Altered Gene Expression Pattern in the Fatty Liver Dystrophy Mouse Reveals Impaired Insulin-mediated Cytoskeleton Dynamics*  

(Received for publication, March 2, 1999, and in revised form, May 14, 1999)

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The mouse fatty liver dystrophy (fld) mutation is characterized by transient hypertriglyceridemia and fatty liver during the neonatal period, followed by development of a peripheral neuropathy. To uncover the metabolic pathway that is disrupted by the fld mutation, we analyzed the altered pattern of gene expression in the fatty liver of fld neonates by representational difference analysis of cDNA. Differentially expressed genes detected include a novel member of the Ras superfamily of small GTP-binding proteins, a novel Ser/Thr kinase, and several actin cytoskeleton-associated proteins including actin, profilin, α-actinin, and myosin light chain. Because these proteins have a potential functional link in the propagation of hormone signals, we investigated cytoskeleton dynamics in fld cells in response to hormone treatment. These studies revealed that preadipocytes from fld mice exhibit impaired formation of actin membrane ruffles in response to insulin treatment. These findings suggest that the altered mRNA expression levels detected in fld tissue represent a compensatory response for the nonfunctional fld gene and that the fld gene product may be required for development of normal insulin response.

Fatty liver dystrophy (fld) is a recessive mutation that arose spontaneously in an inbred mouse strain and is named for the hallmark fatty liver present in neonatal mice and impaired nerve function apparent in adult animals (1). The fatty liver develops as the newborn mice begin to suckle milk and is accompanied by elevated plasma triglyceride levels (1000 mg/dl). However, the fatty liver and hypertriglyceridemia resolve spontaneously when mice reach 2–3 weeks of age, at which time the mutant animals begin to develop a peripheral neuropathy that persists throughout their lifetime. The neuropathy is associated with abnormal myelin formation and axonal degeneration in the peripheral nerve (2). The fld gene has been mapped to mouse chromosome (Chr) 12 (3), but neither the mutant gene nor the biochemical basis for the lipid and nerve defects have been identified (reviewed in Ref. 4).

Previous studies of the fld fatty liver have revealed altered mRNA levels for proteins involved in lipid metabolism, including hepatic lipase (60% reduction), apolipoprotein A-IV (100-fold induction), and apolipoprotein C-II (6-fold induction) (1). Using quantitative two-dimensional gel electrophoresis, we have detected approximately 25 additional proteins that show significantly altered levels in the fatty liver of neonatal fld mice; the majority of these proteins could not be identified on the basis of current two-dimensional protein data base information (5). The goal of the present study was to identify genes with altered expression levels in the fatty liver of fld mice, with the intent of uncovering a specific metabolic pathway affected by the mutation. Toward this end, we have employed representational difference analysis (RDA) to isolate cDNA tags corresponding to mRNAs with altered expression in the fld fatty liver.

The RDA technique was originally developed and applied to isolate differences between complex genomes, such as genetic lesions in tumors that result from DNA deletion, insertion, or rearrangement (6, 7). Subsequently, RDA has been adapted for use at the cDNA level and employed to isolate sequence tags corresponding to mRNA species that are differentially expressed between two cell populations (8–11). In cDNA-RDA, representations generated by PCR from two cDNA populations of interest are compared in successive rounds of subtraction-hybridization, kinetic enrichment, and selective amplification, resulting in sequences corresponding to mRNA species that are expressed at different levels in the two populations.

Using RDA we have isolated 22 mRNA species with altered expression levels in the fatty liver of fld neonates compared with their wild type counterparts. These RDA sequences include several that encode proteins associated with the actin cytoskeleton, a putative novel Ser/Thr protein kinase, and a putative novel member of the Ras superfamily of small GTP-binding proteins. Because members of the Ras and kinase protein families are functionally linked to components of the actin cytoskeleton in the process of hormone signal propagation, we investigated whether hormone-induced changes in the cytoskeleton were impaired in fld cells. Indeed, it was found that preadipocytes isolated from fld mice fail to form actin membrane ruffles in response to insulin. These results establish that fld mouse tissues exhibit altered expression levels of cytoskeleton-associated and putative signal transduction proteins, which is associated with impaired cytoskeleton response to hormones such as insulin.
Representational Difference Analysis

Representational difference analysis was performed as described below, based on the protocol by Hubank and Schatz (11).

Amplicon Generation—RNA was prepared from liver wild type and fld littermates at 6 days of age by the acid phenol-guanidium method (Trizol; Life Technologies, Inc.). Total RNA (400–500 μg) obtained from two isogenic littermates was combined for poly(A)+ RNA isolation (Poly(A)-Tract IV, Promega, Madison, WI), and 5 μg of the resulting poly(A)+ RNA was used for cDNA synthesis (Superscript Choice System, Life Technologies, Inc.). 1 μg of cDNA was digested with DpnII (New England Biolabs, Beverly, MA), extracted with phenol-chloroform, and R-Bgl2 adaptors were reused in RDA (11). PCR amplification was performed using one-tenth of the resulting cDNA in a total volume of 50 μl containing 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 2 mM MgCl 2, 0.001% gelatin, 200 μM dNTPs, 0.01 μg/μl forward and reverse primers, and 1 unit of AmpliTaq (Perkin-Elmer, Foster City, CA). PCR cycling conditions consisted of a hot start “touchdown” protocol in which the annealing temperature was gradually decreased over a 10 °C range (from 63 to 53 °C) to reduce nonspecific priming (15), and products were analyzed by agarose gel electrophoresis. The initial reaction conditions were denaturation at 94 °C for 1 min, annealing at 65 °C for 2 min, and extension at 72 °C for 1 min. The annealing temperature was decreased by 0.5 °C at each cycle for 20 cycles and maintained at 53 °C for an additional 12 cycles. Products were analyzed by agarose gel electrophoresis. The gene specific primers employed were as follows: Ifld-1, forward, 5′-TGGGGAACACCTTGAGATC-3′, and reverse, 5′-ACTACGCCCGCGAGTGTTTGA-3′; Ifld-2, forward, 5′-ATGACCGCGTCTGGGAGAAC-3′, and reverse, 5′-CTGGCAGCAGCATGTTGAAG-3′; HPBT, forward, 5′-CAGGACAGTAAAGCATGC-3′, and reverse, 5′-CCTGGTGGAAAAGGACCTT-3′, and PMP-22, forward, 5′-ACAGTGCTACCTCCTGACCTGAG-3′, and reverse, 5′-CAGGTACATATGATGACAATG-3′.

Chromosomal Localization of RDA Sequences

Selected RDA sequences were mapped in the mouse genome using a [c57BL/6J × Mus musculus] F 1 interspecific backcross (16). Isolated inserts were prepared from RDA plasmids, radiolabeled, and hybridized to restriction-digested genomic DNA from C57BL/6J and M. musculus to identify restriction fragment length variants. Distinct variants were then scored in 67 backcross mice that had been typed for more than 350 genetic markers. Linkage was detected with Map Manager version 2.6.5 (17).

Primary Cell Culture and Actin Cytoskeleton Staining

Preadipocytes were released from inguinal fat pads of 4-week-old wild type and fld mice by collagenase digestion followed by filtration through 62-μm nylon mesh (18). Preadipocytes were recovered by centrifugation at 800 × g for 5 min at room temperature and plated onto sterile coverslips at a density of 4 × 10 4 cells/coverslip in 6-well culture dishes and maintained for 3 days in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 15 nm insulin. After 24 h, cells were approximately 50% confluent.

For hormone stimulation studies, cells were incubated in Dulbecco’s modified Eagle’s medium devoid of serum and insulin for 18 h to bring them to a quiescent state. Cells were subsequently treated for 10 min at 37 °C with phosphate-buffered saline alone, with 1% fetal calf serum, or with 40 ng/ml insulin and then fixed and stained with tetramethylrhodamine isothiocyanate-phallolidin (Sigma) as described (19). Stained cells were observed with a Zeiss Axioskop microscope at an excitation wavelength of 540 nm and an emission wavelength of 590 nm.

RESULTS

Isolation of Differentially Expressed Sequence Tags from fld Liver Using RDA—RDA was performed using RNA from the liver of fld and wild type littermates at 6 days of age, at which time the fld mice exhibit an enlarged fatty liver. Both possible conditions were employed to allow isolation of sequences expressed at higher abundance in fld compared with wild type (“F clones”), as well as those expressed at higher abundance in wild type compared with fld (“W clones”). Following three or four rounds of RDA, the resulting difference products were cloned into plasmids, and approximately 150 of these were characterized by sequencing. Based on DNA sequence,
clones were segregated into 22 groups, and a representative of each group was used to probe Northern blots to confirm differential expression in liver of fld and wild type mice. As shown in Fig. 1 and Table I, these RDA clones correspond to mRNA species expressed at low, moderate, and high abundance and include examples that exhibit diminished (i.e. insulin-like growth factor-binding protein 4, Igfbp4), moderately increased (i.e. profilin), or vastly increased levels in the fld fatty liver (i.e. novel sequences Ifld1, Ifld2, and Ifld3). Some of the RDA sequences are expressed only at ages during which the fatty liver phenotype is apparent (i.e. Ifld1, Ifld2, and Ifld3), whereas others are also expressed in adult mouse liver (i.e. profilin and Igfbp4).

The F clones could be classified into 16 unique groups based on DNA sequence alignments, and the W clones could be segregated into 6 groups (Table I). These sequence groups were evaluated by comparison with nucleic acid sequence data bases using the BLAST algorithm (14). 14 of 22 of the sequences evaluated by comparison with nucleic acid sequence data bases. The remainder either represent novel sequences with no match in data bases (i.e. F1 and F23) or sequences that are related but not identical to known genes (i.e. F4, F9, and F24).

Expression levels of the RDA clones were evaluated in RNA isolated from liver of 6-day-old and adult mice via Northern blot analysis. As summarized in Table I, 13 of the 16 F clones hybridized to mRNA species with elevated expression levels in liver from 6-day-old fld compared with wild type mice; among these was apo A-IV, which is known to be expressed at dramatically elevated levels in the fld fatty liver (1). Three of the F clones (F7, F15, and F20) produced no detectable signal on Northern blots performed with 10 μg of total liver RNA, indicating that they are expressed at low levels. The W clones corresponding to insulin-like growth factor-binding protein 4 and troponin C hybridized to mRNA with reduced levels in the fld liver, whereas four of the W clones could not be detected on blots containing RNA from 6-day-old fld and wild type liver. Interestingly, all of the W clones in this latter group (myosin light chain, myosin heavy chain, troponin I, and α-2 actinin) were expressed in the liver of adult (3-month-old) wild type and fld mice and exhibited elevated expression levels in adult fld compared with wild type liver (denoted in Table I by mRNA level measurements enclosed in parentheses). Of the RDA sequences isolated, only four (one F clone and three W clones) were confirmed as false positives that were expressed in wild type and fld liver at similar levels; these sequences are not included in Table I.

The three cDNA tags that exhibited the greatest level of mRNA induction in fld compared with wild type liver (Fig. 1) represented previously undescribed sequences and were given the gene symbols Ifld1, Ifld2, and Ifld3 (induced in fld -1, -2, and -3). Ifld1 exhibits 60% amino acid sequence identity with members of the Ras family of small guanosine triphosphatases, including conservation of two GTP-binding sites and an effector domain (Fig. 2a). Ifld2 exhibits 75% amino acid identity with a putative serine/threonine protein kinase and contains protein kinase C and casein kinase II phosphorylation recognition motifs (Fig. 2a). Ifld3 was found to have no significant match to sequences in current releases of nucleic acid or protein data bases. To determine whether any of these three novel sequences correspond to the fld gene on proximal Chr 12, we mapped the genes in a mouse backcross panel. Ifld1 maps to Chr 5, Ifld2 maps to Chr 2, and Ifld3 is located on Chr 3 (Fig. 2b). These results suggest that Ifld1 and Ifld2 represent novel Ras-related and serine/threonine kinase genes but excludes the three RDA clones as candidates for the fld gene itself. Additional RDA clones shown in Table I that were mapped and excluded as fld candidates include the genes for tricarboxylate transport protein (distal Chr 12, distinct from the fld locus), profilin (Chr 11), ubiquitin (Chr 11), androgen-withdrawal apoptosis protein (Chr 5), Igf/H DNA with homology to bcl2 (Chr 4), and insulin-like growth factor-binding protein 4 (Chr 11).

Although genetic mapping results excluded Ifld1 and Ifld2 as fld gene candidates, their similarity to known proteins involved in signal transduction and the striking induction of the corresponding mRNAs in the fld fatty liver suggested that they might represent an important secondary response to the underlying defect and serve as markers for the metabolic pathway(s) disrupted by the fld mutation. We therefore examined expression of Ifld1 and Ifld2 mRNA in two other tissues that exhibit abnormal phenotypes in fld mice, sciatic nerve, and adipose tissue. After resolution of the fatty liver, fld mice exhibit reduced myelination and altered protein expression in sciatic nerve and drastically reduced adipose tissue mass (2, 4). Because of the small amount of tissue available from nerve and adipose, we used reverse transcriptase-coupled PCR to examine gene expression in these tissues from wild type and fld mice. The reverse transcriptase-coupled PCR results from liver recapitulated those seen by Northern blot, with vastly increased expression in the liver of fld neonates (6 days of age), low levels in the liver of both wild type and fld adult mice (6 weeks of age), and no detectable products in samples not treated with reverse transcriptase (Fig. 3a). Both Ifld1 and Ifld2 were also expressed in sciatic nerve, with somewhat lower levels detected in sciatic nerve from adult fld compared with wild type mice when normalized to levels of HPRT (a ubiquitously expressed mRNA) and PMP22 (a specific marker for peripheral nerve) (Fig. 3b). Ifld1 and Ifld2 mRNA were detected in inguinal and epididymal adipose tissue at similar levels in adult wild type and fld mice. Thus, Ifld1 and Ifld2 are expressed in each of the three tissues currently known to exhibit phenotypic alterations in fld mice.

Abnormal Insulin Response in fld Cells—We next considered the putative functions of the Ifld1 and Ifld2 proteins, together with those encoded by other RDA sequences that we had isolated (Table I). Both Ras-related proteins (Ifld1) and Ser/Thr kinases (Ifld2) are potentially involved in hormone signal transduction; interestingly, insulin-like growth factor-binding protein-4 (RDA clone W18), the G protein β-subunit (F26), and

| 6 day | 3 mo |
|-------|------|
| Ifld1 |       |
| Ifld2 |       |
| Ifld3 |       |
| Profilin |   |
| Igfbp4 |     |
the F24 sequence with similarity to a guanine nucleotide regulatory protein could also potentially function in signal transduction. A number of RDA clones with altered expression in fld liver are known to be associated with the actin cytoskeleton, including actin (F27), profilin (F5), α-2 actinin (W2), and myosin light chain (W1). Because GTP-binding proteins, kinases, and the actin network are all intimately involved in the propagation of hormone signals (20, 21), we hypothesized that the altered expression levels of these mRNAs in fld cells might be associated with impaired capacity for hormone-induced cytoskeleton reorganization.

To test this, we directly examined the actin cytoskeleton response to acute treatment with serum and insulin in primary cells isolated from wild type and fld mice. Both serum and insulin induce characteristic changes in cytoskeleton architecture in normal cells (22, 23). The lysophosphatidic acid present in serum induces the formation of elongated actin stress fibers, whereas insulin induces the formation of protrusions around the periphery of cells known as membrane ruffles. To determine whether cytoskeletal response to either of these stimuli was impaired in fld cells, we isolated preadipocytes from fld and wild type mice, serum starved them to produce a quiescent state, and then treated them with 1% serum or 40 ng/ml insulin for 10 min to induce cytoskeletal rearrangement (22, 23). The actin cytoskeleton was then visualized by staining with fluorescently tagged phalloidin, a fungal toxin that specifically binds actin. As shown in Fig. 4, the addition of serum to preadipocytes from both wild type and fld mice resulted in the formation of elongated actin stress fibers visible within the cytosol (Fig. 4, a and b). In contrast, treatment of wild type cells with insulin elicited the formation of membrane ruffles, which result from polymerization of a meshwork of actin filaments at the cell surface (Fig. 4c). Remarkably, membrane ruffles were not formed upon insulin treatment of preadipocytes from fld mice (Fig. 4d). These results suggest that fld cells are not generally deficient in actin polymerization but that the propagation of an insulin signal through the cytoskeleton is impaired and provides a functional link with the observed alterations in mRNA levels for actin and associated proteins. Further studies will be required to determine whether the impaired hormone response of the cytoskeleton represents a direct or secondary manifestation of the underlying fld mutation.

DISCUSSION

The phenotype of an organism or tissue can ultimately be traced back to the set of genes expressed in individual cells. Thus, the characterization of differences in mRNA expression between cell types provides a window into the fundamental differences in cellular function that may exist between them. Using the RDA technique, we have isolated several mRNAs with differential expression patterns in the fatty liver of fld mutant mice compared with their wild type counterparts. RDA has previously been utilized for comparison of expression patterns in cultured cells undergoing different treatments (8–11); the current study further establishes RDA as a reliable technique to isolate differentially expressed sequences from animal tissues as a means to compare wild type and mutant phenotypes.

The expression levels for mRNAs isolated by RDA included species of high, moderate, and low abundance and both large and modest differences in hepatic expression levels between fld and wild type mice (summarized in Table 1). Overall, differential mRNA expression levels were confirmed for ~70% of the RDA clones isolated here, indicating a success rate for RDA that compares favorably to related techniques for differential mRNA cloning (24, 25). Most of the remaining clones isolated corresponded to mRNAs that could not be detected on Northern blots containing total RNA, indicating that they may be ex-

### Table I

| Contig | Size<sup>a</sup> | mRNA level<sup>b</sup> | GenBank<sup>™</sup> accession<sup>c</sup> | Identification | BLAST<sup>d</sup> p value | Mouse Chr<sup>e</sup> |
|--------|----------------|------------------------|----------------------------------|----------------|--------------------------|-----------------|
| F1     | 374            | —/+/+/+                 | No match, Ifld3                  | 3.5e-10        | 4.8e-45                  | 11              |
| F9     | 188            | —/+/+/+                 | U02019, Human AU-rich element RNA-binding protein, Ifld2 | 4.6e-45        | 2.1e-2           | 3               |
| F3     | 301            | —/+/+/+                 | X84325, Mouse rho D (also known as rho M), Ifld1 | 7.8e-38        | 5.3e-2           | 3               |
| F23    | 113            | —/+/+/+                 | AA245957, EST, similar to tricarboxylate transport protein precursor | 5.2e-138       | 1.2e-2           | 3               |
| F24    | 535            | —/+/+/+                 | U02082, Human guanine nucleotide regulatory protein (tim1) | 5.0e-59        | 1.4e-2           | 3               |
| F25    | 133            | —/+/+/+                 | M64248, Mouse apolipoprotein A-IV | 7.0e-56        | 9.1e-2           | 3               |
| F14    | 199            | —/+/+/+                 | M11690, Mouse ubiquitin           | 1.2e-76        | 1.1e-2           | 3               |
| F27    | 195            | —/+/+/+                 | J04181, Mouse A-X actin mRNA     | 4.7e-51        | 1.4e-2           | 3               |
| F26    | 248            | —/+/+/+                 | D29802, Mouse mRNA for G protein β subunit homologue | 1.0e-52        | 1.4e-2           | 3               |
| F2     | 362            | —/+/+/+                 | X03369, Rat mRNA for β-tubulin   | 1.6e-129       | 1.7e-2           | 3               |
| F5     | 263            | —/+/+/+                 | X14425, Mouse Profilin           | 4.1e-97        | 1.1e-1           | 3               |
| F13    | 231            | —/+/+/+                 | M74067, Rat androgen-withdrawal apoptosis protein RVP1 | 1.8e-55        | 1.5e-2           | 3               |
| F7     | 297            | —/+/+/+                 | AA063442, EST, similar to mitochondrial HMG Coa synthase | 1.1e-90        | 1.3e-2           | 3               |
| F15    | 292            | —/+/+/+                 | A511092, Mouse EST               | 1.6e-65        | 1.6e-2           | 3               |
| F20    | 240            | —/+/+/+                 | X12660, Human Ig JH DNA, with scattered homology to bcl2 | 3.6e-75        | 4.2e-2           | 3               |
| W17    | 204            | —/+/+/+                 | J00793, Mouse fast skeletal troponin C | 4.4e-47        | 1.4e-2           | 3               |
| W18    | 240            | —/+/+/+                 | X51582, Mouse insulin-like growth factor binding protein 4 | 2.8e-27        | 1.1e-1           | 3               |
| W1     | 248            | —/+/+/+                 | K02243, Mouse alkalai myosin light chain | 5.3e-83        | 1.2e-2           | 3               |
| W6     | 252            | —/+/+/+                 | U32574, Rabbit myosin heavy chain | e-105          | 1.2e-2           | 3               |
| W12    | 212            | —/+/+/+                 | J04992, Mouse fast fiber troponin I | e-105          | 1.4e-2           | 3               |
| W14    | 248            | —/+/+/+                 | M69406, Human skeletal muscle α-2 actinin | 2.0e-12         | 1.3e-2           | 3               |

<sup>a</sup> Contig refers to the sequence group to which each RDA sequence was assigned; order is arbitrary. F contigs contain sequences isolated when RDA was performed using wild type RNA as tester. W contigs contain sequences isolated when RDA was performed using wild type RNA as tester.

<sup>b</sup> Size refers to the length of the sequence tag contained in RDA clones, given in base pairs.

<sup>c</sup> The relative mRNA levels in wild type compared to fld liver (wt/fld) was determined using Northern blots containing 10 μg of total RNA from 6-day-old wild type and fld liver. ND indicates not detectable in the Northern blots with 6-day liver samples; levels given in parentheses indicate levels that were detectable in Northern blots performed with RNA from adult liver.

<sup>d</sup> GenBank™ accession number of the best sequence match with the corresponding RDA sequence as detected by BLAST search.

<sup>e</sup> Mouse chromosome map positions are from the Mouse Genome Data Base, except those denoted by the following superscripts: 1, mapping data given in this paper; 2, unpublished data (K. Reue, P. Xu, Y. Xia, and A. J. Lusis); 3, excluded from linkage with fld by segregation in a (BALB/CByJ-fld × M. castaneus)F<sub>2</sub> cross (K. Reue and P. Xu). UN, unknown.
Altered Gene Expression in the fld Mouse

Ifld1

Mouse Ifld-1
Mouse RhoD
Mouse Rac2
Human RhoA

Yeast Rho1

Ifld2

Mouse Ifld-2
Dog Phosphoprotein
Human Ser/Thr PK
Ustilago cAMP-dep PK
Yeast cAMP-dep PK

Yeast cAMP-dep PK

b

2

3

4

5

6/65, 12.3 ± 4.1
6/63, 9.5 ± 3.7
8/63, 12.7 ± 4.2
3/56, 5.4 ± 3.0
1/56, 1.8 ± 1.0
3/46, 4.6 ± 3.8
0/66 (3.4)
8/66, 12.1 ± 4.0
4/66, 6.1 ± 2.9
cum = 90

D2Mit6
D2cde
D2Mit65
Pg51
Gp330
Acr
D2Mit35
D2Mit14
D2Mit30
D2cld
D2Mit19
D2cld2
D2cld1
D2cld12
D2cld1
D2Mit25

1/66, 1.7 ± 1.2
4/60, 6.7 ± 3.2
cum = 78

D3Mit227
D3Mit229
D3Mit23
D3Mit29

D3Uca4
D3Uca1
D3Mit9

D3Mit24
D3Mit26
D3Uca5
D3Mit18
D3Mit19

L/pap1

D5Mit54
D5Mit5

D3Uca1
D5Uca5
D5Uca2
D5Mit9
D5Uca4
D5Uca3
Mvk
Lbc
D5Mit65
Srb1
Ifld1

Gus
pressed at low levels. Preliminary experiments performed with primers designed to two of these sequences have allowed detection using reverse transcriptase-coupled PCR (data not shown). Most striking in terms of relative mRNA levels in fld versus wild type were the three sequences Ifld1, Ifld2, and Ifld3, which exhibited vastly elevated expression levels in fld in contrast to barely detectable expression in wild type tissue.

A potential limitation of the RDA technique is that it does not reveal whether differential expression at the mRNA level is ultimately reflected in the corresponding protein levels. Clearly it is not feasible to directly examine protein levels for the majority of RDA products, many of which are novel, but we have confirmed that protein levels for two of the RDA products do indeed increase in parallel with the elevated mRNA levels.

Using quantitative two-dimensional gel electrophoresis, we previously demonstrated that apo A-IV and actin protein levels are increased 7- and 2-fold, respectively, in liver from neonatal fld compared with wild type mice (5). In these two-dimensional electrophoresis studies, we detected nearly two dozen additional unidentified proteins with altered expression levels in fld in contrast to barely detectable expression in wild type tissue.

Of primary significance in our analysis of differential gene expression in fld cells is the novel insight it has provided into the nature of the metabolic defect resulting from the fld gene mutation. Our studies revealed altered gene expression levels for several proteins that may be implicated in insulin signaling and cytoskeleton dynamics. For example, Ifld1, which is dramatically induced in the fld fatty liver, shows approximately 60% amino acid identity to a mouse Rac protein, including conservation of GTP-binding and effector motifs, and Ifld2 was identified as a putative novel Ser/Thr protein kinase based on 75% identity to members of this family (Fig. 2). Both Rac and Ser/Thr protein kinases are involved in insulin signal transduction and the resulting cytoskeletal rearrangement; Rac is absolutely required for membrane ruffle formation (23), and its effect may be mediated by Ser/Thr kinases such as p21-activated protein kinases (26). Furthermore, a number of additional RDA clones encode proteins associated with the actin cytoskeleton. These include several proteins (i.e. actin, profilin, α-actinin, and myosin) that are known to be localized to membrane ruffles (27–29). Although our genetic mapping results excluded Ifld1, Ifld2, and these cytoskeletal components as the fld gene, the altered mRNA expression levels detected in fld cells may represent a compensatory response for the nonfunctional fld gene, indicating that the fld gene product may be required for development of normal insulin response. Consistent with this interpretation is our recent finding that fld mice are hyperinsulinemic and glucose intolerant, indicating im-

![Fig. 3. Reverse transcriptase-coupled PCR analysis of Ifld1 and Ifld2 expression in liver, sciotic nerve, and white adipose tissue of wild type (wt) and fld mice.](image)

![Fig. 4. Response of actin cytoskeleton to serum and insulin in cells isolated from wild type and fld mice.](image)
paired response of fld tissues to insulin.2

The RDA clones characterized in this study were all isolated from liver, but two of the novel sequences that were isolated (Ifld1 and Ifld2) were also found to be expressed in two other tissues that are clearly affected by the fld mutation, adipose tissue, and peripheral nerve. In both adipocytes and peripheral nerve Schwann cells, signaling pathways triggered by insulin and/or insulin-like growth factors are crucial for the regulation of differentiation and metabolism (30–34). Our novel finding of impaired insulin response in fld cells may therefore represent a direct causal link to the phenotype of fld adipocytes and Schwann cells. The signaling pathways involved in insulin-mediated cytoskeleton dynamics and membrane ruffle formation and the gene products involved in this biochemical cascade have not been fully elucidated. The identification of the fld gene defect may therefore shed light on molecular events involved in development of normal insulin response.

Acknowledgments—We thank Yu-Rong Xia and Jake Luis (UCLA) for assistance in genomic mapping of RDA clones. We thank David Schatz (Yale) for providing a detailed protocol for cDNA-RDA.

REFERENCES

1. Langner, C. A., Birkenmeier, E. H., Ben-Zeév, O., Schotz, M. C., Sweet, H. O., Davison, M. T., and Gordon, J. I. (1989) J. Biol. Chem. 264, 7994–8003
2. Langner, C. A., Birkenmeier, E. H., Roth, K. A., Bronson, R. T., and Gordon, J. I. (1991) J. Biol. Chem. 266, 11955–11964
3. Rowe, L. B., Sweet, H. O., Gordon, J. I., and Birkenmeier, E. H. (1996) Mamman Genome 7, 555–557
4. Reue, K., and Doolittle, M. H. (1996) J. Lipid Res. 37, 1387–1405
5. Rehnmark, S., Giometti, C. S., Slavin, B. G., Doolittle, M. H., and Reue, K. (1999) J. Lipid Res. 40, 2209–2217
6. Lisitsyn, N. A., Lisitsyn, N. M., and Wigler, M. H. (1993) Science 259, 946–951
7. Lisitsyn, N. A., Lisitsyn, N. M., Dalbagni, G., Barker, P., Sanchez, C. A., Gnarra, J., Linehan, W. M., Reib, B. J., and Wigler, M. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 151–155
8. Braun, B. S., Frieden, R., Lessnick, S. L., May, W. A., and Denny, C. T. (1995) Mol. Cell Biol. 15, 4623–4630
9. Cha, C. C., and Paul, W. E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2507–2512
10. Edman, C. F., Prigent, S. A., Schipper, A., and Feramisco, J. R. (1997) Biochem. J. 323, 113–118
11. Hubank, M., and Schatz, D. G. (1994) Nucleic Acids Res. 22, 5640–5648
12. Labarca, C., and Paigen, K. (1980) Anal. Biochem. 102, 344–352
13. Cohen, R. D., Castellani, L. W., Qiao, J.-H., Van Lenten, B. J., Luisis, A. J., and Reue, K. (1997) J. Clin. Invest. 99, 1906–1916
14. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
15. Don, R. H., Cox, P. T., Wainwright, B. J., Baker, K., and Mattick, J. S. (1991) Nucleic Acids Res. 19, 4008
16. Welch, C. L., Xia, Y.-R., Schecter, I., Farese, R., Mehrabian, M., Mehdizadeh, S., Warden, C. H., and Luisis, A. J. (1996) J. Lipid Res. 37, 1406–1421
17. Manly, K. F. (1993) Mamman Genome 4, 303–313
18. Briquet-Laugier, V., Dugail, I., Ardouin, B., Le Liepvre, X., Lavau, M., and Quinnard-Boulang, A. (1994) Am. J. Physiol. 267, E439–E446
19. Ridley, A. J. (1995) Methods Enzymol. 256, 306–313
20. Gupta, D. D., Nandini, R., and Rao, K. S. (1998) Cytobios 86, 75–111
21. Tsakiridis, T., Wang, Q., Taha, C., Grinstein, S., Downey, G., and Klip, A. (1997) Soc. Gen. Physiologists Series 52, 257–271
22. Ridley, A. J., and Hall, A. (1992) Cell 70, 389–399
23. Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992) Cell 70, 401–410
24. Sunday, M. E. (1995) Am. J. Physiol. 269, L273–L284
25. Wan, J. S., Sharp, S. J., Peurier, G. M.-C., Wagaman, P. C., Chambers, J., Pyati, J., Hom, Y.-L., Galindo, J. E., Huvat, A., Peterson, P. A., Jackson, M. R., and Erlander, M. G. (1996) Nature Biotechnol. 14, 1685–1691
26. Dharmawardhane, S., Sanders, L. C., Martin, S. S., Daniels, R. H., and Bokoch, G. M. (1997) J. Cell Biol. 136, 1265–1278
27. Machesky, L. M., and Pollard, T. D. (1993) Trends Cell Biol. 3, 381–385
28. Bretscher, A., and Lynch, W. (1985) J. Cell Biol. 100, 1656–1663
29. Conrad, P. A., Giuliano, K. A., Fisher, G., Collins, K., Matsuda, P. A., and Taylor, L. D. (1991) J. Cell Biol. 120, 1381–1391
30. Spritz, N., Singh, H., and Marinan, B. (1975) J. Clin. Invest. 54, 1049–1056
31. Cheng, H. L., and Feldman, E. L. (1997) J. Cell. Physiol. 171, 161–167
32. Ong, J. M., Kirchgessner, T. G., Schotz, M. C., and Kern, P. A. (1988) J. Biol. Chem. 263, 12923–12928
33. Semenkovich, C. F., Wims, M., Nee, L., Etienne, J., and Chan, L. (1989) J. Biol. Chem. 264, 9036–9038
34. Raynolds, M. V., Awald, P. D., Gordon, D. F., Gutierrez-Hartmann, A., Rule, D. C., Wood, W. M., and Eckel, R. H. (1990) Mol. Endocrinol. 4, 1416–1422
35. Saraste, M., Pyati, J., and Wittinghofer, A. (1990) Trends Biochem. Sci. 15, 430–434
36. Cales, C., Hancock, J. F., Marshall, C. J., and Hall, A. (1988) Nature 332, 548–551
37. Sinn, L. A. (1990) Biochim. Biophys. Acta 1054, 287–284
38. Woodgett, J. R., Gould, K. L., and Hunter, T. (1986) Eur. J. Biochem. 161, 177–184
39. Kishimoto, A., Nishiyama, K., Nakanishi, H., Urataji, Y., Nomura, H., Takeyama, Y., and Nishizuka, Y. (1995) J. Biol. Chem. 260, 12492–12499