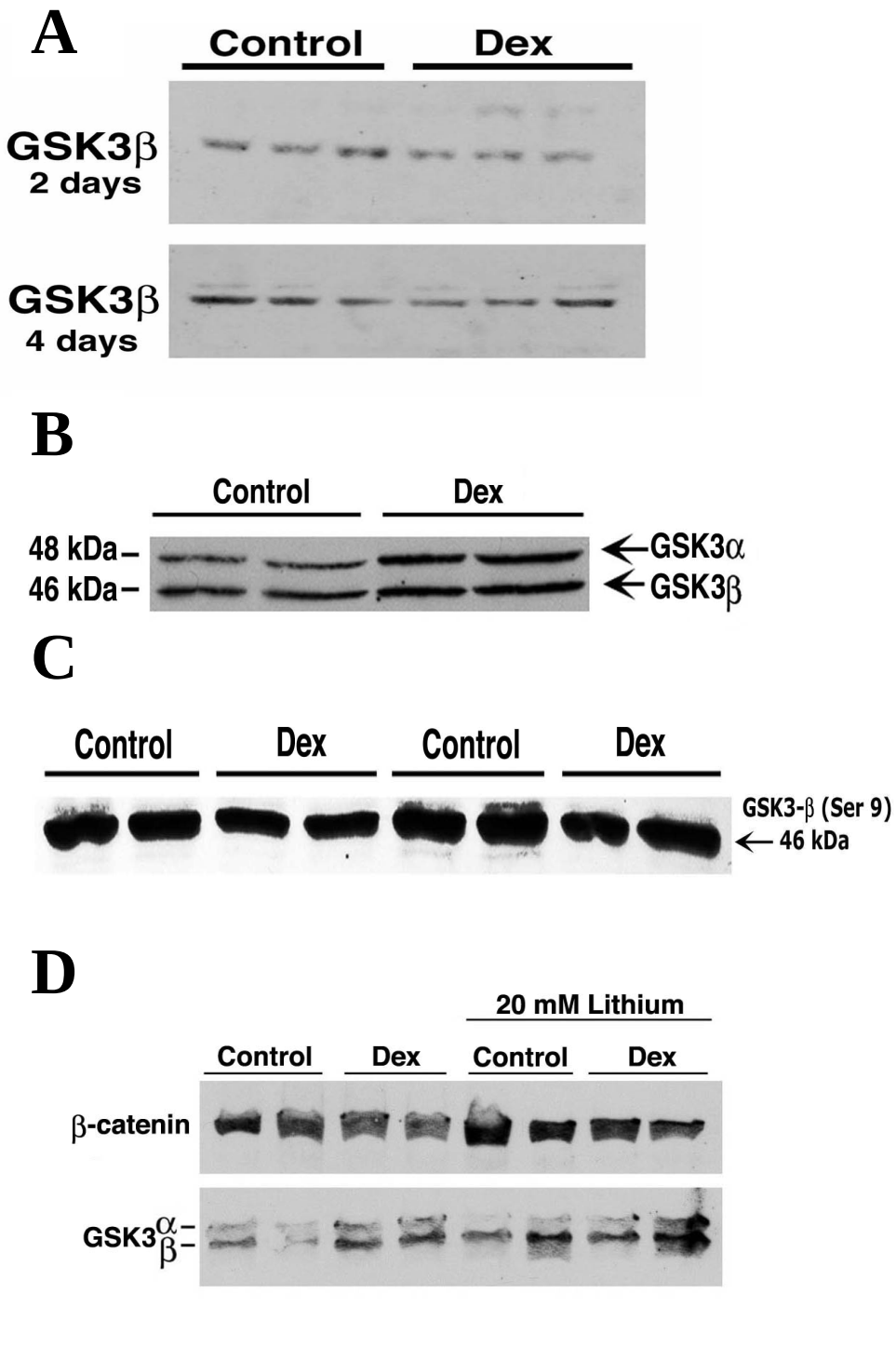

A
Control
Dex
GSK3β
2 days
GSK3β
4 days
B
Control
Dex
48 kDa
46 kDa
GSK3α
GSK3β
C
Control
Dex
Control
Dex
GSK3-β (Ser 9)
46 kDa
D
20 mM Lithium
Control
Dex
Control
Dex
β-catenin
GSK3α
β

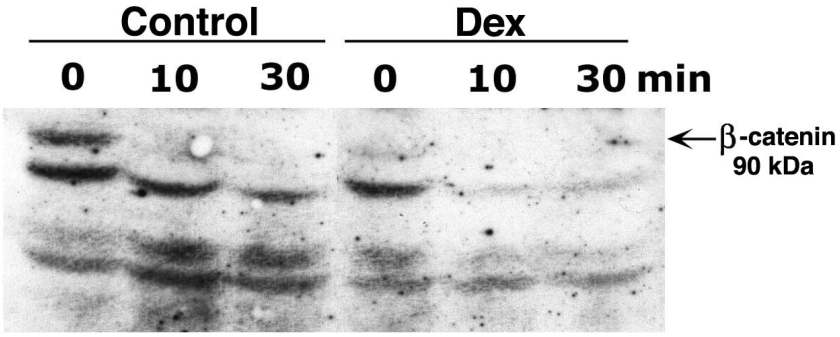
Figure 13: (A) Expression of total GSK3-b in 235-1 cells determined by Western blots of protein isolated from replicate cultures of control or 100 nM Dex-treated cells. Cells were treated for two or four days and then collected. Aliquots of protein (40 µg) were examined using a specific antibody against GSK3-b. A representative blot is shown. [Lanes 1, 2 and 3 control; lanes 4, 5 and 6 Dex]. The top Western blot represents a two day treatment with Dex, while the bottom Western blot represents a four day treatment with Dex. The total level of GSK3-b was not altered in control or Dex-treated cells; however, b-catenin levels are reduced by 40-50% in the Dex-treated cells. (B) Expression of active GSK3-b (Tyr 216) in 235-1 cells. Samples were collected from duplicate cultures of control or 100 nM Dex-treated cells. Cells were treated for four days and Western blots of protein were probed using a phosphospecific antibody against active GSK3-b. Aliquots of cellular protein (40 µg) were analyzed for active enzyme. A representative blot is shown. [Lanes 1 and 2 control; lanes 3 and 4 Dex]. The amount of active GSK3-ß enzyme was doubled in Dex-treated cells; simultaneously, ß-catenin expression was decreased. (C) Expression of Ser 9 phosphorylated GSK3-b. Western blots of protein (30 µg) isolated from the control or 100 nM Dex-treated cells were probed using a phosphospecific antibody for GSK3-b (Ser 9), which is the inhibited form. A representative blot is shown. [Lanes 1, 2, 5 and 6 control; lanes 3, 4, 7 and 8 Dex]. Changes in inhibitory phosphorylation between control and treated cells are visible. Dex-treated cells show a 25% decrease in inhibitory Ser 9 phosphorylation, indicative of increased GSK3-b enzyme activity. (D) Expression of b-catenin and active GSK3-b. Duplicate cultures were analyzed by Western blots of protein isolated from control, 100 nM Dex, 20 mM LiCl or LiCl and Dex-treated cells. Control and Dex-treated cells were treated for four days and LiCl, which inhibits GSK3-b activity, was added to cultures one day prior to cell collection. Samples were probed using a phosphospecific antibody against GSK3-b phosphorylated at Tyr 216 and antiserum against b-catenin. Aliquots of protein (40 µg) were analyzed for b-catenin and active GSK3-b enzyme. A representative blot is shown. [Lanes 1 and 2 control; lanes 3 and 4 Dex; lanes 5 and 6 LiCl; lanes 7 and 8 Dex and LiCl]. The top band represents b-catenin, while the bottom band represents the active GSK3-b enzyme. Active GSK3-ß enzyme was increased in Dex-treated cells; the levels of ß-catenin were decreased

Degradation of ß-catenin occurs by phosphorylation at key N-terminal residues, and this phosphorylation is the key regulator of cytoplasmic ß-catenin levels (Amit et al., 2002; Chunming et al., 2002; Provost et al., 2003). Phosphorylation of ß-catenin was examined to determine the effects of glucocorticoid on the degradation of ß-catenin. As shown in Figure 14A, total levels of phosphorylated ß-catenin were examined by using ?-phosphatase, which removes phosphates nonspecifically from Ser, Thr and Tyr on ß-catenin over the course of 30 minutes. In both control and Dex-treated cells, phosphates were removed in one major dephosphorylation step, evident at 0 minutes. However, control cells needed additional time to remove further phosphates, suggesting that

degradation of ß-catenin is occuring more rapidly in Dex-treated cells, as opposed to control cells.

In light of these results, we examined the specific N-terminal phosphorylation using phosphospecific antibodies. In Figure 14B, we examined Ser 45 phosphorylated ß-catenin. In this experiment cells were treated for four days in the presence of Dex and then, on the third day, 20 mM LiCl was added to inhibit GSK3-ß activity. The results show that glucocorticoid causes an increase in the amount of phosphorylated ß-catenin. Treatment with LiCl induced a slight decrease in the amount of phosphorylated ß-catenin. This is likely due to the specificity of the antibody, which recognizes a GSK3-ß targeted site, Thr 41, as well as the non-GSK3-ß site Ser 45. We also examined the GSK3-ß targeted sites using a phosphospecific antibody that recognizes ß-catenin only when it is phosphorylated at Ser 33/37/Thr41 (Figure 14C). These sites become phosphorylated only after ß-catenin is primed at Ser 45, by Casein Kinase 1. The results from this experiment indicate that there is an increase in the amount of phosphorylated ß-catenin when cells are treated with Dex. However, when LiCl is added to inhibit GSK3-ß activity, there is a dramatic decrease in the amount of Ser 33/37/Thr41 phosphorylated ß-catenin. Together these data suggest that ß-catenin levels are regulated in glucocorticoid-treated cells by increasing the amount of phosphorylated ß-catenin. These results also suggest that there are at least two different kinases that phosphorylate ß-catenin in response to glucocorticoid leading to degradation.

A



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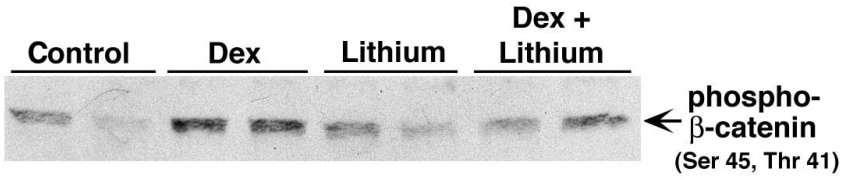

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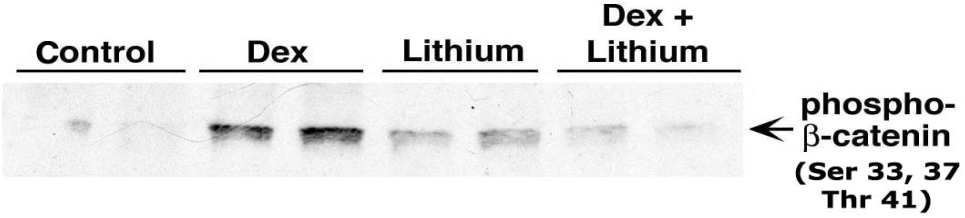


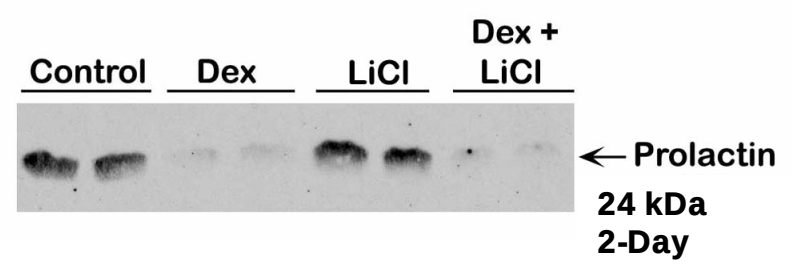
Figure 14: (A) Expression of phosphorylated ß-catenin in 235-1 rat pituitary cells. All samples were treated for four days; protein expression from control and (100 nM) Dex was analyzed by Western blot technique. Treatment of lysates with l Phosphatase for 30 minutes served to remove all phosphate groups. Samples were probed with antiserum against b-catenin. Aliquots of 100 mg of protein were analyzed and a representative blot is shown. [Lanes 1, 2 and 3 represent control samples at indicated time intervals. [Lanes 4, 5 and 6 represent Dex treated cells] The results show a change in the phosphorylation states of b-catenin in both control and Dex-treated cells. l-

phosphatase removed phosphates in one major step in both control and Dex-treated cells. Control cells needed additional time to remove phosphates, as evident from the b-catenin band shifts in lanes 2 and 3. The b-catenin still present on the film indicates remaining nonphosphorylated forms. (B) (C) Expression of phosphorylated b-catenin (Ser 45/Thr 41 and Ser33/37/Thr41). Western blots of protein (40 mg) isolated from control, 100 nM Dex, 20 mM LiCl or Dex + LiCl-treated cells. Control and Dex-treated cells were treated for six days and LiCl was added to cultures one day prior to cell collection. Immunoblots were probed using a phosphospecific b-catenin antibodies, which detects levels of b-catenin when phosphorylated at Ser 45/Thr41 or Ser 33/37/Thr41. Representative blots are shown. [Lanes 1 and 2 control; 3 and 4 Dex; 5 and 6 LiCl; 7 and 8 Dex and LiCl]. In figure B, there is an increase in Ser 45/Thr 41 phosphorylated b-catenin in Dex-treated cells, regardless of the presence of LiCl. The mechanism of Ser 45 phosphorylation occurs by an alternate kinase, CK1, which primes b-catenin for further phosphorylation by GSK3-b. In figure C, there is an increase in Ser 33/37/Thr 41 phosphorylated b-catenin in Dex-treated cells. In LiCl-treated cells, there is a decrease in phosphorylated b-catenin, as lithium functions to inhibit GSK3-b activity.

Previous work in our lab has shown that glucocorticoid treatment reduces prolactin gene expression and causes a decrease in cellular adhesion. When 235-1 cells are treated with glucocorticoid, we see a simultaneous decrease in prolactin and ß-catenin levels. We wanted to determine whether there exists a common pathway relating ß-catenin and prolactin; therefore our experiments were designed to examine the effects of LiCl on prolactin protein levels (Figure 15 A B). Cells were treated for a period of two or four days with Dex, and 20 mM LiCl for one day. After two and four day treatment with glucocorticoid there is a decrease in prolactin levels. Four-day control cells show an increase in prolactin levels, possibly due to the increase in cell-to-cell adhesion. After four days of growth the cells are confluent and they have numerous contacts with each other. In the 235-1 cell line if the contacts between cells are increased then prolactin levels will increase simultaneously. Treatment with LiCl slightly decreased basal prolactin levels and did not alter the response to Dex. One possible reason for the decrease in basal prolactin levels is because cells have been treated with Dex simultaneously, which is known to decrease prolactin gene expression and protein levels.

Treatment with LiCl for 24 hours may not be enough time to restore adhesion, which would ultimately increase prolactin levels in the 235-1 cells.

A



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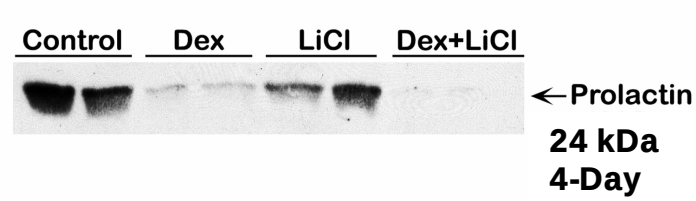

Figure 15: Expression of prolactin in 235-1 cells. Western blots of protein (40 mg) isolated from control, 100 nM Dex, 20 mM LiCl or Dex + LiCl-treated cells. Control and Dex-treated cells were treated for two and four days and LiCl was added to cultures one day prior to cell collection. Immunoblots were probed using antisera against prolactin. A representative blot is shown. [Lanes 1 and 2 control; 3 and 4 Dex; 5 and 6 LiCl; 7 and 8 Dex and LiCl]. Glucocorticoid causes a decrease in prolactin levels. After a four day treatment with Dex, prolactin levels are practically undetectable LiCl treatment also functioned to decrease prolactin levels slightly.

DISCUSSION:

The data presented above support the conclusion that glucocorticoid treatment directs numerous changes in 235-1 cells. Previous work in our lab has shown that these changes consist of reduced prolactin gene expression, reduced prolactin protein levels, decline in the growth rate and decreased cell-to-cell adhesion (Spangler and Delidow, 1998; Delidow et al., 2002). The change in cell-to-cell adhesion suggested that there was a loss of the cadherin-catenin adhesion complex, although previous research was unable to determine which cadherin was responsible in the loss of adhesion. The catenins are partly responsible for the loss of cellular adhesion; however catenin levels are not reduced until two days after treatment with Dex, while the loss of adhesion is evident immediately because cells start to appear more rounded after one day treatment with Dex. We have provided evidence that treatment with glucocorticoid decreases ß-catenin levels by increasing at least two different kinase activities, which cause an increase in the degradation rate of the protein.

ß-catenin is a multifunctional protein involved in the regulation of the cadherin-catenin cell-to-cell adhesion complex and is an important downstream regulator of the Wnt signaling pathway. Experimental data showed that glucocorticoid treatment causes a 50% reduction in ß-catenin protein levels (Figure 10). This reduction was due to an alteration in the half-life of ß-catenin, without altering the mRNA levels. ß-catenin's fate in the cytosol is regulated by the presence of Wnt stimulation and phosphorylation. Without Wnt signaling, ß-catenin is constantly being phosphorylation by CK1 and

GSK3-ß, which targets the protein for degradation. ß-catenin's half-life is twenty minutes in the 235-1 cells before degradation; however treatment with Dex reduces ß-catenin's half-life from twenty to nine minutes. One hypothesis is that treatment with glucocorticoid decreases Wnt stimulation and, therefore, causes a reduction in the cytoplasmic ß-catenin levels (Figure 12). It is also believed that similar effects are taking place in the nucleus and membrane. Membrane-bound ß-catenin is associated in the adhesion complex; however glucocorticoid treatment decreases cellular adhesion while also decreasing the amount of membrane bound ß-catenin (Figure 12). This loss of ß-catenin from the membrane may be in part responsible for the loss of cellular adhesion and the changes in cellular morphology evident in Figure 11. In the presence of Wnt signaling a pool of cytosolic ß-catenin is established, which then translocates ß-catenin to the nucleus where it binds to TCF, initiating gene transcription (Provost et al., 2003; Chunming et al., 2002). In the presence of Dex there is a decrease in the amount of nuclear ß-catenin, due to the fact that ß-catenin's degradation has been increased. Likewise, there is an increase in the phosphorylated forms of ß-catenin also showing that there is an increase in the degradation of ß-catenin (Figure 14BC).

In the absence of Wnt signaling, GSK3-ß phosphorylates ß-catenin, which then forms a complex with the tumor suppressors Axin and adenomatous polyposis coli (APC) leading to proteasomal degradation. Glucocorticoid treatment causes a two-fold increase in the amount of active GSK3-ß which phosphorylates ß-catenin (Figure 13B). When Wnt signaling is turned off GSK3-ß activity is turned on and ß-catenin's translocation to the nucleus is decreased. Our results show that ß-catenin levels are also regulated by the inhibition of GSK3-ß. When GSK3-ß is phosphorylated on Ser 9, the kinase is inactive

and will not phosphorylate ß-catenin. Treatment with glucocorticoid decreased the amount of inhibited GSK3-ß by 25% (Figure 13C). It is believed that both the decrease in inhibitory Ser 9 phosphorylation and increase in active Tyr 216 phosphorylation of GSK3-ß both control the regulation of ß-catenin in the 235-1 cells.

Abnormalities in Wnt signaling or mutations in the N-terminal phosphorylation sites (exon 3) cause an accumulation of cytosolic ß-catenin which initiates gene transcription and can ultimately lead to tumorigensis since the whole signaling process is up regulated. The four serine/threonine residues (Ser 33/37/45 and Thr 41) are conserved from *Drosophilia* to human and mutations in these N-terminal phosphorylation sites are observed in many types of human tumors (Chunming et al., 2002). Research has found that all such mutations are associated with malignant types of tumors, but there are two different mechanisms by which ß-catenin is involved in precipitating colon cancer. Some colon cancer research has found that there is a mutation in the APC complex, while others has found mutations in the specific N-terminal phosphorylation sites. When ß-catenin is mutated in exon three, it is unable to bind to the F box protein ß-Trcp, which is the ubiquitination apparatus (Liu, 2002). Therefore, ß-catenin accumulates in the cytosol eventually turning on gene transcription.

This work has provided further evidence that there are at least two different kinases involved in the phosphorylation of ß-catenin leading to degradation. When cells are treated for a period of four days with Dex and one day with LiCl, there is an increase in the amount of phosphorylated ß-catenin. However, when duplicate cultures are treated with Dex and LiCl, our results indicate that Ser 45 phosphorylation is still detectable (Figure 14B). This suggests that Ser 45 is phosphorylated by a kinase other than GSK3-

ß, since it is not inhibited by LiCl. Research has shown that in vivo Ser 45 is phosphorylated by CK1 and it functions as a priming site on ß-catenin. When CK1 is deleted, ß-catenin is unable to become phosphorylated and ultimately degraded (Chunming et al., 2002; Amite et al., 2002). It has also been found that without CK1 phosphorylation the GSK3-ß targeted sites are unable to become phosphorylated, which can also lead to stabilization of ß-catenin. This demonstrates the important role of at least two different kinases, but also stresses the importance of individual residues in ß-catenin. Furthermore, when cells are treated with Dex, there is an increase in Ser 33/37/Thr 41 phosphorylation. However, when cells are treated with Dex and LiCl few forms of Ser 33/37/Thr41 phosphorylated ß-catenin remain (Figure 14C). These sites are GSK3-ß targeted sites and are inhibited by LiCl. These results provide more evidence to support the conclusion that two kinases regulate ß-catenin activity.

A question stemming from this research is how a corticosteroid can activate a kinase? One potential mechanism could be through Akt signaling, which regulates cell fate between survival and apoptosis and is also involved in glycogen synthesis. Corticosteroids, insulin, and other growth factors have been shown to activate the Akt signaling through tyrosine kinases or G-protein coupled receptors (Langdown, 2001; Burgering and Coffer,1995; Franke et al., 1995) Once Akt is activated it can phosphorylate a number of substrates involved in inhibiting apoptosis. One such substrate phosphorylated by Akt is GSK3-ß (Hajduch et al., 2000; Cross et al., 1995). GSK3-ß regulates ß-catenin's cellular fate, is involved in regulating glycogen synthesis in response to insulin (Welsh et al., 1996), and is involved in cell cycle regulation by preventing phosphorylating of cyclin D1 (Diehl et al., 1998). If GSK3-ß is

phosphorylated by Akt, it demonstrates that cross talk between Wnt signaling and Akt signaling exists. A potential technique to look at the interactions of the two pathways would be to block Akt activity to see what alterations occur with GSK3-ß.

Another area of interest from this research is how CK1 is activated by a glucocorticoid? Our research and others have shown that in order to regulate ß-catenin levels, CK1 must be first activated before GSK3-ß can phosphorylate ß-catenin (Amit et al., 2002; Chunming et al., 2002). Additional studies have shown that CK1 appears to play a role in glycogen metabolism in response to insulin (Bacskai et al., 1993; Tuazon, and Traugh 1991; Osterrieder et al., 1982) and the δ isoform of CK1 has been shown in vitro to play a role in the hyperphosphorylation of Tau therefore playing a role in Alzheimer's disease (Schwab et al., 2000; Ghoshal et al., 1999). The possible connection is that since GSK3-ß is also involved in these processes then there must be an interaction between these two kinases. However the direct mechanism is unknown. One reason why it is difficult to find out how CK1 and GSK3-ß are related is due to the fact that CK1 undergoes autoregulation. This autophosphorylation is main type of regulation for this kinase and therefore it is difficult to determine what affects this autophosphorylation or what signal activates or inactivates this kinase.

CONCLUSIONS:

In conclusion, these data suggest that glucocorticoid acts post-translationally to alter the level of β-catenin in 235-1 cells by increasing at least two kinase activities. β-catenin phosphorylation takes place in two steps: first, it must be primed at Ser 45 by CK1; next, β-catenin is phosphorylated by GSK3-β at Ser 33, 37 and Thr 41, which targets the protein for degradation. Our data show that Dex increases the level of active GSK3-β and decreases the amount of inhibitory GSK3-ß. The mechanism of increased Ser 45 phosphorylation will be the focus of further study to ascertain whether there is an alteration in expression or activation of CK1, the putative kinase for this site. It is important to determine the mechanism by which CK1 regulates GSK3-ß activity, since Ser 45 must be primed first before GSK3-ß can phosphorylate its targeted sites. Future studies also include looking at the gene targets of ß-catenin to determine whether prolactin is one of the targets of expression.

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