The Neuroendocrine Protein 7B2 Is Required for Peptide Hormone Processing In Vivo and Provides a Novel Mechanism for Pituitary Cushing’s Disease

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Summary

The neuroendocrine protein 7B2 has been implicated in activation of prohormone convertase 2 (PC2), an important neuroendocrine precursor processing endopeptidase. To test this hypothesis, we created a null mutation in 7B2 employing a novel transposon-facilitated technique and compared the phenotypes of 7B2 and PC2 nulls. 7B2 null mice have no demonstrable PC2 activity, are deficient in processing islet hor-
mones, and display hypoglycemia, hyperproinsulinemia, and hypoglucagonemia. In contrast to the PC2 null phenotype, these mice show markedly elevated circulating ACTH and corticosterone levels, with adrenal cortical expansion. They die before 9 weeks of severe Cushing’s syndrome arising from pituitary intermediate lobe ACTH hypersecretion. We conclude that 7B2 is indeed required for activation of PC2 in vivo but has additional important functions in regulating pituitary hormone secretion.

Introduction

A wide variety of biologically active proteins and peptides are generated in animal cells via proteolytic processing of inactive or less active precursor proteins. Recently, a eukaryotic family of serine proteases related to subtilisin has been identified and shown to be involved in this processing (reviewed in Rouille et al., 1995; Seidah and Chretien, 1997; Steiner, 1998). Neoproteolytic processing of precursor proteins by these enzymes is followed by exoproteolytic removal of C-terminal basic residues to generate the mature hormonal products. PC1/PC3 and PC2 differ in substrate preference, and this differential specificity coupled with differential expression in various neuroendocrine tissues leads to selective processing of complex precursors such as proglucagon and proopiomelanocortin (POMC) to generate mixtures of bioactive peptides with divergent or even opposing activities (Rouille et al., 1995). Data obtained in neuroendocrine cell lines show that PC2 also differs from other members of this family in that its activation, while autocatalytic like that of the other enzymes (reviewed in Steiner, 1998), appears to require interaction with the neuroendocrine protein 7B2 (Braks and Martens, 1994) in order to generate the enzymatically active mature form (Zhu and Lindberg, 1995; Muller et al., 1997).

Due to the widespread distribution of PC1/PC3 and PC2 in the brain and neuroendocrine system, defects in the expression or activity of these enzymes would be expected to produce complex disturbances of endocrine function, behavior, and metabolism. A human subject with genetic defects in both alleles of PC1/PC3 is obese, hypocortisolic, and hypogonadal (Jackson et al., 1997). In contrast, PC2 null mice show no abnormalities of steroidogenesis or reproduction, although they are hypoglycemic and mildly runted (Furuta et al., 1997). The islets of PC2 null mice contain reduced numbers of β cells and markedly increased numbers of α and δ cells, reflecting the defective processing of proglucagon to glucagon and of somatostatin 28 to somatostatin 14. Proinsulin processing is less completely inhibited because of the coexpression of PC1/PC3 in the β cells, where this enzyme apparently plays a more dominant role than PC2 (Furuta et al., 1998). The hypoglycemia arising from the severe glucagon deficiency accounts for the decreased β cell mass as a response to reduced demand for insulin (Furuta et al., 1998).

7B2, a small acidic protein exclusively localized to neuroendocrine tissues (Iguchi et al., 1984; Marcinkiewicz et al., 1994), was first identified in 1982 by Seidah and colleagues (Hsi et al., 1982) and has since been extensively employed as a neuroendocrine marker (Roebroek et al., 1989; Ohashi et al., 1990; Vieau et al., 1992; Collini et al., 1998). While it is now clear that 7B2 represents a specific binding protein for PC2 (Braks and Martens, 1994; Benjannet et al., 1995a; Zhu and Lindberg, 1995), the mechanism of the interaction of 7B2 with PC2 is not well understood. Recent studies indicate that the two proteins bind early in the secretory pathway (Benjannet et al., 1995a; Muller et al., 1997), and binding determinants have been identified on both 7B2 (Zhu et
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tion of proPC2 to PC2, assisted by 7B2, occurs in the later stages of the secretory pathway (reviewed in Steiner, 1998). While the precise role of 7B2 in this process is not known, it is clear that proPC2 molecules which have not encountered 7B2 prior to secretion can undergo cleavage of the propeptide without acquiring enzymatic activity (Muller et al., 1997; Zhu et al., 1998). An analysis of the role of 7B2 in the regulation of peptide hormone production has been hampered by the lack of regulated neuroendocrine cell lines that do not express 7B2.

In order to provide in vivo evidence of the link between 7B2 and PC2 activity, we generated 7B2 null mice using a novel transposon-based technique (Westphal and Leder, 1997). As predicted, these mice lack PC2 activity and have multiple metabolic derangements, similar but not identical to those found in PC2 null mice (Furuta et al., 1997). Interestingly, in contrast to PC2 null mice, 7B2 null mice develop and die of Cushing’s disease, with multiple sequelae of hypercorticosteronism, indicating a novel role for 7B2 in the normal control of secretion of peptides from the intermediate lobe of the pituitary.

Results

Generation of 7B2 Null Mice Using a Novel Transposon-Based Approach

We have employed in vitro transposition to rapidly generate mouse knockout and knockin constructs (Westphal and Leder, 1997). Using the previously described technique, a construct was generated to target 7B2, a gene which lies within 50 kb of the 3' end of the formin gene, previously studied in our laboratory (Wang et al., 1997) (Figure 1A). Briefly, this approach is based upon random in vitro integration of transposable elements (Devine and Boeke, 1994) and selection of rare transposition events that have integrated into exonic DNA. Note that chromosomal rearrangement at the 7B2 locus upon homologous recombination is excluded in the transposon-based approach, since the integrity of this site is verified by probing both the 5' and 3' ends (Westphal and Leder, 1997).

After electroporation of the gene-targeting construct into embryonic stem cells (Meiner et al., 1996), several ES clones were shown to have undergone homologous recombination (Westphal and Leder, 1997). Two independent targeted ES clones were injected into blastocysts, chimeric mice were derived, and germline transmission was achieved (data not shown). Subsequent matings of chimeric 129 SvEv males to SvEv females allowed us to place the targeted allele on a pure genetic background. All mice presented in this study were derived from two independent mouse lines. Genotyping of heterozygotic matings indicated offspring of all possible genotypes (Figure 1B). In order to analyze 7B2 RNA levels, whole brains were extracted and Northern blots were prepared from 7B2 wild-type and 7B2 null mice. As shown in Figure 1C, 7B2 null mice lack detectable 7B2 RNA transcripts.

7B2 Null Mice Are Deficient in proPC2 Protein Maturation and Devoid of PC2 Activity

Initial demonstrations that the 7B2 and PC2 proteins interact (Braks and Martens, 1994) were followed by studies which indicated that PC2 activity may be totally dependent upon 7B2 expression (Zhu and Lindberg, 1995). We were hence interested to see how PC2 levels and activity might be affected by loss of 7B2 function in vivo. In an initial analysis of PC2 protein expression in brain (where PC2 is expressed at high levels), wild-type and null brains were homogenized and then separated by SDSPAGE and probed with anti-PC2 antisera. In three independent experiments, the maturation of proPC2 was severely inhibited in 7B2 null mice (Figure 2A, right lane). In order to examine the catalytic ability of PC2 in 7B2 null mice, we immunopurified PC2 from detergent extracts of wild-type and null mouse brains with anti-PC2 antibodies and performed fluorometric PC2 activity assays (Figure 2B; each line represents three independent determinations per genotype). Our
Figure 2. ProPC2 Is Incompletely Processed and Is Enzymatically Inactive in 7B2 Null Animals

(A) Western blot of proPC2/PC2 forms in the brains of wild-type or null animals. The maturation of proPC2 (upper band) to PC2 (lower band) is significantly impaired in mutant animals (right).

(B) Enzymatic activity of PC2 immunopurified from the brains of wild-type (solid circles) or 7B2 null mice (open circles) was assayed against a fluorogenic peptide substrate, as described in Experimental Procedures. Specificity of the PC2 assay was verified by assessing inhibition using the PC2 inhibitor CT peptide 1-31 at 1 μM.

results indicate that 7B2 null mice completely lack PC2 activity (Figure 2B), confirming a physiological requirement for 7B2 in PC2 activation (Zhu and Lindberg, 1995).

Immature Glucagon and Insulin and Decreased Enkephalins in 7B2 Null Mice

Since 7B2 null mice exhibited metabolic derangements and lacked PC2 activity, we assessed the production of several PC2-dependent polypeptide hormones. In order to study proglucagon processing, pancreatic islets were pulse-chase labeled, and immunoprecipitated glucagon-related proteins were subjected to SDS-PAGE. As shown in Figure 3A, the generation of mature glucagon is essentially eliminated in 7B2 null islets and only small amounts of intermediate cleavage products are generated, in contrast to the rapid and complete conversion

Figure 3. PC2-Dependent Processing Is Disrupted in 7B2 Null Mice

(A) Proglucagon processing. Islets were labeled for 45 min and chase incubated for 1 hr and 3 hr, respectively, as described in Experimental Procedures. Note marked reduction in processing of proglucagon to glucagon in 7B2 null islets. Similar results were obtained in at least two other independent experiments. Immunoprecipitated glucagon-related peptides were analyzed by gradient SDS-PAGE. Glucagon, proglucagon 33-61; Glicentin, proglucagon 1-69; GRPP-Glu, glicentin-related polypeptide-glucagon, proglucagon 1-61; P, pulsed cells; 1C and 3C, chased cells of 1 hr and 3 hr, respectively; M, combined chase media.

(B) Proinsulin processing. Islets were labeled for 45 min and chase incubated for 1 hr as in (A). Note the rapid conversion of mouse proinsulin I and II into insulin in the wild-type islets and the slower conversion in the 7B2 null islets associated with significant accumulations of des-31,32 intermediates (peaks c and d). Immunopurified insulin-related peptides were resolved by HPLC. Peaks are as follows (based upon assignments given in Figure 1 of Furuta et al., 1998): a, mouse insulin II; b, mouse insulin I; c, des-31,32 mouse proinsulin II; d, des-31,32 mouse proinsulin I; g, mouse proinsulin II; h, mouse proinsulin I.

(C) Analysis of immunoreactive enkephalins in acid extracts prepared from 7B2 null (open circles) or wild-type (solid circles) mouse brains. Note the significant reduction of Met-Enk-Arg-Phe and complete absence of Met-enkephalin in 7B2 null mouse brain.
Metabolic Abnormalities

Since PC2 null mice are hypoglycemic due to lack of mature glucagon, showing some increase in proinsulin levels (Furuta et al., 1997), we analyzed 7B2 null mouse blood samples for insulin and sugar levels. At 4-5 weeks of age, 7B2 null mice are severely runted, hypoglycemic, and have elevated circulating insulin-like material (Figure 4A). Clear clinical signs of abnormality are apparent in these animals even at 4 days of age. Figure 4B depicts two 7B2 null mice flanked by two littermate wild-type mice; the null mice are pale and eczematous (note especially the severe bruising of the left null). Many 7B2 null mice suffer from significant bleeding into the abdomen, leading to their pale appearance (data not shown). In fact, only 11% of mutant mice survive to weaning (i.e., we obtained 12 nulls in a total of 109 offspring of heterozygotic matings at weaning). Despite initial runting, however, those null mice that survive to weaning show abnormal fat deposition, especially on the back and around the neck.

The observed defects in sugar and fat metabolism in the 7B2 null mice prompted us to study the pancreas in greater detail. As shown in Figure 4C, both β and non-β endocrine cell mass are significantly expanded in the pancreas of 7B2 null mice, as measured by morphometric analysis. These differences are demonstrated in a representative manner in Figure 4D, a 150× magnification of a pathological specimen from a 7B2 null compared to a wild-type mouse at 5 weeks of age, which indicates both enlarged and abnormal islets. Note that the mutant islets are hyperplastic, and also show disordered architecture, with disruption of the normal eccentric location of non-β cells (stained brown in both wild-type and mutant sections).

7B2 Mutant Mice Manifest Severe Metabolic Abnormalities

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7B2 Mutant Mice Develop a Novel Form of Pituitary Cushings Disease

Given the abnormal pattern of fat deposition observed in 7B2 null animals, we suspected a problem with the pituitary/adrenal axis leading to elevated blood steroid levels. As a first step in investigating this possibility, we measured total pituitary ACTH in wild-type and 7B2 null animals and found a 10- to 20-fold elevation of intact ACTH, with none of the ACTH cleavage product CLIP (corticotropin-like intermediate peptide) detected in 7B2 null animals (Figure 5A). Similar results were seen in three other experiments. In agreement, biosynthetic studies of POMC processing in isolated whole pituitaries showed markedly elevated production of intact ACTH and minimal conversion of this peptide to α melanocyte-stimulating hormone (α-MSH), a processing event normally mediated by PC2 in the intermediate lobe (Zhou et al., 1993) (Figure 5B). Immunohistochemical studies showed marked reduction in anterior lobe staining for ACTH in 7B2 nulls (Figure 5C, lower panels). In the intermediate lobe, cross-reaction of the ACTH antisera with the PC2-mediated cleavage product CLIP (see Figure 5A) would be expected to obscure differences between the 7B2 null and control mice. Alterations in ACTH processing are best detected using an antisera to the amidated cleavage product α-MSH, which (by virtue of being amide specific) does not react with intact ACTH. Figure 5C (upper panels) shows that the intermediate lobe of the 7B2 null exhibits markedly reduced α-MSH staining compared to wild-type intermediate lobe. These data confirm the lack of ACTH cleavage detected in the biosynthetic studies, and, taken together with radioimmunoassay data demonstrating high levels of intact ACTH in the intermediate lobe of the 7B2 null (unpublished observations), confirm that the increase in total pituitary ACTH seen in the 7B2 null pituitary resides solely in the intermediate lobe.

Consistent with the increased pituitary levels of ACTH, circulating ACTH in plasma obtained from 7B2 null animals was greatly elevated over controls (Figure 6A; the animal to animal variation is most likely due to stress in handling). We therefore assayed serum corticosterone in 7B2 null and wild-type mice. As expected, serum corticosterone was elevated approximately 4-fold in 7B2 mutant mice (Figure 6A). Given the raised levels of steroids, the pathology of the adrenal cortex was examined. In agreement with the known trophic influence of ACTH on the adrenal cortex, 7B2 null mice exhibit cortical hyperplasia compared to age-matched controls (Figure 6B). The presence of high plasma ACTH and steroids coupled with cortical hyperplasia supports a diagnosis of Cushings syndrome in these animals.

Multiple Histologic Signs of Hypercorticosteronism

Several other clinical symptoms exhibited by the 7B2 null animal are also consistent with severe Cushings syndrome. These include changes in skin; compared to samples derived from wild-type mice, 7B2 null mice exhibit marked thinning of the skin and dermal atrophy with hyperkeratosis (Figure 7, compare [A] and [B]). Even more strikingly, 7B2 null liver is markedly abnormal, with destruction of the normal liver architecture and severe fat vacuolation (Figures 7C and 7D). 7B2 null spleen is roughly one-fifth the size of wild-type spleen, with loss of normal architecture and generalized lymphoid atrophy (Figures 7E and 7F). Lastly, the development of a typical

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Figure 4. Metabolic Parameters, Appearance, and Generalized Islet Hyperplasia in 7B2 Null Mice

(A) 7B2 null mice are runted, hypoglycemic, and hyperproinsulinemic. Average values are indicated; n = 6 mice for each genotype (4- to 5-week-old mice).

(B) Appearance of 7B2 null mice at 4 days of age. Two 7B2 null mice (center) are pale and have significant bruising when compared to WT littermate controls (outside two mice).

(C) Morphometric analysis reveals hyperplasia of both \( \beta \) and non-\( \beta \) cell mass in islets of 7B2 null mice (performed as previously described; Withers et al., 1998). Mice were 4-5 weeks of age.

(D) Magnification (150×) of WT versus mutant pancreatic tissue reveals generalized islet hyperplasia and abnormal morphology in 7B2 null mice. Islets are enlarged, with disordered appearance of the normally eccentrically located non-\( \beta \) cells (stained brown).

dorsal cervical “buffalo hump” of fat, a classic symptom of Cushing’s syndrome in humans, is clearly visible in the null animals at 7 weeks (Figures 7G and 7H). All of these features are characteristic of full-blown Cushing’s syndrome.

Discussion

We report here the generation and characterization of 7B2 null mice produced using a transposon-based technique (Westphal and Leder, 1997). The 7B2 null mouse completely lacks PC2 activity, providing in vivo confirmation of the link between PC2 activation and 7B2 expression (Zhu and Lindberg, 1995). In general agreement with a previously reported PC2 null mouse (Furuta et al., 1997), the 7B2 null mouse is hypoglycemic and hyperproinsulinemic, with generalized islet cell expansion and altered islet cell morphology. The islet cell changes differ from those in the PC2 null mouse in that \( \beta \) cell mass is also increased, possibly indicative of direct stimulation by elevated circulating corticosterone (see below) or an indirect effect of steroid-induced insulin.
resistance. Like the PC2 null, the 7B2 null mouse also shows severely depressed levels of bioactive peptides such as mature enkephalins and glucagon, known to be synthesized by PC2-specific mechanisms (Rouille et al., 1994, 1997; Johanning et al., 1998). However, the most important derangement of PC2-mediated peptide processing in the 7B2 nulls involves the synthesis of the corticotropin ACTH.

Both the anterior as well as the intermediate lobes of the pituitary synthesize the ACTH precursor POMC. In the anterior lobe, cleavage of POMC into full-length ACTH—the end product in this lobe—should be unaffected by deletion of either PC2 or 7B2, since this cleavage occurs primarily through the action of PC1/PC3 rather than PC2 (Benjannet et al., 1991; Bloomquist et al., 1991; Thomas et al., 1991; Zhou et al., 1993). However, in the intermediate lobe, which expresses high levels of PC2 (Day et al., 1992), this enzyme normally acts to internally cleave ACTH into the noncorticotropic peptides α-MSH and CLIP (Benjannet et al., 1991; Thomas et al., 1991; Zhou et al., 1993). In the 7B2 null pituitary, which lacks functional PC2, this cleavage event is severely impaired and intermediate lobe ACTH (generated by PC1/PC3 from POMC) remains intact, resulting in extremely high levels of ACTH in this lobe. Preliminary experiments in which intact ACTH was specifically measured by a two-site immunoassay in separate pituitary lobes of 7B2 nulls and littermate controls confirm that the high levels of intact ACTH normally found in the anterior lobe are instead located in the intermediate lobe.
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Figure 6. Pituitary Cushing's Syndrome in 7B2 Null Animals: Increased Circulating Glucocorticoids Derive from Adrenal Hyperplasia Resulting from Greatly Elevated Plasma ACTH

(A) Plasma levels of ACTH and corticosterone are greatly elevated in 7B2 null mice. (B) 7B2 null mice (right) manifest a markedly expanded adrenal cortex (marked C) when compared to 7B2 WT mice (left). Magnification, 20×.

Surprisingly, the 7B2 null animals contrast sharply with the PC2 nulls in that they exhibit high levels of plasma ACTH and go on to develop severe pituitary Cushing's disease, surviving at most to 9 weeks after birth. The adrenal cortical hyperplasia observed in these animals, a consequence of continuous trophic stimulation by increased circulating ACTH, results in high serum levels of corticosterone. Elevated levels of circulating steroids are then responsible for a number of phenotypic changes observed in the 7B2 null, such as atrophic skin, lipodystrophy, and splenic lymphoid atrophy. The running of 7B2 mutant pups may also be due to chronically increased circulating corticosterone. None of these changes are observed in PC2 null mice, which exhibit no detectable dysfunction of the pituitary/adrenal axis. These data imply that profound differences exist in the release of pituitary peptide hormones in the two null animals.

The release of ACTH from anterior lobe corticotrophs is predominantly controlled by hypothalamic corticotropin-releasing hormone (CRH), while the secretion of POMC products from the intermediate lobe—densely innervated by neurons arising from the basolateral hypothalamus—is under the influence of neurotransmitters such as dopamine and GABA (reviewed in Autelitano et al., 1989). Although in the normal mouse maintenance of plasma levels of ACTH is largely a function of anterior lobe secretory activity, in the 7B2 and PC2 null animals, plasma levels might be expected to reflect secretion from both the anterior and intermediate lobes. In fact, the elevated circulating corticosterone in the 7B2 nulls acts to suppress hypothalamic secretion of CRH and anterior lobe synthesis of POMC, since POMC synthesis is highly responsive to transcriptional inhibition by steroids in the anterior but not in the intermediate lobe (reviewed in Autelitano et al., 1989). Consistent with this interpretation, our data show that ACTH staining is absent, and POMC mRNA is undetectable, in the anterior lobe of the 7B2 null (Figure 5; B. Peng and J.E. Pintar, unpublished results). Coupled with the radioimmunoassay data showing increased intact ACTH in the intermediate lobe, these data implicate the intermediate lobe as the sole source for the increased circulating ACTH in the 7B2 null. Indeed, plasma levels of ACTH in the PC2 mutant might also be expected to be increased, since ACTH secretion probably continues from the anterior as well as from the intermediate lobe. Intriguingly, however, the PC2 null animal shows no signs of increased circulating ACTH, and corticosterone levels are within the normal range (M. Furuta, unpublished data). These data indicate that the 7B2 null animal exhibits essential differences from both wild-type and PC2 null animals in the control of intermediate lobe pituitary secretion. Differential findings in the 7B2 as opposed to the PC2 mutant mice point to important additional functional roles for 7B2 not related to PC2-mediated effects.
Figure 7. Abnormal Skin, Liver, and Spleen Morphology in 7B2 Null Mice

(A and B) 7B2 null mouse skin (B) is atrophic, hyperkeratotic, and shows marked epidermal thinning when compared to wild type (A). Magnification, 20×.

(C and D) 7B2 null liver lacks lobular architecture and shows fat vacuolation (D) as compared to wild type (C). Magnification, 20×.

(E and F) Splenic lymphoid atrophy in 7B2 null (F) versus wild-type spleen (E). Magnification, 5×.

(G and H) Typical Cushingoid “buffalo hump” of fat in 7B2 null (right) as opposed to wild-type (left).
The hypothesis of additional roles for 7B2 is strengthened by recent findings that 7B2 is found in brain areas lacking PC2, while the converse has never been observed (Seidel et al., 1998). With respect to the increased secretion of ACTH from the pituitary of 7B2 null animals, these novel effects of 7B2 might be due to developmental changes during maturation of the intermediate lobe affecting the number and size of melanotrophs; changes in the innervation of this lobe affecting secretory activity; or to effects of 7B2 on the secretory activity of melanotrophs.

Developmental proliferation and differentiation of the various pituitary cell types are known to be under the control of transcription factors such as Pit-1 (reviewed in Rosenfeld et al., 1996); growth factors (Borrelli et al., 1992; McAndrew et al., 1995); and oncogenes such as Rb (Hu et al., 1994). In the anterior lobe, maturation and proliferation of corticotrophs are at least in part controlled by hypothalamic CRH and are normally feedback inhibited by circulating corticosterone (reviewed in Autelitano et al., 1989). On the other hand, in the intermediate lobe, highly innervated by dopaminergic fibers, dopamine plays an important role in regulating the proliferation of intermediate lobe melanocytes. The D2 receptor blocker haloperidol increases the number of melanocytes, thereby causing intermediate lobe hyperplasia, while bromocriptine and other D2 receptor agonists produce opposite effects (Chronwall et al., 1987; reviewed in Autelitano et al., 1989). In agreement with the notion of dopaminergic control of intermediate lobe growth, a recent report has shown that 4- to 6-month-old D2 receptor null mice exhibit mild intermediate lobe hyperplasia and increased plasma ACTH, resulting in a partial Cushings syndrome (Saiardi and Borrelli, 1998; see also Kelly et al., 1997). Alterations in pituitary morphology are also seen in the dopamine transporter mutant mouse, which exhibits anterior and intermediate lobe hypoplasia (Bosse et al., 1997). The D2 receptor null animal also exhibits significant upregulation of PC1 and PC2 expression, most likely due to both the hyperplastic changes as well as to the known transcriptional upregulation of these enzymes by dopaminergic antagonists (Bloomquist et al., 1991; Day et al., 1992; Oyarce et al., 1996), which receptor blockade would be expected to mimic. However, we have not been able to demonstrate either significant intermediate lobe hyperplasia or elevated levels of either PC1 or PC2 in the 7B2 null pituitary.

Hypersecretion from the intermediate lobe constitutes the most likely mechanism for the elevated plasma ACTH observed in the 7B2 null animal. Secretion from this lobe is controlled by dopaminergic and GABAergic innervation. The increase in plasma ACTH resulting from intermediate lobe hyperplasia seen in the dopamine receptor null animal (Saiardi and Borrelli, 1998) indicates that intact ACTH can be secreted from the intermediate lobe. In agreement with this notion, intact ACTH is also elevated in plasma in an equine Cushings syndrome model in which tumors derive from the intermediate lobe (Orth and Nicholson, 1982). However, the lack of severe Cushings syndrome in the dopamine receptor and transporter nulls implies that while developmental alterations in dopaminergic innervation can potentially contribute to the pathogenesis of this disease, other secretory deficits must also be present in the 7B2 null which culminate in the exceptionally high circulating ACTH levels in this animal. In support of this idea, preliminary results indicate that basal release of intact ACTH is greatly enhanced in isolated pituitaries of 7B2 null animals compared to controls (unpublished results), suggesting that isolated pituitaries retain the property of hypersecretion even when removed from direct dopaminergic influence. Further study of the synthesis, secretion, and regulatory roles of 7B2 in pituitary cells may provide clues as to novel controlling mechanisms for peptide hormone secretion.

Cushings disease in humans results from excessive trophic stimulation of the adrenal cortex by increased plasma ACTH (Meier and Biller, 1997). Clinically, most pituitary adenomas expressing ACTH are found within the anterior lobe, though involvement of the intermediate lobe, thought to be vestigial in humans, has been proposed (Lamberts et al., 1980). Genetic models for pituitary Cushings syndrome include the D2 receptor null cited above (Saiardi and Borrelli, 1998) and the CRH transgenic mouse (Stenzel-Poore et al., 1992). Interestingly, despite presenting qualitatively similar steroid-induced tissue changes as the 7B2 null, the CRH transgenic mouse and the D2 receptor null mouse both exhibit a much less severe Cushings phenotype, with normal life spans. These data highlight the fact that loss of 7B2 expression affects pituitary secretory activity in a much more profound manner than loss of CRH regulation, dopamine receptors, or PC2. A detailed study of pituitary development and function in the 7B2 null mouse should further our understanding of the control of ACTH and α-MSH secretion, and it may shed light upon the pathogenesis of some forms of Cushings syndrome—and potentially of other diseases of secretory hormone production—in humans.

Experimental Procedures

Generation of Mice

Mice were generated by ES targeting technology, using a transposon-based system to make the targeting construct, and were genotyped as described previously (Devine and Boeke, 1994; Westphal and Leder, 1997). Whole RNA was extracted from mouse brains, and Northern were probed with an exon 2 probe (Barral et al., 1996).

Corticosterone Assays

Corticosterone assays, performed on serum obtained from 5- to 6-week-old animals by retroorbital puncture, were carried out as described previously (Meiner et al., 1996). Animals were housed together, and blood samples were taken between 10 am and 12 pm.

Plasma ACTH, Glucose, and Insulin Assays

EDTA plasma was prepared from blood obtained from 5- to 6-week-old animals; 10 and 30 μl of 7B2 null plasma and 30 μl of WT control plasma (nonlittermate) were assayed in duplicate using the Nichols human ACTH 1-39 assay kit (San Juan Capistrano, CA), which is specific for intact ACTH 1-39. For glucose analyses, 4- to 5-week-old mice were weighed and blood was analyzed using a standard glucometer. Plasma insulin levels were measured using a Linco RIA kit (St. Charles, MO) that does not distinguish between insulin and proinsulin. Animals were housed together, and blood samples were taken between 10 am and 12 pm (glucose and insulin) or 1 and 3 pm (ACTH).

Pathology and Pancreatic Morphometry

Tissues from mice between 5-6 weeks of age were removed and fixed in Optimal Fix (American Histology Reagent, Lodi, CA), blocked in paraffin, sectioned at 10 μm, and stained with hematoxylin and
eosin. Pancreatic tissue was excised, fixed in Bouin's solution, and embedded in paraffin. Sections (5-7 μm) were immunoperoxidase labeled for the non-C cell hormones using a cocktail of antibodies and counterstained with hematoxylin. β and non-β endocrine mass were quantitated by point-counting morphometry as described previously (Withers et al., 1998).

PC2 Western Blotting

Brains were homogenized in 2 ml of boiling Laemmli sample buffer containing 6 M urea for 5 min. Proteins were then separated by SDS-PAGE on 8.8% gels. Immunoblotting was performed as described previously, employing antiserum directed against PC2 carboxyl terminus (Muller et al., 1997). In a parallel experiment, brains were homogenized in 2 ml of 0.1 N HCl, frozen at -80°C, thawed, and briefly sonicated. Precipitated proteins were pelleted at 10,000 g for 20 min at 4°C. Pellets were solubilized in boiling Laemmli sample buffer before separation of proteins by SDS-PAGE and immunoblotting. Both extraction procedures yielded identical results.

PC2 Enzyme Assay

Brains were homogenized on ice in 50 mM Tris-HCl (pH 7.5), containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, and protease inhibitors (1 μM pepstatin, 1 μM antipain, 1 μM trans epoxysuccinic acid, 280 μM tosylphenylalanine chloromethyl ketone, 140 μM tosyllysyl chloromethyl ketone, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 15,000 × g for 20 min at 4°C, and the supernatant was collected. The brain extracts were incubated for 3-4 hr with 7.5 μl of 50% anti-PC2 IgG-coupled protein A-Sepharose. Antisera used were either directed against the carboxyl terminus of PC2 (Zhu and Lindberg, 1995) or were preimmune (results not shown). Immunopurified PC2 assay was washed once with the extraction buffer and twice with PBS. The beads were then resuspended to a 25% solution in PBS. PC2 assay was measured in 20 μl of this solution as previously described (Lindberg et al., 1995). Briefly, the assay was performed in 100 mM sodium acetate (pH 5.0), containing 5 mM CaCl₂, 0.1% Brij, and protease inhibitors (1 μM pepstatin, 1 μM trans epoxysuccinic acid, 280 μM tosylphenylalanine chloromethyl ketone, and 140 μM tosylseryl chloromethyl ketone). The 7B2 carboxy-terminal domain (CT peptide), a PC2-specific inhibitor (Lindberg et al., 1995), was added at 1 μM final concentration in some experiments. The incubations were conducted at 37°C in the presence of Pyr-Glu-Arg-Thr-Lys-Arg-methylcoumarinamide (MCA) as a substrate. Fluorescence of the enzyme product was measured in a fluorometer (Cambridge, MA), and the amount of this solution as previously described was used to visualize the complex. The primary antibodies were anti-PC2 antiserum (gift of Dr. Peng Loh, NIH, Washington, D.C.) (Castro et al., 1989) and was used at a final dilution of 1:24,000. Results depict total immunoreactive ACTH per HPGC fraction for one control and one 7B2 null pituitary. The entire experiment was repeated three times with different sets of pituitaries with similar results.

Pituitary ACTH Analysis

Pituitaries from three 7B2 null and three wild-type mice were individually homogenized via sonication in 150 μl ice-cold 1 N acetic acid. The samples were stored frozen at -70°C prior to thawing. The samples were subjected to centrifugation for 10 min at 13,000 rpm in a microfuge at 4°C. The clear supernatants were transferred to new tubes. One hundred microliter aliquots of each sample were sequentially injected onto a high-pressure gel permeation chromatography column consisting of 250 mm × 5 mm TSK-250 columns preceded by a guard column (Ohannesian et al., 1996). The eluant was 32% acetonitrile, 0.1% trifluoroacetic acid, and the flow rate was 0.5 ml/min. Fifty 1 min samples were collected. BSA (100 μg) was immediately added to each as a carrier protein, and the samples were stored frozen prior to analysis by radioimmunoassay (RIA). Fifteen microliter of each fraction was subjected to RIA for ACTH-immunoreactive peptides. The polyclonal anti-ACTH antiserum DPS was raised against residues 1-39 of human ACTH (gift of Dr. Peng Loh, NIH, Washington, D.C.) (Castro et al., 1989) and was used at a final dilution of 1:240,000. Results depict total immunoreactive ACTH per HPGC fraction for one control and one 7B2 null pituitary. The entire experiment was repeated three times with different sets of pituitaries with similar results.

Pituitary Immunocytochemistry

Pituitaries from wild-type and null animals were removed, fixed in formaldehyde, embedded in paraffin, and sectioned at 5 μm. A standard immunohistochemistry protocol using an avidin-biotin complex kit from Pierce was then followed; alkaline phosphatase was used to visualize the complex. The primary antibodies were ACTH C-terminal-specific antiserum Kathy, used at a final dilution of 1:2,000,000 (Schmabel et al., 1989) and α-MSH antiserum Wanda, used at 1:50,000 (gifts of R. E. Mains, Johns Hopkins University School of Medicine, Baltimore, MD).

Pituitary Biosynthetic Labeling and Analysis of POMC-Derived Peptides

Immediately after decapitation of mice, pituitaries were removed and rinsed with 20 vol of serum-free DMEM medium without methionine (starvation medium) containing 2 mg/ml FAF-BSA. The tissues were then labeled with 35S-methionine (2 μCi/ml in starvation medium, Amersham, 1000 Ci/mmol) for 3 hr, followed by another 3 hr chase incubation in medium supplemented with nonradioactive methionine. To ensure a continuous supply of 35S-methionine during the pulse labeling period, the labeling medium was replaced once. Pituitary tissues were extracted with 5 N acetic acid containing 2 mg/ml FAF-BSA, frozen and thawed three times, and sonicated. After brief centrifugation, the supernatants were lyophilized and resuspended in Lib buffer. Chase media were diluted with the same buffer. POMC-related peptides were immunoprecipitated with an antiserum against the N-terminal residues of ACTH (gift of Dr. Dick Mains, Johns Hopkins) and protein A-Sepharose. Immunoprecipitated materials were separated on SDS-PAGE tube gels, sliced, and radioactivity in each slice estimated by scintillation counting.

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