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Androgen regulation of the human FERM domain encoding gene *EHM2* in a cell model of steroid-induced differentiation

Sanjay Chauhan^a, Ritu Pandey^b, Jeffrey F. Way^a, Thomas C. Sroka^c, Manolis C. Demetriou^c, Susan Kunz^a, Anne E. Cress^{c,d,e}, David W. Mount^{b,e}, and Roger L. Miesfeld^{a,e,*}

^aDepartment of Biochemistry and Molecular Biophysics, University of Arizona, Tucson, AZ 85721, USA

^bDivision of Bioinformatics, The Arizona Cancer Center, University of Arizona, Tucson, AZ 85721, USA

^cCancer Biology Graduate Program, University of Arizona, Tucson, AZ 85721, USA

^dDepartment of Cell Biology and Anatomy, University of Arizona, Tucson, AZ 85721, USA

^eDepartment of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721, USA

Abstract

We have developed a cell model to investigate steroid control of differentiation using a subline of HT1080 cells (HT-AR1) that have been engineered to express the human androgen receptor. Dihydrotestosterone (DHT) treatment of HT-AR1 cells induced growth arrest and cytoskeletal reorganization that was associated with the expression of fibronectin and the neuroendocrine markers chromogranin A and neuron-specific enolase. Expression profiling analysis identified the human FERM domain-encoding gene *EHM2* as uniquely induced in HT-AR1 cells as compared to 16 other FERM domain containing genes. Since FERM domain proteins control cytoskeletal functions in differentiating cells, and the human *EHM2* gene has not been characterized, we investigated *EHM2* steroid-regulation, genomic organization, and sequence conservation. We found that DHT, but not dexamethasone, induced the expression of a 3.8 kb transcript in HT-AR1 cells encoding a 504 amino acid protein, and moreover, that human brain tissue contains a 5.8 kb transcript encoding a 913 amino acid isoform. Construction of an unrooted phylogenetic tree using 98 FERM domain proteins revealed that the human *EHM2* gene is a member of a distinct subfamily consisting of nine members, all of which contain a highly conserved 325 amino acid FERM domain.

Keywords

FERM domain; Band 4.1; HT1080; Dihydrotestosterone; Chromogranin; Fibronectin; Neuroendocrine; EPB41L4B; Yurt

Epithelial cell migration from one region of the embryo to another is a central theme in developmental biology and numerous genetic mutants affecting these processes have been identified [1]. Moreover, the reorganization of intracellular cytoskeletal structures, and

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*Corresponding author. Fax: 1-520-621-1697. E-mail address: RLM@u.arizona.edu (R.L. Miesfeld).

Note added in proof

Genotyping of HT-AR1 cells and an HT-1080 cell stock obtained directly from American Type Culture Collection showed that the two cell lines match at 11/11 loci, thereby confirming that the HT-AR1 and HT-1080 cell lines are isogenic (AEC and RLM, unpublished data).

mechanisms of cell attachment to extracellular matrices, are among the many epithelial cell processes that can be investigated using cancer cell line models [2,3]. Proteins involved in mediating cell migration and attachment have been identified using molecular genetic and biochemical approaches and many of these proteins contain highly conserved protein interaction domains. One such family of proteins are those containing a FERM (Four.1 protein, ezrin, radixin, moesin) domain which functions as a protein docking surface with the cytosolic tail of transmembrane proteins such as CD44 [4].

Based on sequence similarities, FERM domain proteins have previously been divided into five subfamilies [5] which have a conserved molecular structure consisting of three subdomains that form a cloverleaf with lobes of ~100 amino acids each (F1, F2, and F3) [6–10]. The F3 domain has been shown to be important in target protein binding [11] and is the site of intramolecular regulation by the C-terminal domain of some FERM proteins [7]. Activation of some FERM proteins can occur by phosphorylation of threonine residues in the C-terminal domain [12], or through binding of phosphoinositides to the FERM domain [13], both of which alter the intramolecular folding of the protein and expose target protein binding sites in the FERM domain [14]. Most FERM domain proteins contain one or more functional components in the C-terminal half of the protein that encode an actin or spectrin binding domain [4], a protein tyrosine phosphatase activity [15], or even a Rho GEF function [16]. Activated ezrin, for example, binds to the cytosolic tail of the membrane protein CD44 through its FERM domain, and to actin molecules through an actin binding domain, thereby tethering actin filaments to the plasma membrane [17].

We are interested in understanding how steroid receptors control cell-specific responses through modulation of intracellular signal transduction pathways and have developed a molecular genetic model to investigate androgen signaling in the human cancer cell line HT1080 [18]. The human HT1080 cell line expresses endogenous glucocorticoid receptor (GR), but not the closely related androgen (AR), progesterone (PR) or mineralocorticoid (MR) receptors [19], all of which have the potential to regulate similar genes through a highly conserved DNA binding domain [20]. Importantly, it has been shown that glucocorticoid treated HT1080 cells undergo a well-characterized morphological change in culture that involves increased fibronectin expression [21–23] and alterations in matrix attachment properties [24]. Based on our earlier studies of AR and GR signaling in cell lines derived from mouse thymocytes [25], rat hepatocytes [26], and rat prostate epithelial cells [27,28] suggesting that some, but not all, steroid responses are shared between AR and GR, we wondered if ectopic expression and ligand activation of human AR in HT1080 cells would elicit the same cytoskeletal reorganization observed with activated GR [21–24].

We report here that sublines of HT1080 cells expressing physiological levels of AR and GR are growth arrested by treatment with dihydrotestosterone (DHT) but not with dexamethasone (Dex). More importantly, we found that DHT-treatment induced a rapid alteration in cell morphology characterized by cytoskeletal reorganization and expression of neuroendocrine differentiation markers. Based on the proposed role of FERM domain proteins in mediating such cytoskeletal changes in differentiating cells [17], we analyzed DHT-regulated expression of known FERM domain encoding genes and identified the human *EHM2* gene as a uniquely induced early response gene in HT-AR1 cells. Finally, since the genomic organization and expression of the human *EHM2* gene have not been previously characterized, we performed bioinformatics analysis and found that *EHM2* is exceptional amongst FERM domain containing genes in both its androgen-regulated profile and distinct 325 amino acid FERM domain which is highly conserved in humans, flies, and worms.

Experimental procedures

Generation of HT1080-derived HT-AR cell lines

The human AR cDNA expression vector pDC-hAR-PAC was made by subcloning a 3.1 kb *Bgl*III–*Bam*HI fragment from pCMV5-hAR [29] into the *Bam*HI site of the pBluescript SK phagemid (Stratagene, La Jolla, CA). The AR cDNA insert from the pBSK+ plasmid was then excised using *Not*I–*Xho*I and subcloned into pDC-PAC, a derivative of pDC-Hyg [26] to create pDC-hAR-PAC. HT1080 cells were obtained from Dr. Ray Nagle and stably transfected with pDC-hAR-PAC or the empty vector pDC-Puro using DOTAP cationic lipid (Avanti Polar Lipids, Alabaster, AL) as described [26]. Puromycin-resistant HT1080 sub-clones were isolated after 10–14 days in selection media which were made by first combining DMEM with defined calf bovine serum (CBS) (Hyclone, Logan, Utah) to 10% (v/v), streptomycin (Sigma Chemical, St. Louis, MO) to 1% (w/v), penicillin G (Sigma) to 1% (w/v), and 0.3 µg/ml puromycin (Sigma), and then filter sterilized with a 0.22 µm filter membrane. Puromycin-resistant subclones were characterized using a DHT-dependent transactivation assay based on a mouse mammary tumor virus reporter plasmid containing the firefly luciferase gene (MMTV-Luc) [27]. Expression of functional AR protein in selected HT-AR subclones was confirmed by Western blotting using an anti-AR antibody. HT-AR cell lines were cultured in puromycin selection media 5 days prior to performing experiments in 10% CBS or 5% C/S CBS DMEM culture media.

Cell proliferation analysis

Cell proliferation was measured by plating HT1080-derived sublines in culture media in six-well plates (Falcon) at 10,000 cells/well 4 h prior to adding dihydrotestosterone or dexamethasone at the indicated concentrations. Triplicate wells were used for each data point and cells were harvested at 3, 5, or 7 days after hormone treatment. Cells were washed with PD, trypsinized, and stained with trypan blue, and viable cells were counted using a hemacytometer (Fischer Scientific).

Microscopy

Cells were seeded at high (40,000/dish) or low (1200/dish) density on a Delta T dish (0.15 mm; Bioprotech, Butler, PA) and grown overnight in DMEM selection media containing 10% normal calf serum or charcoal stripped (C/S) calf serum at 37 °C. The following morning, fresh media were added with or without DHT (10^{-6} M) and the cells were incubated for 1 h at 37 °C. Images were captured using a grayscale CCD camera (ORCA-100, Hamamatsu, Japan) mounted on an inverted Olympus IMT2 microscope (Olympus America, Melville, NY) equipped with a Bioprotech Delta T live cell system (Bioprotech, Butler, PA) under a humidified (5% CO₂ balanced with ultrazero air) atmosphere.

Immunocytochemical localization

Detection of protein expression in HT-AR1 cells by immunostaining was performed using antibodies specific for cytokeratin 5 (mouse monoclonal antibody RCK 103, 1:10 dilution, BD PharMingen). For these experiments, cells were grown on 12 mm coverslips coated with human fibronectin, allowed to adhere overnight, and then supplemented with fresh media ± DHT. After 4 days, the coverslips were harvested and dipped in PBS followed by fixation in chilled 100% methanol for 10 min. The coverslips were subsequently dipped six times in cold acetone and air-dried. Diluted primary antibodies were applied to cells for 30 min at room temperature and then the coverslips were washed three times in PBS for 5 min each. Primary antibodies were detected with anti-rabbit Alexa 568 (Molecular Probes, Eugene, OK) using dilution 1:400 and the same incubation and wash procedure. Cell nuclei were stained with Hoechst 33258 (Sigma) using a stock of 2 mg/ml diluted to 1:100 for use, rinsed twice in PBS for 2–3 min

each, and post-fixed in 100% ethanol, and mounted using Mowial (Sigma). Stained cells were visualized using appropriate filters on a Zeiss Axiophot 200 microscope and imaged using Axiovision software.

Membrane blotting

Northern blots were performed using total RNA that was isolated from untreated and DHT treated HT-AR1 cells (4, 12, 24, and 48 h) using the RNA-Bee™ RNA isolation reagent (Tel-test, Friendswood, TX, USA). Human RNA from various tissues was obtained from BD Biosciences (Clontech, Palo Alto, CA). Equal amounts of RNA (20 µg) from HT-AR1 cells or human tissues were run on denaturing formaldehyde gels and transferred to Duralon-UV membranes (Stratagene, La Jolla, CA) by standard capillary methods using 10× SSC. Subsequently, RNA was UV cross-linked to the membrane using a UV Stratalinker (Stratagene) instrument. Prime-a-Gene labeling system (Promega) was used to radioactively label fibronectin or *EHM2* cDNA probes according to manufacturer's instructions. Membranes were hybridized in 50% deionized formamide, 10% dextran sulfate, 1% (w/v) SDS, 1 M NaCl, and 100 µg/ml denatured salmon sperm DNA at 42 °C for 16 h using a Gene Roller hybridization chamber (Savant Instruments, Holbrook, NY). Blots were washed twice at 42 °C in 2× SSC, 0.1% SDS, and twice at 42 °C in 0.1 × SSC, 0.1% SDS.

Western blots were performed using protein isolated from cells that were lysed on the plate with RIPA buffer (150 mM NaCl, 50 mM Tris, 5 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) deoxycholate, and 0.1% (w/v) SDS, pH 7.5) plus protease inhibitors (PMSF, 2 mM; leupeptin and aprotinin, 1 µg/ml). The harvested lysate was briefly sonicated on ice and protein concentration was determined by the BCA assay (Pierce, Rockford, IL). Each sample was prepared by adding 20 µg of protein with equal volume of 2× reducing sample buffer. Samples were boiled for 5 min and after a quick chill on ice they were loaded onto 8% or 12% SDS–PAGE gels. Following electrophoresis, proteins were electrotransferred to Millipore Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) and incubated with antibodies specific for androgen receptor (Santa Cruz Biotechnology C-19), fibronectin (Accurate Chemical and Scientific Corporation), α-tubulin (Sigma B512), chromogranin A (NeoMarkers LK2H10) or neuron-specific enolase (NeoMarkers E27). Secondary antibodies used in Western blotting were conjugated to horseradish peroxidase and visualized by chemiluminescence (ECL Western Blotting Detection System, Amersham, Arlington Heights, IL, USA).

Expression profiling

Total RNA was isolated from untreated and DHT-treated HT-AR1 cells using the RNA-Bee™ RNA isolation reagent (Tel-test, Friendswood, TX, USA). Fluorescently labeled cDNA was prepared from 30 µg of total RNA using Micromax direct cDNA microarray system (NEN Life Science Products, Boston, MA) and Cy3 (control) or Cy5 (experimental) fluorescent dyes. Labeled cDNA was purified using Qiagen QIAquick PCR purification kit (Qiagen, Valencia, CA), combined into one tube, and precipitated at –20 °C overnight using 3 M sodium acetate (pH 5.2) and isopropanol. Expression profiling analysis using a human cDNA microarray slides containing 5300 human gene sequences obtained from the Microarray Core Service of Arizona Cancer Center (<http://www.azcc-microarray.arl.arizona.edu>) was performed as previously described [30]. Slides were scanned for Cy3 and Cy5 fluorescent emission using Gene Pix 4000A microarray Scanner and analyzed by Gene Pix Pro 4.0 software (Axon Instruments). Cluster and TreeView analysis [31] was used to analyze the fluorescence intensities and to format the intensity data as log-transformed ratio values. The Arizona Cancer Center BioResource for Gene Array analysis (Biorag) was used for initial screening of differentially expressed genes to identify candidates most likely involved in cytoskeletal functions. This online resource can be accessed at <http://www.biorag.org>.

RT-PCR amplification of EHM2 fragment

Poly(A)⁺ RNA was isolated from HT-AR1 cells treated with DHT (10⁻⁶ M) for 48 h using poly(A)Ttract mRNA isolation system III (Promega, Madison, WI). Complementary DNA was prepared using random primer mix and SuperScript II RNase H-Reverse Transcriptase in 20 µl reaction mixture according to supplier's protocol (Invitrogen, Carlsbad, CA). PCR was performed using a human *EHM2* primer set (5'-AGGATTGTTTGATCAGATTG-3' and 5'-AATGGAAGAGAGATGCTTC-3') based on GenBank sequences shared by the sequence files AF153416, AF153418, and AB032179 using standard conditions (hot start; 3 min at 94 °C, 1 min at 94 °C, 1 min at 55 °C, and 1 min at 68 °C for 34 cycles). The *EHM2* PCR product was visualized by electrophoresis on a 0.8% agarose gel stained with ethidium bromide.

Analysis of protein families

BlastP searches were performed against the GenBank protein database to identify proteins that are similar to Band 4.1 family. Proteins and domains were aligned using ClustalW [32], edited with Genedoc (<http://www.psc.edu/biomed/genedoc/>), and an unrooted phylogenetic tree was constructed by the distance method using the neighbor-joining algorithm implemented in the program Neighbor in the PHYLIP (3.5) package [33]. The Dayhoff PAM model of protein evolution was used to compute the distances between the sequences [34] using the PROTDIST program. This analysis allowed the identification of the most similar protein sequences in the same or different organism based upon protein sequence similarity in the multiple sequence alignment. Assignments of likely orthology were based upon the presence of a strongly supported cluster of highly similar sequences from diverse organisms. A Bootstrap analysis was performed using PHYLIP to assess how well the branch patterns were supported in the predicted phylogenetic tree. This method resampled columns in the multiple sequence alignment to generate 500 new alignments, each of which was used to produce a new tree. The number of alignments that support each branch pattern in the tree was then assessed and is reported in Fig. 6. When a clear majority of bootstrap trees (>70%) were in agreement, support was considered to be good. In many cases, bootstrap support was excellent, in the 95–100% range.

Results

DHT treatment of HT-AR1 cells induces a neuroendocrine cell differentiation phenotype

To take advantage of the well-characterized steroid response in HT1080 cells that had previously been studied using the glucocorticoid analog dexamethasone (Dex) [21–24], and to test the possibility that AR and GR signaling pathways in HT1080 cells may be partially overlapping, we stably integrated human AR cDNA into HT1080 cells using the eukaryotic expression vector pDC-PAC. Puromycin-resistant sublines were isolated that expressed near physiological levels of AR protein (HT-AR1 and HT-AR2) based on Western blots that included a protein sample from the human prostate cell line LNCaP [35] (Fig. 1A). A puromycin-resistant vector-only control subline (HT-VC1) was also isolated, which like the parental HT1080 cell line [19], did not express AR protein. Steroid treatment of these HT1080 sublines with Dex or DHT showed that while the growth of HT-VC1 cells was not effected by either DHT or Dex (Fig. 1B), growth of both the HT-AR1 and HT-AR2 cell cultures was >90% inhibited (Figs. 1C and D) by DHT, but not Dex, treatment. This differential steroid response was not due to the levels of functional AR and GR protein in the HT-AR1 and HT-AR2 cell lines, since transactivation assays using a mouse mammary tumor virus reporter gene showed that both cell lines responded similarly to Dex and DHT (SK and RM, unpublished data). Based on the similar level of AR protein in HT-AR1 cells and the well-characterized LNCaP human prostate cell line (Fig. 1A), we chose to use the HT-AR1 cell line for all subsequent studies.

Dexamethasone treatment of HT1080 cells has been shown to induce high levels of fibronectin expression [21–23] without altering cell proliferation rates or cell morphology [21]. We observed a stark difference between the effect of Dex and DHT on the growth of AR expressing HT1080 sublines (Figs. 1C and D) and therefore determined if DHT treatment led to differences in the steady-state levels of fibronectin RNA and protein. As shown in Fig. 1E, fibronectin RNA levels were significantly increased after 24 and 48 h of DHT treatment as measured by Northern blots. Western blots using an anti-fibronectin antibody showed a coinciding increase in fibronectin protein levels in DHT treated HT-1080 cells (Fig. 1F). These data indicated that the shared induction of fibronectin expression by GR [21–23] and AR (Fig. 1F) in HT-AR1 cells was likely to be distinct from the observed AR-specific growth arrest phenotype (Fig. 1D).

During the course of these experiments, we noticed that DHT treatment of HT-AR1 cells led to the appearance of neuronal-like membrane structures, suggesting that the growth arrest phenotype in HT-AR1 cells may involve activation of a differentiation pathway. As shown in Fig. 2, DHT treatment of HT-AR1 cells cultured in normal serum resulted in the appearance of multiple extensions protruding from the plasma membrane (panel b). In addition, when HT-AR1 cells were cultured in charcoal-stripped (C/S) serum, cell surface structures resembling secretory granules were readily apparent in the DHT-treated cultures (panel d). To better visualize DHT-dependent changes in the cytoskeleton, we cultured the cells on coverslips coated with fibronectin and performed immunostaining with an anti-keratin 5 antibody and Hoechst dye. As can be seen in panel f of Fig. 2A, the organization of keratin networks was also dramatically altered in HT-AR1 cells, suggesting that androgens activate a signaling pathway that controls cytoskeletal organization in these cells. Finally, as shown in Fig. 2B, Western blotting experiments using antibodies directed against the neuronal-specific proteins chromogranin A (CgA) and neural specific enolase (NSE) revealed that HT-AR1 cells constitutively express both of these proteins (Fig. 2B), and moreover, that DHT treatment increases CgA and NSE protein levels relative to that of tubulin (Fig. 2B). Both CgA and NSE are considered neuroendocrine cell markers that have been shown to be expressed in numerous tissues including prostate [36].

Although the parental HT1080 cell line had been originally described as an outgrowth of an undifferentiated fibrosarcoma isolated from the acetabulum of a 35-year-old male [18], and it is often compared to fibroblast-derived cell lines [37] and normal human fibroblasts [22], the results presented in Fig. 2 suggest that the AR-mediated growth arrest and neuronal differentiation phenotype of HT1080 cells was similar to reconstituted human prostate cancer cell lines that stably express AR cDNA [38–41]. Indeed, except for a rat prostate epithelial cell line we generated that expresses integrated AR cDNA [28], this striking phenotype has not been observed in other AR-expressing cell lines we have characterized, including a rat hepatocyte cell line [28], a mouse thymoma cell line [25], or Rat-1 and HeLa cells that were transfected with AR cDNA [26]. Taken together, these data suggest that the differentiated state of HT1080 cells may be more complex than that of a transformed fibroblast, and in fact, may be indicative of a neuroendocrine, or even prostatic, origin based on its unusual sensitivity to reconstituted androgen signaling (see Discussion).

DHT-induction of EHM2 expression in HT-AR1 cells

FERM domain proteins are often associated with the type of cytoskeletal reorganization seen in HT-AR cells [17]. To determine if altered expression of FERM domain containing genes was associated with cell differentiation in this model, we used a 5k human cDNA array to analyze androgen-regulated genes in DHT-treated HT-AR1 cells. This array contains cDNA sequences of 17 human FERM domain genes (see Experimental procedures) which we analyzed using the Cluster and TreeView algorithms as shown in Fig. 3. The only FERM

domain gene that displayed a pattern of expression indicative of a primary AR target gene in these cells was *EHM2*, a recently described FERM domain encoding gene that was initially identified as an ortholog of the mouse *EHM2* gene [42,43]. Interestingly, the *Drosophila* ortholog of *EHM2*, Yurt, has been shown to be required for epithelial cell migration during embryonic development, and loss of function Yurt mutations are embryonic lethal [44].

To validate DHT-induction of *EHM2* expression, and to determine which of six GenBank predicted alternatively spliced *EHM2* transcripts were expressed in HT-AR1 cells, we used RT-PCR to isolate a 1260 bp fragment of *EHM2* cDNA encompassing the FERM domain region. Fig. 4 shows results of Northern blots using this human *EHM2* cDNA probe to analyze the same RNA samples used in the expression profiling analysis. These data reveal that a major 3.8 kb *EHM2* transcript was rapidly induced in DHT-treated HT-AR1 cells, and moreover, the pattern of *EHM2* expression over 48 h matches what was seen in the cluster diagram (Fig. 3). The Northern blot data in Fig. 4A also showed that a second *EHM2* transcript of 2.3 kb was upregulated by androgens in HT-AR1 cells. Finally, we determined if *EHM2* expression in HT-AR1 cells was DHT-specific, since DHT, but not Dex, causes growth inhibition (Fig. 1) and induction of a neuronal cell-like phenotype (Fig. 2). The results of Northern blot analysis shown in Fig. 4B demonstrate that *EHM2* expression is AR-dependent in HT-AR1 cells, suggesting that this transcriptional response is associated with the differentiation phenotype, rather than a general response to steroid treatment.

Expression of *EHM2* transcripts in human tissues

Shimizu et al. [43] performed tissue expression analysis of the mouse *EHM2* gene and found that a major 3.8 kb transcript was expressed in mouse liver cells, with lower amounts in lung and kidney cells. *EHM2* expression was not detected in adult mouse heart, brain, spleen, skeletal muscle or testis in their study [43]. To analyze expression of the human *EHM2* gene, we used total RNA isolated from human prostate, breast, brain, heart, testes, spleen, and liver cells to analyze steady-state levels of *EHM2* transcripts by Northern blotting as shown in Fig. 5. Expression of the 3.8 kb *EHM2* transcript was highest in testes with much lower amounts in prostate and breast tissue. All three of these tissues also contained the 2.3 kb *EHM2* transcript seen in DHT-treated HT-AR1 cells (Fig. 4). Surprisingly, human brain tissue contained very high levels of a 5.6 kb *EHM2* transcript that was not seen in mouse brain [43].

Bioinformatics analysis of the *EHM2* locus (EPB41L4B) on chromosome 9 using Genome Browser (<http://www.genome.ucsc.edu>) and AceView (<http://www.ncbi.nlm.nih.gov>) suggested that the three *EHM2* transcripts identified by Northern blotting encode two *EHM2* protein isoforms. As diagrammed in Fig. 5B, the 5.6 kb brain-specific transcript corresponds to the 5584 bp GenBank sequence AF153416, encoding a protein of 913 amino acids, whereas, the 3.8 and 2.3 kb transcripts represent the GenBank sequences AF153418 and AB032179, respectively. The 3.8 and 2.3 kb *EHM2* transcripts differ only in the length of the 3' UTR and encode the same 504 amino acid protein. Importantly, the 5.6 kb *EHM2* transcript AF153416 contains the same open reading frame sequence for the first 504 amino acids as AF153418 and AB032179, however, the carboxy terminal region includes an additional 409 amino acids of unknown function.

Identification of a distinct *EHM2* family within the band 4.1 superfamily

To understand the evolutionary relationship between the *Drosophila* Yurt protein and other Band 4.1 proteins, Hoover and Bryant [44] performed a limited phylogenetic tree analysis using the full-length sequence of 19 FERM domain proteins. Their analysis suggested that *EHM2*, Yurt, and a FERM domain protein from *Caenorhabditis elegans* (T04C9) constitute a subfamily that is most closely related to NBL4 proteins as originally proposed by Shimizu et al. [43]. Since their study included only a limited number of the nearly 100 FERM domain

containing gene sequences in the GenBank database, and was based on comparisons using sequences outside of the FERM domain, we performed a much more extensive study to determine if EHM2-related proteins were indeed a separate subgroup. For our analysis we limited the comparison to a ~220 amino acid segment of the FERM domain identified in 98 protein sequences contained in the GenBank database. This segment encompasses the more highly conserved F1 and F2 lobes within the FERM domain protein structure [7]. These 98 protein sequences were analyzed with an unrooted phylogenetic tree that was constructed by the distance method using a neighbor-joining algorithm implemented the PHY-LIP (3.5) package [33].

As shown in Fig. 6, this phylogenetic tree analysis identified 11 separate FERM domain subfamilies, of which the two largest were ERM (ezrin/radixin/moesin) with 28 genes, and Band 4.1, with 14 genes. More importantly, it was clear from this analysis that the human *EHM2* gene belongs to a separate subfamily consisting of at least nine members from seven different species. Note that in addition to the human *EHM2* gene located on chromosome 9, an EHM2-related protein (Hs14916925) is encoded by the EPB41L5 gene on chromosome 2. *EHM2* orthologs of these two human genes were found in the mouse genome, one of which is Mn9506567 that is related to human *EHM2*, and the other is Mn13278193, the ortholog of the EHM2-related protein located on human chromosome 2. These data revealed that the genomes of *C. elegans* (Ce17553794), *Drosophila melanogaster* (Dm7299771), and *Anopheles gambiae* (AgEAA08568) encoded a single ancestral *EHM2*-related gene. The genomes of *Rattus norvegicus* (Rn27675822) and *Canis familiaris* (Cf8979743) appear to encode only the ortholog of the human *EHM2*-related gene (Hs14916925), however, the genomic sequence of these two species is incomplete at this time.

The data in Fig. 6 also show that we identified three minor FERM protein subfamilies represented by MIR (myosin regulatory light chain interacting protein) [45], CDEP (chondrocyte-derived ezrin-like domain containing protein) [46], and GRSP (general receptor for phosphoinositides) [47]. In addition, we found that the PTP proteins can be further subdivided into three separate subfamilies based solely on sequence differences within the ~220 amino acid core FERM domain. Interestingly, Andersen et al. [15] used phylogenetic tree analysis to compare sequences within the catalytic domain of 113 PTP genes in the public databases. They classified the PTP family of enzymes into nine non-transmembrane subtypes (PTP-NT) and eight receptor-like subtypes (PTP-R). Within the PTP-NT subtypes, three branches contained PTPs that encoded N-terminal FERM domain sequences named NT5, NT6, and NT7. These subfamilies are labeled PTP-NT5, PTP-NT6, and PTP-NT7 in Fig. 6 and are represented by the PTP proteins Hs131530, Hs5902032, and Hs16550298, respectively. The independent sorting of these PTP proteins into three subfamilies on the basis of sequences in the catalytic domain [15], or the FERM domain (Fig. 6), provides corroborative evidence to suggest that our phylogenetic tree analysis of the FERM domain protein family was complete.

Amino acid sequence comparisons of the EHM2 subfamily proteins we identified in the GenBank database were analyzed using ClustalW as shown in Fig. 7. These data shown that EHM2 proteins encode an extended F3 subdomain, which together with the F1 and F2 subdomains, constitutes a ~325 amino acid FERM domain spanning residues 70–407. The predicted positions of the F1, F2, and F3 lobes in human EHM2 are indicated in Fig. 7 based on alignment with FERM domain residues mapped to these regions in the molecular structure of ezrin [10], moesin [7], radixin [8], and merlin [9].

Discussion

In this report, we describe functional genomic studies of the human *EHM2* gene that we found by expression profiling using a 5k human cDNA array to be the most rapidly induced gene in

DHT-treated HT-AR1 cells. In light of new studies showing that FERM domain proteins modulate intracellular signaling pathways by directly activating target proteins [48–52], often through interactions with the more variable F3 subdomain [52], DHT-induction of *EHM2* expression in HT-AR1 cells could represent a possible initiating event in cell differentiation. Interestingly, expression analysis of other FERM domain proteins in HT-AR1 cells, including ezrin which has been reported to be androgen-regulated in the rat ventral prostate [53], did not show significant changes in transcript levels within the first 24 h following DHT treatment (Fig. 3). These results suggest that *EHM2* may have a unique role amongst FERM domain proteins in mediating neuroendocrine-like cytoskeletal changes associated with steroid-induced differentiation of HT1080 cells. Interestingly, the observed high level expression of the larger *EHM2* isoform in human brain tissue (Fig. 5) would be consistent with a role for *EHM2* family members in cytoskeletal functions in neuronal cells.

Our phylogenetic tree analysis using only the core FERM domain of nearly a hundred protein sequences revealed that *EHM2*-related proteins constitute a distinct subfamily, of which only two members have been characterized to date. One is the mouse *EHM2* gene which was found to be highly expressed in a mouse tumor model [42] and the other is the *Drosophila* Yurt gene [44]. Hoover and Bryant [44] used Yurt antibodies to analyze Yurt protein in wild-type and mutant *Drosophila* embryos. They found that Yurt was localized to the apical and lateral domains of the plasma membrane based on double-staining with phosphotyrosine and *Dlg* (Discslarge) antibodies. This membrane association would be consistent with other FERM domain proteins [17].

Unlike many FERM domain proteins that have been shown to encode additional protein functions in the C-terminal region [4], including an autoregulatory function that inhibits FERM domain activity [7], *EHM2* appears to encode only an extended FERM domain located 70 amino acids from the N-terminus (Fig. 7). Based on its small size and lack of recognizable protein motifs in the ~100 amino acid C-terminus of *EHM2*, it is possible that the *EHM2* protein functions as a constitutively active FERM signaling protein that is controlled by transcriptional regulation, rather than autoregulation involving intramolecular folding of subdomains. If this were the case, it might explain why *EHM2* expression is so tightly controlled by DHT in HT-AR1 cells, whereas most other FERM domain proteins were unaffected by steroid treatment (Fig. 3). In this regard, it is tempting to speculate that the activity of the 913 amino acid brain-specific *EHM2* protein, which has 409 more amino acids in the C-terminal region than does the *EHM2* isoform expressed in HT1080 cells, might be subjected to autoregulation similar to other large FERM domain proteins.

What might explain the AR-selective induction of *EHM2* and what function might *EHM2* have in the HT-AR1 cell differentiation pathway? The answer to the first question could be related to the observation that both GR and AR upregulate fibronectin RNA and protein levels, but only AR activation leads to growth arrest and neuroendocrine-like cell differentiation in the HT-AR1 cell line. This result would suggest that a subset of AR-regulated genes in this model system is not shared by GR, and moreover, that these genes have either gained, or retained, sensitivity to androgen signaling when functional AR is provided by stable transfection. One possibility is that the HT1080 cell line established by Rasheed et al. [18] could have been derived from an androgen-sensitive primary cancer, for example, a myofibroblastoma that originated in smooth muscle tissue [54]. In support of this idea, DHT treatment of HT-AR1 cells induces the expression of the motor protein myosin 1 B (MYO1B) (Chauhan et al., submitted), which could indicate that HT1080 cells originated from muscle tissue. Interestingly, unconventional myosins such as MYO1B have been shown to function in vesicle transport in neurons [55] which would be consistent with our observation that androgen treatment leads to the appearance of secretory granules and induces the expression of chromogranin A and neuron-specific enolase in HTAR1 cells (Fig. 2). Since six independent

AR-expressing subclones of HT1080 cells we have established share this same DHT-induced neuronal cell-like phenotype (Way and Miesfeld, unpublished data), we suggest that HT1080 cells possess previously unknown stem cell properties that may have been retained during cancer cell progression or reactivated by subsequent genomic rearrangements.

In summary, we found that activation of androgen signaling in the human HT1080 fibrosarcoma cell line resulted in dramatic cytoskeletal reorganization and neuroendocrine-like cell differentiation that was associated with induction of the human FERM domain encoding gene *EHM2*. The unique expression profile of *EHM2* in DHT-treated HT-AR1 cells, and its distinct 325 amino acid FERM domain sequence, suggests that this recently discovered gene may have a regulatory role in mediating cytoskeletal reorganization in steroid-regulated cell differentiation pathways.

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References

1. Schneiderman HA, Bryant PJ. Genetic analysis of developmental mechanisms in *Drosophila*. *Nature* 1971;234:187–194. [PubMed: 4943085]
2. Gautreau A, Louvard D, Arpin M. ERM proteins and NF2 tumor suppressor: the Yin and Yang of cortical actin organization and cell growth signaling. *Curr. Opin. Cell Biol* 2002;14:104–109. [PubMed: 11792551]
3. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. *Nat. Rev. Mol. Cell Biol* 2003;4:33–45. [PubMed: 12511867]
4. Sun CX, Robb VA, Gutmann DH. Protein 4.1 tumor suppressors: getting a FERM grip on growth regulation. *J. Cell Sci* 2002;115:3991–4000. [PubMed: 12356905]
5. Takeuchi K, Kawashima A, Nagafuchi A, Tsukita S. Structural diversity of band 4.1 superfamily members. *J. Cell Sci* 1994;107(Part 7):1921–1928. [PubMed: 7983158]
6. Han BG, Nunomura W, Takakuwa Y, Mohandas N, Jap BK. Protein 4.1 R core domain structure and insights into regulation of cytoskeletal organization. *Nat. Struct. Biol* 2000;7:871–875. [PubMed: 11017195]
7. Pearson MA, Reczek D, Bretscher A, Karplus PA. Structure of the ERM protein moesin reveals the FERM domain fold masked by an extended actin binding tail domain. *Cell* 2000;101:259–270. [PubMed: 10847681]
8. Hamada K, Shimizu T, Matsui T, Tsukita S, Hakoshima T. Structural basis of the membrane-targeting and unmasking mechanisms of the radixin FERM domain. *EMBO J* 2000;19:4449–4462. [PubMed: 10970839]
9. Shimizu T, Seto A, Maita N, Hamada K, Tsukita S, Hakoshima T. Structural basis for neurofibromatosis type 2. Crystal structure of the merlin FERM domain. *J. Biol. Chem* 2002;277:10332–10336. [PubMed: 11756419]
10. Smith WJ, Nassar N, Bretscher A, Cerione RA, Karplus PA. Structure of the active N-terminal domain of ezrin. *J. Biol. Chem* 2003;278:4949–4956. [PubMed: 12429733]
11. Hamada K, Shimizu T, Yonemura S, Tsukita S, Hakoshima T. Structural basis of adhesion-molecule recognition by ERM proteins revealed by the crystal structure of the radixin-ICAM-2 complex. *EMBO J* 2003;22:502–514. [PubMed: 12554651]
12. Nakamura F, Amieva MR, Furthmayr H. Phosphorylation of threonine 558 in the carboxyl-terminal actin-binding domain of moesin by thrombin activation of human platelets. *J. Biol. Chem* 1995;270:31377–31385. [PubMed: 8537411]
13. Hirao M, Sato N, Kondo T, Yonemura S, Monden M, Sasaki T, Takai Y, Tsukita S. Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible

- involvement of phosphatidylinositol turnover and Rhodependent signaling pathway. *J. Cell Biol* 1996;135:37–51. [PubMed: 8858161]
14. Mangeat P, Roy C, Martin M. ERM proteins in cell adhesion and membrane dynamics. *Trends Cell Biol* 1999;9:187–192. [PubMed: 10322453]
 15. Andersen JN, Mortensen OH, Peters GH, Drake PG, Iversen LF, Olsen OH, Jansen PG, Andersen HS, Tonks NK, Moller NP. Structural and evolutionary relationships among protein tyrosine phosphatase domains. *Mol. Cell. Biol* 2001;21:7117–7136. [PubMed: 11585896]
 16. Kubo T, Yamashita T, Yamaguchi A, Sumimoto H, Hosokawa K, Tohyama M. A novel FERM domain including guanine nucleotide exchange factor is involved in Rac signaling and regulates neurite remodeling. *J. Neurosci* 2002;22:8504–8513. [PubMed: 12351724]
 17. Bretscher A, Edwards K, Fehon RG. ERM proteins and merlin: integrators at the cell cortex. *Nat. Rev. Mol. Cell Biol* 2002;3:586–599. [PubMed: 12154370]
 18. Rasheed S, Nelson-Rees WA, Toth EM, Arnstein P, Gardner MB. Characterization of a newly derived human sarcoma cell line (HT-1080). *Cancer* 1974;33:1027–1033. [PubMed: 4132053]
 19. Bulens F, Merchiers P, Ibanez-Tallon I, De Vriese A, Nelles L, Claessens F, Belayew A, Collen D. Identification of a multihormone responsive enhancer far upstream from the human tissue-type plasminogen activator gene. *J. Biol. Chem* 1997;272:663–671. [PubMed: 8995310]
 20. Miesfeld, RL. Glucocorticoid action: biochemistry. In: DeGroot, LJ.; Jameson, JL., editors. *Endocrinology*. Philadelphia: W.B. Saunders; 2000. p. 1647-1654.
 21. Dean DC, Newby RF, Bourgeois S. Regulation of fibronectin biosynthesis by dexamethasone, transforming growth factor beta, and cAMP in human cell lines. *J. Cell Biol* 1988;106:2159–2170. [PubMed: 2454932]
 22. Oliver N, Newby RF, Furcht LT, Bourgeois S. Regulation of fibronectin biosynthesis by glucocorticoids in human fibrosarcoma cells and normal fibroblasts. *Cell* 1983;33:287–296. [PubMed: 6678610]
 23. Brenner KA, Corbett SA, Schwarzbauer JE. Regulation of fibronectin matrix assembly by activated Ras in transformed cells. *Oncogene* 2000;19:3156–3163. [PubMed: 10918570]
 24. Kondoh N, Shuda M, Arai M, Oikawa T, Yamamoto M. Activation of anchorage-independent growth of HT1080 human fibrosarcoma cells by dexamethasone. *In Vitro Cell Dev. Biol. Anim* 2002;38:111–117. [PubMed: 11928993]
 25. Chapman MS, Askew DJ, Kuscuglu U, Miesfeld RL. Transcriptional control of steroid-regulated apoptosis in murine thymoma cells. *Mol. Endocrinol* 1996;10:967–978. [PubMed: 8843413]
 26. Gordon DA, Chamberlain NL, Flomerfelt FA, Miesfeld RL. A cell-specific and selective effect on transactivation by the androgen receptor. *Exp. Cell Res* 1995;217:368–377. [PubMed: 7698238]
 27. Whitacre DC, Karnas KJ, Miesfeld RL. Analysis of glucocorticoid and androgen receptor gene fusions delineates domains required for transcriptional specificity. *Endocrine* 2001;15:111–118. [PubMed: 11572317]
 28. Whitacre DC, Chauhan S, Davis T, Gordon D, Cress AE, Miesfeld R. Androgen-induction of in vitro prostate cell differentiation. *Cell Growth Differ* 2002;13:1–11. [PubMed: 11801526]
 29. Chamberlain NL, Driver ED, Miesfeld RL. The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. *Nucleic Acids Res* 1994;22:3181–3186. [PubMed: 8065934]
 30. Chauhan S, Leach CH, Kunz S, Bloom JW, Miesfeld RL. Glucocorticoid regulation of human eosinophil gene expression. *J. Steroid Biochem. Mol. Biol* 2003;84:441–452. [PubMed: 12732289]
 31. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc. Natl. Acad. Sci. USA* 1998;95:14863–14868. [PubMed: 9843981]
 32. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673–4680. [PubMed: 7984417]
 33. Felsenstein J. PHYLIP-Phylogeny inference package version (3.2). *Cladistics* 1989;5:164–166.
 34. Schwartz, RM.; Dayhoff, MO.; Orcutt, BC. A model of evolutionary change in proteins. In: Dayhoff, MO., editor. *Atlas of Protein Sequence and Structure*. Washington, DC: National Biomedical Research Foundation; 1978.

35. Tilley WD, Wilson CM, Marcelli M, McPhaul MJ. Androgen receptor gene expression in human prostate carcinoma cell lines. *Cancer Res* 1990;50:5382–5386. [PubMed: 2386943]
36. Wilson EM, Oh Y, Hwa V, Rosenfeld RG. Interaction of igf-binding protein-related protein 1 with a novel protein, neuroendocrine differentiation factor, results in neuroendocrine differentiation of prostate cancer cells. *J. Clin. Endocrinol. Metab* 2001;86:4504–4511. [PubMed: 11549700]
37. Schiemann WP, Blobe GC, Kalume DE, Pandey A, Lodish HF. Context-specific effects of fibulin-5 (DANCE/EVEC) on cell proliferation, motility, and invasion. Fibulin-5 is induced by transforming growth factor-beta and affects protein kinase cascades. *J. Biol. Chem* 2002;277:27367–27377. [PubMed: 12021267]
38. Zhau HY, Chang SM, Chen BQ, Wang Y, Zhang H, Kao C, Sang QA, Pathak SJ, Chung LW. Androgen-repressed phenotype in human prostate cancer. *Proc. Natl. Acad. Sci. USA* 1996;93:15152–15157. [PubMed: 8986779]
39. Ling MT, Chan KW, Choo CK. Androgen induces differentiation of a human papillomavirus 16 E6/E7 immortalized prostate epithelial cell line. *J. Endocrinol* 2001;170:287–296. [PubMed: 11431162]
40. Yuan S, Trachtenberg J, Mills GB, Brown TJ, Xu F, Keating A. Androgen-induced inhibition of cell proliferation in an androgen-insensitive prostate cancer cell line (PC-3) transfected with a human androgen receptor complementary DNA. *Cancer Res* 1993;53:1304–1311. [PubMed: 8443809] [published erratum appears in *Cancer Res.* 55 (3) (1995) 719]
41. Heisler LE, Evangelou A, Lew AM, Trachtenberg J, Elsholtz HP, Brown TJ. Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. *Mol. Cell. Endocrinol* 1997;126:59–73. [PubMed: 9027364]
42. Hashimoto Y, Shindo-Okada N, Tani M, Takeuchi K, Toma H, Yokota J. Identification of genes differentially expressed in association with metastatic potential of K-1735 murine melanoma by messenger RNA differential display. *Cancer Res* 1996;56:5266–5271. [PubMed: 8912867]
43. Shimizu K, Nagamachi Y, Tani M, Kimura K, Shiroishi T, Wakana S, Yokota J. Molecular cloning of a novel NF2/ERM/4.1 superfamily gene, eh2, that is expressed in high-metastatic K1735 murine melanoma cells. *Genomics* 2000;65:113–120. [PubMed: 10783258]
44. Hoover KB, Bryant PJ. *Drosophila* Yurt is a new protein-4.1-like protein required for epithelial morphogenesis. *Dev. Genes Evol* 2002;212:230–238. [PubMed: 12070613]
45. Koyano Y, Kawamoto T, Kikuchi A, Shen M, Kuruta Y, Tsutsumi S, Fujimoto K, Noshiro M, Fujii K, Kato Y. Chondrocyte-derived ezrin-like domain containing protein (CDEP), a rho guanine nucleotide exchange factor, is inducible in chondrocytes by parathyroid hormone and cyclic AMP and has transforming activity in NIH3T3 cells. *Osteoarthritis Cartil* 2001;9:S64–S68. [PubMed: 11680691]
46. Olsson PA, Korhonen L, Mercer EA, Lindholm D. MIR is a novel ERM-like protein that interacts with myosin regulatory light chain and inhibits neurite outgrowth. *J. Biol. Chem* 1999;274:36288–36292. [PubMed: 10593918]
47. Klarlund JK, Holik J, Chawla A, Park JG, Buxton J, Czech MP. Signaling complexes of the FERM domain-containing protein GRSP1 bound to ARF exchange factor GRP1. *J. Biol. Chem* 2001;276:40065–40070. [PubMed: 11445584]
48. Takahashi K, Sasaki T, Mammoto A, Takaishi K, Kameyama T, Tsukita S, Takai Y. Direct interaction of the Rho GDP dissociation inhibitor with ezrin/radixin/moesin initiates the activation of the Rho small G protein. *J. Biol. Chem* 1997;272:23371–23375. [PubMed: 9287351]
49. Takahashi K, Sasaki T, Mammoto A, Hotta I, Takaishi K, Imamura H, Nakano K, Kodama A, Takai Y. Interaction of radixin with Rho small G protein GDP/GTP exchange protein Dbl. *Oncogene* 1998;16:3279–3284. [PubMed: 9681826]
50. Perez OD, Kinoshita S, Hitoshi Y, Payan DG, Kitamura T, Nolan GP, Lorens JB. Activation of the PKB/AKT pathway by ICAM-2. *Immunity* 2002;16:51–65. [PubMed: 11825565]
51. Speck O, Hughes SC, Noren NK, Kulikauskas RM, Fehon RG. Moesin functions antagonistically to the Rho pathway to maintain epithelial integrity. *Nature* 2003;421:83–87. [PubMed: 12511959]
52. Di Paolo G, Pellegrini L, Letinic K, Cestra G, Zoncu R, Voronov S, Chang S, Guo J, Wenk MR, De Camilli P. Recruitment and regulation of phosphatidylinositol phosphate kinase type 1 gamma by the FERM domain of talin. *Nature* 2002;420:85–89. [PubMed: 12422219]

53. Pang ST, Dillner K, Wu X, Pousette A, Norstedt G, Flores-Morales A. Gene expression profiling of androgen deficiency predicts a pathway of prostate apoptosis that involves genes related to oxidative stress. *Endocrinology* 2002;143:4897–4906. [PubMed: 12446617]
54. Morgan MB, Pitha JV. Myofibroblastoma of the breast revisited: an etiologic association with androgens? *Hum. Pathol* 1998;29:347–351. [PubMed: 9563783]
55. Wu X, Jung G, Hammer JA 3rd. Functions of unconventional myosins. *Curr. Opin. Cell Biol* 2000;12:42–51. [PubMed: 10679359]

Fig. 1.

Growth arrest and fibronectin expression in HT1080 sublines expressing human AR. (A) Western blot of protein extracts from puromycin-resistant HT1080 sublines transfected with the pDC-PAC vector (HT-VC1), or with the AR expression vector pDC-AR-PAC (HT-AR1, HT-AR2). The blot also contains a protein extract isolated from the human prostate cancer cell line LNCaP [35], for comparison. The membrane was probed with anti-AR or anti-tubulin antibodies. Results of cell proliferation assays are also presented using HT-VC1 (B), HT-AR1 (C) or HT-AR3 (D) cells grown in culture media with or without 1 μ M DHT or Dex for 3, 5 or 7 days. The number of viable cells in each triplicate well was determined by trypan blue exclusion. (E) Northern blot of fibronectin expression using total RNA isolated from HT-AR1 cells cultured in DHT for 0, 4, 12, 24, and 48 h. The level of L7 ribosomal protein (L7 RP) RNA was used as a loading control. (F) Western blot of fibronectin protein levels in HT-AR1 cells following DHT treatment as described in (E). The level of α -tubulin protein was used as a loading control.

Fig. 2.

DHT treatment of HT-AR1 cells induces cellular differentiation. (A) Morphology of HT-AR1 cells grown in normal serum $\pm$ DHT (a,b) or C/S serum $\pm$ DHT (c,d) as visualized by Nomarski microscopy. Panels (e) and (f) show HT-AR1 cells grown on fibronectin coated coverslips in normal serum $\pm$ DHT and immunostained with an anti-keratin 5 antibody (RCK103). The cells were counterstained with Hoechst dye to visualize nuclei. (B) Western blots of protein extracts made from HT-AR1 cells cultured in $\pm$ DHT media for 0, 4, 12, and 24 h and analyzed with antibodies specific for chromogranin A, neuron-specific enolase or α -tubulin.

Fig. 3.
EHM2 induction by DHT in HT-AR1 cells is unique compared to other FERM domain encoding genes. Selective hierarchical cluster analysis of FERM domain encoding genes using data obtained from expression profiling of DHT-treated HT-AR1 cells. The color code representing the fold-differences in expression level is shown at the bottom. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Androgen regulation of *EHM2* expression in HT-AR1 cells. (A) Northern blot using total RNA isolated from HT-AR1 cells treated with DHT for the indicated times. The membrane was hybridized with an *EHM2* cDNA probe made from the 1260 bp RT-PCR amplified segment of the human *EHM2* open reading frame as described in Experimental procedures. (B) Northern blot of RNA obtained from HT-AR1 cells treated with Dex or DHT for 48 h and hybridized with the same *EHM2* cDNA probe as in (A). The L7 RP probe was used as a loading control for these blots.

Fig. 5.

Expression of alternative *EHM2* transcripts in various human tissues. (A) Results of a Northern blot using total RNA from human tissues hybridized with the same 1260 bp *EHM2* probe as in Fig. 4. (B) Schematic diagram showing the genomic organization of the human *EHM2* gene on chromosome 9 based on data obtained from Genome Browser (<http://www.genome.ucsc.edu>) and AceView (<http://www.ncbi.nlm.nih.gov>). The relative position of the 1260 bp RT-PCR product within the *EHM2* coding sequences contained in exons 3–13 is shown. The GenBank accession numbers of the three *EHM2* sequences corresponding to the 5.6, 3.8, and 2.3 kb transcripts observed in HT-AR1 cells and human tissues are shown. Note that the only difference between the nucleotide sequences of AF153418 and AB032179 GenBank files is in the 3' UTR, whereas, the sequence of AF153416 indicates that the 5.6 kb *EHM2* transcript observed in brain corresponds to an alternative product encoding 409 additional amino acids.

Fig. 6.

Phylogenetic tree analysis of the FERM domain superfamily. Unrooted neighbor-joining tree of 98 core FERM domain superfamily sequences was made. Confidence levels of the branching patterns are: filled circle, excellent support (>90% of bootstrap replicas); and empty square, good or >70%; empty circle, majority support of >50%. The protein gi numbers are mentioned in the figure after the abbreviated species names. Abbreviation of species are: *Anopheles gambiae* (Ag), *Biomphalaria glabrata* (Bg), *Bos taurus* (Bot), *Caenorhabditis elegans* (Ce), *Canis familiaris* (Cf), *Ciona intestinalis* (Ci), *Dictyostelium discoideum* (Dd), *Drosophila melanogaster* (Dm), *Danio rerio* (Dr), *Echinococcus multilocularis* (Em), *Gallus gallus* (Gg), *Homo sapiens* (Hs), *Lytechinus variegatus* (Lv), *Macaca fascicularis* (Mf), *Mus musculus*

(Mm), *Podocoryne carnea* (Pc), *Rattus norvegicus* (Rn), *Schistosoma japonicum* (Sj), *Sus scrofa* (Ss), *Taenia saginata* (Ts), and *Xenopus laevis* (Xl). The PTP-NT5, PTP-NT6, and PTP-NT7 are three subtypes of phosphotyrosine phosphatases previously identified by Andersen et al. [15]. The scale bar shows the branch length for the distance computed for the tree that is scaled in units of expected fraction of amino acids changed as described in Experimental procedures.

Fig. 7.

Sequence Alignment of the *EHM2* subfamily. (A) Diagram showing the relative amino acid positions of the predicted F1, F2, and F3 subdomains within the *EHM2* FERM domain based on alignment with the corresponding structural features identified in the ezrin, radixin, moesin, and merlin/NF-2 proteins by X-ray crystallography. The dotted lines in F3 (residues 355–407) denote an extended homology with some FERM domain proteins. (B) ClustalW sequence alignment of *EHM2* subfamily proteins from seven different species identified the same as in Fig. 5 along with the first four characters of the GenBank file name. The F1, F2, and F3 subdomains are indicated using the same gray shading as in (A). The amino acid numbering within the region being compared is shown for each of these proteins based on the

corresponding GenBank files. The total number of amino acids for each predicted EHM2 protein is shown at the end of sequence block being compared. Note that no other significant matches were found amongst these nine proteins outside of the FERM domain (data not shown).