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Increased Expression of the Gene for α-Interferon-Inducible Protein in Cardiomyopathic Hamster Heart

Eileen M. Denovan-Wright, Gregory R. Ferrier, Harold A. Robertson, and Susan E. Howlett[1]
Department of Pharmacology, Faculty of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

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Cardiomyopathic (CM) hamsters have a disruption in the δ-sarcoglycan gene which leads to progressive cardiac necrosis by 30 to 40 days of age, hypertrophy by 120 days, and heart failure by 250 days. We used differential display to detect other changes in mRNA levels in 30-, 60-, and 90-day-old wild-type and CM hamsters. We identified a 400-bp cDNA with sequence similarity to the human α-interferon-inducible protein (p27). This cDNA annealed with a 570-base mRNA whose steady-state levels were increased in 30-, 60-, and 90-day-old CM compared to wild-type heart. Increased expression of this hamster homolog of p27 (p27-h) was detected in CM hamster cardiac and skeletal muscle at 60 days of age but not in liver, kidney, or brain. Thus, an inherited defect in CM hamsters leads to increased expression of p27-h in advance of the development of hypertrophy and heart failure.

The cardiomyopathic (CM) hamster is a genetic model of heart failure and muscular dystrophy (1). In this model, cardiomyopathy leads to the development of cardiac hypertrophy and overt congestive heart failure (2–4). The cardiomyopathy is characterized by focal cardiac cell necrosis, which begins at 30 to 40 days of age (5, 6). By 120 days of age, the surviving cells start to hypertrophy. Heart failure develops around 250 days and the animals die prematurely by about one year of age (5, 6). In contrast, wild-type hamsters have an average life span of 525 days (7).

The primary defect responsible for cardiomyopathy in the CM hamster is a mutation in the gene encoding δ-sarcoglycan, which eliminates production of this protein in striated muscle (8, 9). It is believed that δ-sarcoglycan is a cytoskeletal protein which associates with the dystrophin-dystroglycan complex (reviewed by 10). It has been proposed that loss of δ-sarcoglycan may impair sarcolemmal integrity in cardiac and skeletal muscles of CM hamsters (11). However, the link between the defect in δ-sarcoglycan and the development of heart disease is not known. In other models of hypertrophy and failure (12–14), changes in the expression of several genes have been observed. It is possible that the inherited defect in CM hamsters also leads to changes in gene expression. Although some of these changes in gene expression may contribute to the development of the disease others may be compensatory and thus beneficial to cardiac function.

Previous studies have documented changes in mRNA levels at various stages of disease development in CM hamster hearts. Most of the studies which have reported alterations in mRNA levels in CM hamsters have been prospective studies designed to determine whether the levels of specific gene products change (see for example 15–17). Although very useful, this approach will detect only changes predicted by current concepts of disease development. In contrast, the technique of differential display is a PCR-based screening method that allows unbiased detection of changes in steady-state levels of mRNA in tissues from different experimental conditions (18–20). Importantly, differential display will potentially detect changes in previously unidentified genes. Several investigators have used this approach to identify genes that are differentially expressed in other genetic models of heart disease (21–23), during normal heart development (24), during rejection in transplanted hearts (25) and in response to drug treatment (26). The objectives of this study were (i) to identify changes in mRNA levels in CM hamster hearts with differential display, (ii) to utilize this technique to establish the time course of changes in gene expression early in disease in CM hamster, and (iii) to combine this technique with Northern blot analysis to assess the tissue specificity of these changes. The present study utilizes differential display to compare changes in gene expression in cardiac ventricles from 30-, 60-, and 90-day-old wild-type and CM hamsters.

[1] To whom correspondence should be addressed. Fax: (902) 494-1388. E-mail: Susan.Howlett@dal.ca.

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MATERIALS AND METHODS

Animals. All experiments were performed in accordance with the guidelines published by the Canadian Council on Animal Care. For differential display, RNA was isolated from the ventricles of 30-, 60-, and 90-day-old CM (CHF 146) and genetically-matched wild-type (CHF 148) male hamsters purchased from Canadian Hybrid Farms (Halls Harbour, Nova Scotia, Canada). The ventricles were dissected from the heart and rinsed in sterile saline to remove excess blood. Tissues were also isolated from brain, liver, kidney and skeletal muscle of 60-day-old CM and wild-type hamsters. All tissues were rapidly frozen in liquid nitrogen and stored at −70°C prior to RNA isolation.

Differential Display RT-PCR and cloning. The techniques used for differential display RT PCR and cloning have been described (20, 25, 27). In brief, total cellular RNA was isolated from the ventricles of individual 30-, 60-, and 90-day-old wild-type and CM hamsters and from other tissues of 60-day-old wild-type and CM hamsters using Trizol reagent (Gibco BRL) and the manufacturer's protocol. For differential display of the RNA samples derived from the ventricles, 10-μg aliquots of total RNA were treated with RQ1 RNase-free DNase (Promega) in the presence of RNasin (Promega) RNase inhibitor to remove trace genomic DNA. The RNA was then converted to single-stranded cDNA using M-MLV reverse transcriptase (Gibco BRL). Single-stranded cDNA was used as the template for PCRs. The radio-labeled PCR products were fractionated on a denaturing acrylamide sequencing gels using a Genomyx LRJ sequencing apparatus, transferred to 3MM filter paper and dried. The dried acrylamide gels were exposed to autoradiography film (BioMax MR) overnight. One differential display band, amplified with primers P7 (5′-ATT AAC CCT CAC TAA ATG CTG TAT G-3′) and T6 (5′-CAT TAT GCT GAG TGA TAT CTT TTT TTT TCG-3′), was identified. The approximately 400-bp PCR product was excised from the dried gel and rehydrated in 40 μl of H_2O for 10 min at room temperature. The eluted DNA was subjected to PCR reamplification using the P7 and T6 primers, rTaq polymerase (Pharmacia) and the following conditions: 60 s @ 94°C; 19 × (30 s @ 94°C, 30 s @ 58°C, 120 s @ 68°C + 4 s per cycle); 7 min @ 68°C. The PCR was subjected to agarose gel electrophoresis and the 400 bp band was removed from the gel, extracted from the agarose using the Qiagen gel extraction protocol, radiolabeled and used as a hybridization probe in Northern blot analysis (see below). Following Northern blot hybridization, the DNA that annealed with a low molecular weight transcript in the RNA samples derived from CM hamster ventricles was eluted from the immobilized RNA by placing the blot in direct apposition to a piece of H_2O-saturated 3MM filter paper, incubating the blot/filter paper for 60 min at 37°C in a humid chamber and cutting out the piece of 3MM filter paper corresponding to the position of the differentially hybridizing RNA species on the Northern blot. The single-stranded DNA was eluted from the filter paper, reamplifed using the original PCR primers and cloned into the pGem-T (Promega) plasmid vector using the methods described previously (20, 27). Plasmid DNA was isolated from selected transformants using spin columns (Qiagen). The 396-bp sequence of the insert of the clone designated as CM1.3 was determined using M13 universal forward and reverse primers and the T7 sequencing kit (Pharmacia).

Northern blot hybridization analysis. Total RNA was electrophoretically separated on a 1% denaturing formaldehyde–agarose gel and transferred to Zetaprobe (Bio-Rad) membrane using standard techniques (28). The concentration of RNA in each sample was determined by spectrophotometry and 5 μg of RNA were loaded onto each lane of the Northern blot. The blot was incubated for 4 hr in pre-hybridization buffer (50% formamide, 5× SSC, 1× Denhardt's reagent, 20 mM sodium phosphate, pH 6.8, 0.2% SDS, 5 mM EDTA, 10 μg/ml poly A, 50 μg/ml sheared salmon sperm DNA, 50 μg/ml yeast RNA) at 42°C. The 400-bp PCR product was radio-labeled with [α-^{32}P]dCTP (3000 Ci/mmol) using the Ready-to-Go dCTP beads (Pharmacia). Prior to use in the hybridization reaction, unincorporated radionucleotides were removed from the labeled probe using a Sephadex G-25 spin column (Pharmacia). The blot was allowed to anneal with 2×10^6 cpm/ml of the radio-labeled probe in hybridization buffer (50% formamide, 5× SSC, 10% dextran sulfate, 1× Denhardt's reagent, 20 mM sodium phosphate, pH 6.8, 0.2% SDS, 5 mM EDTA, 10 μg/ml poly A, 50 μg/ml sheared salmon sperm DNA, 50 μg/ml yeast RNA) overnight at 42°C. Following the hybridization, the blot was washed 4× 15 min in 1× SSC @ 55°C, 4× 15 min in 0.5× SSC @ 55°C, 2× 15 min in 0.25× SSC @ 55°C, 2× 15 min in 0.25 × SSC @ room temperature and then exposed to Biomax MS (Kodak) film with an intensifying screen at −70°C for 5 days. Following the elution of the hybridizing cDNA from the Northern blot, the remaining radio-labeled was removed (28). The blot was subjected to a second hybridization using the conditions described above with the exception that the radio-labeled probe was the purified insert of clone CM1.3.

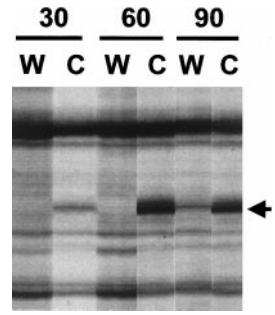

FIG. 1. Differential display RT PCR of CM hamster ventricle mRNA. Comparison of the products generated by differential display RT PCR using single-stranded cDNA of 30-, 60-, and 90-day-old wild-type (W) and CM (C) hamster ventricles as a template. The PCR products generated using primers P7 and T6 were fractionated on a denaturing polyacrylamide gel. A portion of the autoradiogram is shown. The band of approximately 400 bp (arrow), in the samples derived from 60 day old CM hamster ventricles, was selected for further analysis.

RESULTS

Differential display RT PCR of CM hamster ventricle mRNA. Differential display RT PCR was employed to detect differences between RNA populations derived from 30-, 60-, and 90-day-old wild-type and CM hamster ventricles. Each of the five primer combinations produced approximately 200 different sized PCR products per reaction. The vast majority of the PCR products were common in all of the samples. One PCR product was observed in the lanes corresponding to the samples derived from each of the CM ventricles but not the 30- or 60-day-old wild-type ventricles (Fig. 1). The 400-bp band appeared to be more abundant in the samples derived from the 60- and 90-day-old CM hamster ventricles than in the 30-day-old CM hamster ventricles. A 400 bp PCR product was also observed in the samples derived from the 90-day-old wild-type hamster ventricle, however, the amount of the product was less than that observed in CM hamster ventricles at any age.

Northern blot analysis of total RNA from CM ventricles at 30, 60, and 90 days. The PCR product present in the lane corresponding to 60 day old CM hamster ventricle was excised from the dried acrylamide gel and reamplified using the original differential display primers. The reamplified band was used as a hybridization probe in Northern blot analysis of total RNA isolated from 30-, 60-, and 90-day-old wild-type and CM hamster ventricles. The 400-bp PCR-generated probe annealed with a large number of transcripts in each of the total RNA samples (data not shown). Most of the hybridizing RNA species were common to all of the samples. However, a single low molecular weight RNA species in all samples derived from CM hamster heart hybridized with the probe. The relative abundance of this hybridizing band appeared greatest in the 60-day-old cardiomyopathic ventricle samples compared to the 30- and 90-day-old samples. The multiplicity of hybridizing bands suggested that either the PCR product contained several different cDNA species with unique sequence or that the sequence of a single cDNA species hybridized with a large number of different sized transcripts. To rapidly isolate the cDNA of interest, we eluted the hybridizing band directly from the Northern blot (27). The single-stranded cDNA fragment was reamplified using the differential display primers and cloned. One clone, designated CM1.3, was selected for further analysis. The cloned insert of CM 1.3 annealed with a single RNA species of approximately 570 bases as judged by its relative mobility to RNA molecular weight standards (Fig. 2). The abundance of this 570-base RNA species was greatest in the sample derived from the 60-day-old compared to the 30- and 90-day-old CM hamster ventricles. No hybridization of this probe to the samples derived from the 30- and 60-day-old wild-type animals was observed. A minor hybridization signal generated from the CM1.3 probe to a 570-base RNA in the 90-day-old wild-type hamster ventricle was observed.

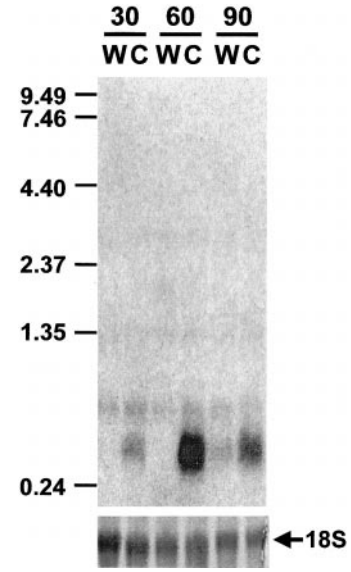

FIG. 2. Northern blot analysis of total RNA isolated from wild-type (W) and CM (C) hamster ventricles using the insert of clone CM1.3 as a hybridization probe. Ten micrograms of total RNA was fractionated on a denaturing formaldehyde gel, transferred to Zeta-probe (Bio-Rad) membrane and stained with methylene blue to access the relative amount of total RNA transferred to the membrane. A transcript of approximately 570 bases found in the RNA isolated from ventricles of 30-, 60-, and 90-day old CM hamsters annealed with the CM1.3 probe. The relative abundance of the hybridizing RNA was greatest in the 60 day old CM hamster sample. The relative mobility of RNA molecular weight standards (0.24- to 9.8-kb RNA ladder; Gibco, BRL) is indicated on the left of each blot. Below the Northern blot is the methylene stained blue 18S rRNA demonstrating that equivalent amounts of RNA were loaded on each lane of the Northern blot.

Sequence analysis of clone CM1.3. The sequence of CM1.3 was determined using forward and reverse sequencing primers (GenBank Accession No. AF212039). The unique sequence between the differential display primer sequences (Fig. 3) was compared to GenBank entries using BLASTn (29). The only significant match was an 84% nucleotide similarity over 88 base pairs to the human p27 mRNA (Accession No. X67325). Alignment of the nucleotide sequence demonstrated that nucleotides 2–176 of CM1.3 could be aligned without introducing gaps with nucleotides 172–346 of the p27 cDNA sequence. Alignment of the predicted amino acid sequences showed that the open-reading frames of CM1.3 and p27 could be aligned over a block of contiguous amino acids. Predicted amino acids 1–58 of CM1.3 directly aligned with amino acids 40–97 of p27. Over the aligned 83 amino acids, the CM1.3 and p27 open reading frames share 48 identical amino acids. Seventy of the 83 amino acids are identical or conservative amino acid substitutions. Of the 22 conservative substitutions, 21 involve the substitution of small hydrophobic amino acids. The other conservative substitution involves two polar but uncharged amino acids. Both of the open-reading frames have a large proportion of hydrophobic amino acids. A polyadenylation signal was identified at position 368–373 of the CM1.3 cDNA. The relative position of the polyadenylation signal in CM1.3 is similar to the position of the polyadenylation signal in the p27 cDNA. Based on the nucleotide and amino acid sequence similarity, we have named this hamster homolog of p27, p27-h.

Expression of p27-h in representative tissues from CM hamsters. To determine whether p27-h was expressed in other tissues, Northern blot analysis of total RNA isolated from brain, liver, kidney and skeletal muscle tissue of 60-day-old wild-type and CM hamsters was performed (Fig. 4). Tissues from 60-day-old hamsters were examined because the expression of the mRNA encoded by p27-h was maximal in ventricles at this age. The hybridization signal of p27-h was ob-

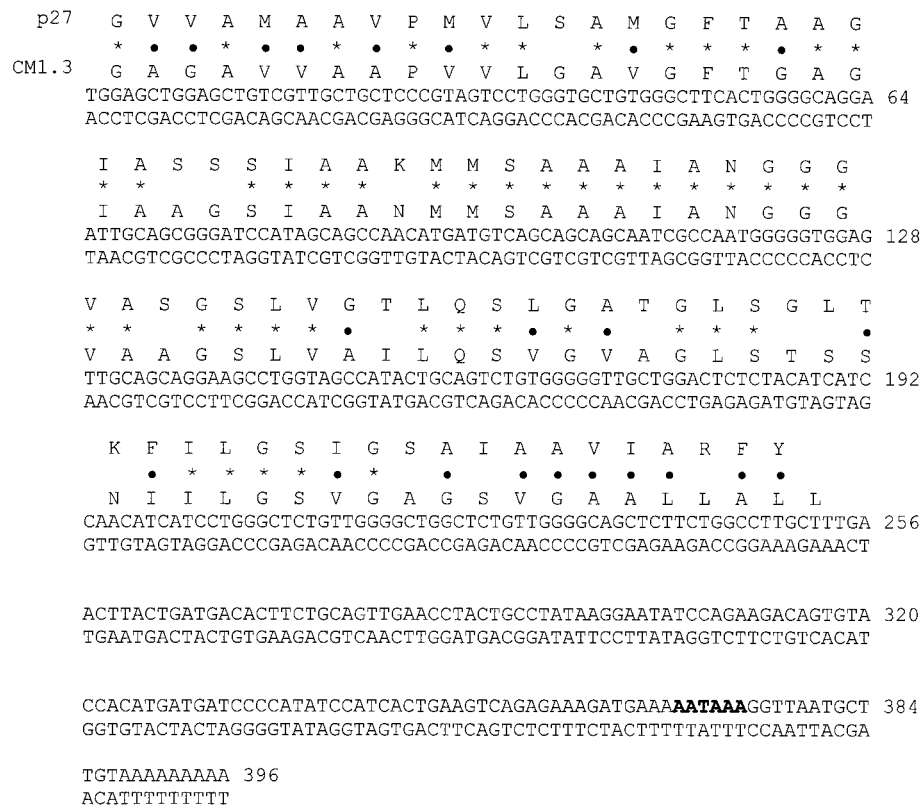

FIG. 3. Identification of CM1.3 as the hamster homolog of the α-interferon inducible protein p27. The nucleotide and predicted amino acid sequence of clone CM1.3 are presented with the comparable portion of the aligned amino acids of the human p27 α-interferon-inducible protein. The asterisk between the aligned protein sequences indicate sequence identity. The filled circles between the aligned protein sequences indicate positions with conservative amino acid substitutions. The numbers at the end of each line refer to the CM1.3 cloned sequence. The DNA sequence was determined using M13 universal forward and reverse primers and the T7 sequencing kit (Pharmacia). BLASTn searches of GenBank revealed that the nucleotide sequence of CM1.3 was similar to the human p27 clone. A polyadenylation signal (in bold type) is present within the 3′ untranslated region of the CM1.3 cDNA.

served only in the skeletal muscle of CM hamsters and not any other RNA samples derived from wild-type or CM hamsters.

DISCUSSION

We have used differential display to identify and isolate a partial cDNA clone which corresponds to an mRNA with increased expression in CM hamster heart. This cDNA, which we have called p27-h (hamster homolog of p27), has nucleotide sequence similarity to the cDNA for an α-interferon inducible protein (p27) previously characterized in human breast carcinoma cells (30). Differential display also allowed us to compare the time-courses of changes in expression of this gene in CM and wild-type hamsters. A difference in gene expression was detectable in 30-day-old CM hamsters and levels were further increased in hearts from 60-day-old animals and remained elevated at 90 days of age. Additional analysis with Northern blots demonstrated that the expression of p27-h was also increased in CM hamster skeletal muscle, but not liver, kidney or brain.

The size of the mRNA that anneals with the p27-h cDNA from CM hamsters is almost identical to the p27 mRNA characterized by Rasmusson *et al.* (30). The α-interferon-induced protein p27 is a relatively small (11 kDa), highly hydrophobic protein (30). The predicted amino acid sequence of p27-h is also highly hydrophobic. The stop codon and the potential polyadenylation signal are in similar positions in the p27 gene and the p27-h cDNA. Furthermore, the size of the 3′ untranslated region of the p27-h and the p27 genes (134 and 174 nucleotides, respectively) is similar. The slight difference in length may account for the small difference in the estimated size of the p27-h mRNA and the reported size of the cDNA of p27 (30).

The steady-state levels of p27-h mRNA were increased at 30, 60, and 90 days in the CM hamster ventricles compared to age- and genetically-matched wild-type controls. Interestingly, we found increased levels of p27-h in ventricles from very young (30-day-old) CM hamsters. Necrotic damage first appears at this stage in the ventricles of CM hamsters (5, 6). Thus, it appears that the increase in p27-h expression coincides with the onset of necrosis in CM hamster heart.

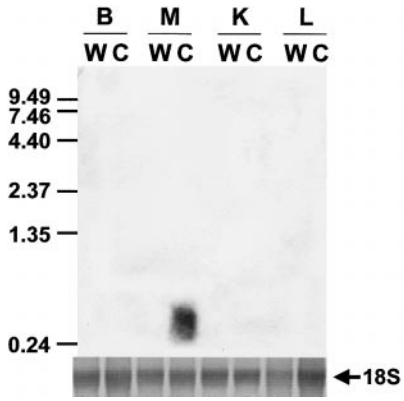

FIG. 4. p27 is expressed in CM hamster ventricle and skeletal muscle but not in brain, kidney or liver. Northern blot analysis of total RNA isolated from brain (B), kidney (K), skeletal muscle (M) and liver (L) of 60 day old wild-type (W) and CM (C) hamsters. Five micrograms of total RNA was fractionated on a denaturing formaldehyde gel, transferred to (Bio-Rad) membrane and stained with methylene blue to access the relative amount of total RNA transferred to the membrane. The methylene blue staining of the 18S rRNA on the membrane is shown beneath the autoradiogram. After pre-hybridization, the blot was allowed to anneal with 1×10^6 cpm/ml of radio-labeled insert of clone CM1.3. A transcript of approximately 570 bases found in the RNA isolated from transverse muscle of CM hamsters annealed with the CM1.3 probe. The relative mobility of RNA molecular weight standards (0.24- to 9.8-kb RNA ladder; Gibco, BRL) is indicated on the left of the blot.

In addition, we found that expression of p27-h further increased in hearts from 60- to 90-day-old CM hamsters, which corresponds to the period of maximal cell necrosis in these animals (5, 6). Thus, changes in expression of p27-h appeared very early in disease in CM hamster, prior to the onset of hypertrophy and heart failure. Therefore, increased expression of p27-h cannot be due to advanced disease and cardiac dysfunction.

The expression of p27-h appeared to be maximal at 60 days of age in CM hamster ventricle. Therefore, we examined the expression of this transcript in other tissues of 60-day-old wild-type and CM hamsters. We did not detect the p27-h mRNA in liver, kidney and brain from wild-type or CM hamsters by Northern blot analysis. Levels of p27-h were undetectable in wild-type skeletal muscle but were markedly increased in skeletal muscle from 60 day old CM hamsters. CM hamster skeletal muscle is also undergoing active cell necrosis at 60 days of age (5, 11). Therefore, it appears that only tissues exhibiting degenerative changes in response to the primary mutation in CM hamsters express p27-h.

p27 was originally described as a protein induced by α-interferon in human neuroblastoma cells (31) and in several different human breast carcinoma cell lines (30). In addition to being inducible, p27 is present at basal levels in neuroblastoma cells (31). Similarly, we found low levels of expression of p27-h in 90-day-old wild-type hamster heart. Examination of the EST database in GenBank revealed that cDNAs with sequence similarity to p27 also have been isolated from various tissues, including human aorta endothelial cells treated with tumor necrosis factor (GenBank Accession No. AA303476 and others), human colon (AA327547), human fetal lung (AA358277) and human pineal gland (AA365448). The p27 mRNA is, therefore, present at low levels in multiple tissues in different species, including hamsters, suggesting that the expression of this gene is not restricted to cancerous or pathologic tissues.

In addition to basal expression, p27 mRNA levels increase in neuroblastoma cell lines in response to α-interferon or the protein synthesis inhibitor cyclohexamide (31). Our results demonstrate that p27-h mRNA increased specifically in those tissues undergoing cell necrosis in CM hamsters. Thus, it is tempting to speculate that the expression of p27-h is linked to inflammation which accompanies the process of necrosis in CM hamsters. The function of p27, however, remains to be determined.

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