Dexamethasone Rapidly Induces a Novel Ras Superfamily Member-related Gene in AtT-20 Cells* (Received for publication, November 7, 1997) Robert J. Kemppainen‡ and Ellen N. Behrend From the Department of Physiology and Pharmacology, Auburn University College of Veterinary Medicine, Auburn, Alabama 36849

Differential display was used to identify a new Ras superfamily gene (Dexras1) induced by dexamethasone (Dex) in AtT-20 cells. Treatment of AtT-20 cells with Dex for 30 min resulted in increased mRNA for Dexras1; the highest concentrations appeared after 2 h of treatment. The gene was also identified in mouse heart, brain, liver, and kidney and furthermore was induced in these tissues after Dex treatment. The deduced protein shows regions of homology characteristic of members of the Ras superfamily of small GTPases. Highest homology (36% identity, 57% positives) was found with human Rap-2b, followed closely by a number of other Ras subfamily members, suggesting that Dexras1 is probably a member of the Ras subfamily of GTPases (members include Ras and Rap). Dexras1 is the first Ras superfamily member identified that is induced in response to steroids. The function of this gene is unknown; however, its wide distribution and rapid induction by Dex suggests the possibility of a role in glucocorticoid action in a variety of tissues.

The intracellular mechanism mediating glucocorticoid inhibition of stimulated corticotropin (ACTH) release from the corticotropes in the anterior pituitary gland in the early time domain is unknown. Exposure of corticotropes to glucocorticoids for periods ranging from approximately 10 min to 3 h (early time domain) inhibits ACTH secretion induced by a variety of secretagogues, including corticotropin-releasing hormone and arginine vasopressin (1). Results of several studies indicate that the inhibitory effect in this time domain is mediated through induction of new protein synthesis, because this suppression can be blocked by inhibitors of transcription and translation (1, 2). Several proteins have been proposed as mediating feedback (1); however, it is presently unclear if they are involved in the process.

Differential display is a method for identifying and cloning induced genes (3). The method involves synthesis of cDNA from mRNA by reverse transcription followed by PCR amplification of 3′-termini of the cDNA fragments using combinations of downstream (oligo(dT)) and upstream arbitrary primers. The labeled, amplified fragments are separated on polyacrylamide gels, and induced fragments are identified by comparison with bands originating from RNA isolated from noninduced cells or tissues.

In an attempt to identify the gene(s) and protein(s) mediating early feedback, we used differential display to isolate dexamethasone (Dex)-induced genes in AtT-20 cells. These mouse-derived corticotroph tumor cells have been shown to be an appropriate model for study of feedback regulation of ACTH secretion (2, 4).

EXPERIMENTAL PROCEDURES

Differential Display—Total RNA was collected from AtT-20/D16–16 cells cultured using standard methods (4). Cells were cultured in 75-cm² flasks and treated either with Dex at 100 nM for 2–24 h or with an equivalent volume of ethanol (vehicle). RNA was collected using phenol/guanidine isothiocyanate (TRIzol, Life Technologies, Inc.) and was treated with DNase I to remove chromosomal DNA. Differential display was performed using the RNAimage system (GenHunter, Nashville, TN) using combinations of three one-base-anchored oligo(dT) primers (-G, -A, and -C) and 80 upstream primers for PCR. 32P-Labeled PCR products were separated on 6% denaturing polyacrylamide gels and subjected to autoradiography.

Isolation and Cloning of Induced Genes—Unique cDNAs originating from Dex-treated cells were cut from the dried gel, eluted from the blotting paper by boiling in water, and precipitated and washed using sodium acetate and ethanol. The cDNAs were reamplified by PCR using the same set of primers and cloned into the pCR-TRAP plasmid (GenHunter).

cDNA Library Construction and Screening—A directional cDNA library was constructed in the AZipLoox vector (Life Technologies, Inc.) using poly(A)+ RNA obtained from AtT-20 cells treated for 2 h with 100 nM Dex. The library was screened using standard hybridization methods with random prime labeled cDNA.

Northern Analysis—Northern blots were performed using 2–5 µg of poly(A)+ RNA (Poly(A)Tract System, Promega, Madison, WI) loaded onto formaldehyde containing agarose gels. RNA was transferred to charged nylon membranes (Ambion Inc., Austin, TX) by capillary transfer. Membranes were hybridized (42 °C) with random prime labeled cDNA for 12–16 h and then washed with 1 × saline sodium citrate with 0.1% SDS twice for 15 min at 25 °C, followed by two washes with 0.25 × saline sodium citrate containing 0.1% SDS for 20 min at 60 °C. Autoradiographs were prepared exposing membranes to BioMax MR film (Eastman Kodak Co., Rochester, NY) for 0.5–4 days.

Sequencing—The gene was sequenced using Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science).

Mice—Male Hsd:ICR mice (30–35 g) were injected intraperitoneally with Dex (10 µg/mouse) or an equivalent volume of vehicle. 1 h later, mice were anesthetized with sodium pentobarbital and killed by exanguination. Liver, heart, brain (excluding pituitary), and kidneys were collected and immediately frozen in liquid nitrogen. RNA was obtained as described above for Northern analysis.

RESULTS

Differential Display—One cDNA was identified using differential display (from screening 240 primer combinations) that was strongly and consistently induced by Dex (replicated in three experiments) in AtT-20 cells (Fig. 1). The induced band was excised from the gel and reamplified by PCR using the same primer set, and the resulting band (approximately 260 base pairs) was cloned into pCR-TRAP. Ten clones were par-
Differentially sequenced and were found to represent three different cDNAs. Representative cDNAs from each clone type were excised from the plasmid and used to probe Northern blots containing poly(A)\(^+\) RNA from vehicle and Dex-treated AtT-20 cells. Hybridization with one of the three cDNAs identified a 1.6-kilobase pair band strongly induced by Dex (Fig. 2A).

Identification and Sequencing of Dexras1—The Dex-induced 3'-cDNA fragment was used to screen a phage cDNA library made using RNA obtained from Dex-treated AtT-20 cells. Several positive clones were identified, and two were selected for sequencing. The nucleotide sequence and deduced protein sequence are shown in Fig. 3. Comparison of the deduced protein (Dexras1) to the Swiss Protein data base using gapped BLAST analysis (5) showed the highest homology to a variety of Ras superfamily members. The highest homology was to human Rap-2b (36% identity, 57% positives, over 180 amino acids) followed closely by a large number of Ras-related proteins. The deduced Dexras1 protein shows several conserved regions specific to members of the Ras superfamily. Alignment analysis (Fig. 4) of Dexras1 with human Rap-2b and human R-Ras (37% identity, 54% positives, over 172 amino acids) showed the highest homology over a core of amino acids, extending approximately from amino acids 28 to 188 of Dexras1 between the three proteins. At a predicted length of 280 amino acids, Dexras1 is considerably longer than Rap-2b or R-Ras (and most other Ras subfamily members).

Tissue Distribution and Dex Induction of Dexras1—To evaluate distribution of the gene, tissues were collected from mice treated with Dex or vehicle. Northern analysis showed Dexras1 expression in multiple tissues and strong mRNA induction following Dex treatment (Fig. 2B). A time course study (Fig. 2C) using poly(A)\(^+\) RNA from AtT-20 cells treated with Dex for varying periods showed that the steroid induced Dexras1 mRNA within 30 min of exposure (earliest time studied) with the highest induction occurring at 2 h. The Dexras1 signal gradually diminished over the period from 4 to 24 h, despite the continued presence of Dex. A final experiment (data not shown) determined if the gene is transcriptionally induced by Dex. Pretreatment of AtT-20 cells with an inhibitor of transcription (5,6-dichlorobenzimidazole riboside), but not with an inhibitor of translation (puromycin) (2, 4), totally blocked the ability of Dex to induce Dexras1 mRNA.

DISCUSSION

Differential display was used to identify a new Ras superfamily member, Dexras1, which was rapidly induced by glucocorticoids (Dex) in AtT-20 cells. The induction appears to be mediated at the transcriptional level. The gene is expressed in...
FIG. 4. Alignment analysis (Clustal method, Lasergene Software, DNASTAR Inc.) of Dexras1, human Rap-2b (GenBank™ accession number P17964), and human R-Ras (GenBank™ accession number P10301). The boxed groups show amino acids identical across the three proteins. H, human.

several tissues in mice, and interestingly, its expression appears to be regulated positively by Dex in these tissues.

The Dexras1 gene is, to our knowledge, the first member of the Ras superfamily that is induced in response to glucocorticoids specifically or steroids in general. A relatively newly described subfamily within the Ras superfamily consisting of Rad, Gem, Kir, and Rem is unique in that its members are also transcriptionally regulated (6, 7). For example, the Gem gene was induced by mitogen treatment of T cells (6), whereas Rem expression was repressed in several tissues in mice following lipopolysaccharide treatment (7). The activity of most Ras proteins is modified by other regulatory proteins including guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), which enhance the release of GDP bound to Ras and the rate of GTPase hydrolysis, respectively (8). Because Ras proteins act as molecular switches, active when GTP is bound and inactive when GDP is bound, GEFs and GAPs serve important roles in controlling Ras protein activity (8, 9). It is apparent from data related to the Rad/Gem/Kir/Rem subfamily and now Dexras1 that an additional level of regulation is present for some Ras superfamily members. Specifically, extracellular signals can alter the expression of genes for these molecules and likely affect intracellular concentrations of their proteins. In addition, regulatory proteins such as GEFs and GAPs likely exist to affect their activity, as well (10).

The deduced structure of the Dexras1 protein contains several characteristic Ras superfamily motifs, including the phosphate/magnesium binding regions GXXGXXGK(S/T) (the P-loop) and three C-terminal amino acids (11, 12). This process is necessary for membrane localization of many Ras proteins (12, 13). The Ras superfamily of small GTPases currently consists of six subfamilies: Ras, Rho, Rab, Ran, ARF, and Rad/Gem/Kir (7, 13). Rap is considered a member of the Ras subfamily (13). By virtue of motif and BLAST comparison, Dexras1 appears most closely related to members of the Ras subfamily. However, the predicted length of the Dexras1 protein (280 amino acids) is longer than most Ras subfamily members (180–220 amino acids) (13), and amino acid differences exist between Dexras1 and typical Ras subfamily members within some of the GTPase characteristic motifs (e.g., in regions G-2 and G-3) (9, 11). Interestingly, members of the other transcriptionally regulated GTPases, the Rad/Gem/Kir/Rem subfamily, also code for larger proteins (295–310 amino acids) (6, 7).

Classically, Ras proteins are thought to act to link signals from receptor tyrosine kinases to a variety of intracellular processes controlling proliferation and differentiation (13, 14). Other data support an even wider possible range of Ras effectors (14). Rap proteins, for example, have been identified tightly associated with the cytoplasmic face of secretory vesicles in rat parotid gland, suggesting a role for these proteins in vesicular transport and exocytosis (15). Glucocorticoids have myriad effects on diverse processes, affecting activity of virtually all tissues in the body (16). Because Dexras1 appears to be rapidly induced by glucocorticoids in several body tissues, it will be of interest to determine how and where this gene participates in glucocorticoid action.

In summary, a new Ras superfamily small GTPase gene, Dexras1, has been identified in AtT-20 cells. The gene is induced in these cells and in several tissues in mice in response to Dex. Dexras1 is the first member of the Ras superfamily induced by steroids or glucocorticoids. Roles for this gene in pituitary cells or other tissues in mediating the effects of glucocorticoids await investigation. In particular, we are interested in investigating whether Dexras1 is involved in negative feedback regulation of pituitary ACTH secretion. Thus far, the only evidence supporting such a role is that the mRNA for Dexras1 is induced rapidly in AtT-20 cells following Dex treatment, in a time frame consistent with the appearance of early glucocorticoid inhibition of stimulated ACTH secretion.

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