A Mutation in Glyceraldehyde 3-Phosphate Dehydrogenase Alters Endocytosis in CHO Cells

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Abstract. The CHO cell mutant FD1.3.25 exhibits both increased accumulation and altered distribution of endocytosed fluid phase tracers. Neither the rate of tracer internalization nor the kinetics of recycling from early endosomes was affected, but exocytosis from late endocytic compartments appeared to be decreased in the mutant. Endocytosed tracer moved more rapidly to the cell poles in FD1.3.25 than in wild type cells. An abundant 36-kD polypeptide was found associated with taxol-polymerized microtubules in preparations from wild type and mutant; in the former but not the latter this polypeptide could be dissociated by incubation of the microtubules in ATP or high salt. The 36-kD polypeptide co-electrophoresed in two dimensions with the monomer of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Analysis of cDNA clones showed that the mutant is heterozygous for this enzyme, with ~25% of the GAPDH RNA containing a single nucleotide change resulting in substitution of Ser for Pro234, a residue that is conserved throughout evolution. Stable transfectants of wild type cells expressing the mutant monomer at ~15% of the total enzyme exhibited the various changes in endocytosis observed in FD1.3.25.

There are numerous examples in the literature of the presence of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in unlikely contexts. Whereas phosphorylation of glyceraldehyde 3-phosphate requires the tetrameric form of the enzyme, the 37-kD monomer functions as a uracil DNA glycosylase (Siegler et al., 1991). Consistent with a role in DNA repair, GAPDH is found not only in the cytosol, where it is an abundant protein, but also in the nucleus (Cool and Sirover, 1989; Morgenneg et al., 1986). A second possible role of nuclear GAPDH is in the export of tRNAs—binding of various tRNAs by the enzyme was shown to correlate with their competence for nuclear export (Singh and Green, 1993).

GAPDH has also been observed to associate with the plasma membrane. In erythrocytes this reflects binding of the enzyme to the cytoplasmic segments of two transmembrane proteins, glucose transporter 1 (Lachaal et al., 1990) and anion exchange protein 1, band 3, (Kliman and Steck, 1980). The latter complexes could be demonstrated in situ in erythrocytes and type A intercalated cells by immuno-colocalization performed with sections of kidney medulla (Ercolani et al., 1992). In studies of skeletal muscle, GAPDH was shown to promote formation of triad junctions from isolated transverse tubules and terminal cisternae of the sarcoplasmic reticulum (Caswell and Corbett, 1985). This activity has been ascribed to the binding of GAPDH to both the dihydropyridine receptor of the transverse tubules and to the junctional foot protein of the terminal cisternae (Brandt et al., 1990). Recent demonstration of binding of two low molecular weight GTP binding proteins to GAPDH associated with the transverse tubules has led to the suggestion of a possible regulatory role of the enzyme in the triad junction (Doucet and Tuana, 1991).

In vitro binding studies have shown that GAPDH associates with filamentous actin (Arnold and Pette, 1970; Méjean et al., 1989), tubulin and microtubules (Kumagai and Sakai, 1983; Durrieu et al., 1987; Walsh et al., 1989; Launay et al., 1989; Somers et al., 1990). Immunostaining of cells permeabilized prior to fixation revealed GAPDH staining consistent with association of the enzyme with stress fibers (Minaschek et al., 1992).

Given the abundance of GAPDH in the cytosol and the dissociation constants measured for the interactions described above, existence of each of these complexes within the cell is plausible, leading one to wonder what the consequences for the cell might be if any of these interactions were altered. In this paper we describe a CHO cell mutant, isolated on the basis of its increased accumulation of a fluid phase endocytic tracer. This mutant exhibits altered distribution of endocytosed tracer and unusual tubulo-
vesicular endocytic compartments. We show that these
phenotypic changes result from a single amino acid substi-
tution in a minor fraction of the GAPDH polypeptides,
which alters the binding of that enzyme to taxol-polymer-
ized microtubules.

Materials and Methods

Cells and Cell Culture

Cells were routinely grown at 34°C under 5% CO₂ in DME (Biofluids,
Inc., Rockville, MD) supplemented with glutamine, non-essential amino
acids, penicillin, and streptomycin (all from Biofluids, Inc.) and 5% ultra-
centrifuged fetal bovine serum (Innovar Laboratories, Inc., Gaithersburg,
MD). The isolation of WT, the parent cells (Thompson and Baker, 1973)
and of the ouabain-resistant, thioguanine-resistant derivative of WT
used for hybridization have been previously described (Robbins et al.,
1984).

The mutant F1D1.3.25 was isolated as follows: WTB was mutagenized
with 200 μg/ml ethyl methanesulfonate (Aldrich Chemical Company, Inc.,
Milwaukee, WI), subcultured for 3 d, and then plated on 10.35 mm dishes
(80,000 cells/dish) containing a heat absorbent film lining (Meridian In-
struments, Okemos, MI). 3 d later cells were incubated overnight with
growth medium containing 2 mg/ml dialyzed FITC-dextran, 40,000 mol wt
(Sigma Chemical Co., St. Louis, MO); to chase the label, medium was
removed, cells were washed three times and incubated for 2 h in medium
without tracer. Cells were then washed with MOPS-FBS buffer (30 mM
MOPS, 115 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, pH 7.4, containing 0.1%
glucose and 5% FBS). A 2 cm² area of each dish was scanned (excitation
of radius 250 μm, laser power, 200 mW; stage speed, 0.25 mm/s.
A 2 cm² area of each dish was scanned (excitation filters for excitation and
emission, respectively). Monensin was added to 25 μM and fluorescence
remasured. Cell number was
determined for each sample; fluorescence values were corrected by subtrac-
tion of background measured with unlabeled cells and data was normal-
zated to cell number.

The assay of transferrin recycling was a minor variation of previously
described procedures (Klausner et al., 1984; Yamashiro et al., 1984). Cells
grown for 3–4 d in six-well trays, 35-mm wells, were rinsed three times in
serum-free growth medium containing 0.1% BSA, and then incubated
with 225-HT 10 μg/ml, for 60 min at 34°C. Cells were rapidly washed three
times in tracer-free medium, then medium containing 1 mg/ml transferrin
and 100 μM demecoloxamine (Ciba-Geigy Corp., Greensboro, NC) was
added to initiate the assay. At intervals cells were rinsed three times
quickly with cold PBS, then solubilized in 0.1 M NaOH and counted in a
Beckman 9000 gamma counter. Values were corrected for non-specific
binding and uptake, determined by incubation in the presence of 1 mg/ml
transferrin, then normalized for cell protein, determined using the BCA™
protein assay reagents (Pierce, Rockford, IL).

Internalization was measured using HRP type VI (Sigma Chemical
Co.). Cells in six-well trays (35-mm wells) were equilibrated in three
changes of Hepes-buffered Ringer’s solution, pH 7.2, containing glucose
and BSA (formulated as described in Heuser, 1989) for 15 min in a CO₂-
free incubator (37°C). Pre-warmed solutions of HRP, 2 mg/ml in Ringer’s,
were added to the cells to initiate the assay. At various time points cells
were washed, lysed and assayed following the procedures of West et al.
(1989). Enzyme activity was linear over the range of 0.25–10 ng/ml in the
final assay mixture. Internalized tracer was normalized to cell protein.
Background activity (HRP bound to the cell surface or the dish) was
measured both by incubation of cells with the tracer at 0°C and by adding
and immediately removing HRP at 37°C; the two procedures gave virtually
identical results, 0.84 ± 0.05 mg/100 μg cell protein, with the various cell
types assayed.

Fluorescence Microscopy

Cells were plated on Permanox® two chamber slides 3–4 d prior to experi-
ments. Prior to incubation with tracer, cells were equilibrated in an
incubator without CO₂ by washing three times over 15 min in Hepes-buffered
Ringer’s solution, pH 7.2, containing BSA and glucose (formulated as de-
scribed in Heuser, 1989). Incubation with endocytic tracers and chase of
the label was also performed in this solution. To remove extracellular
tracer following the pulse, cells were rapidly rinsed three times. For fixa-
tion cells were quickly rinsed two times in PBS, 37°C, and then 2% formal-
inaldehyde in PBS, 37°C, was added for 30 min at room temperature. Follow-

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fixation cells were rinsed four times in PBS. Coverslips were mounted in Fluormount-G (Southern Biotechnology Associates, Inc., Birmingham, AL) that had been diluted in PBS. Cells were viewed with a Nikon Microphot-FX fluorescence microscope.

**Electron Microscopy**

Cells were plated in six-well trays 3 d prior to the experiments. Cells were rinsed three times in serum-free growth medium supplemented with 0.2% BSA then incubated for 30 min in 2 mg/ml HRP (type VI) at 34°C. Following three quick rinses in serum-free medium and two in PBS cells were fixed in 1% glutaraldehyde - 1% formaldehyde for 60 min at room temperature. The samples were then rinsed three times in PBS, once in 0.1 M glycine, pH 7, and then incubated for 45 min in a 0.01% hydrogen peroxide-dry ice and stored at -80°C. Electrophoresis in the second dimension was on a Tris-Glycine polyacrylamide 8-12% exponential gradient gel (O'Farrell, 1975), with a 4% stacking gel, both containing 0.1% SDS. Gels were fixed in 15% trichloroacetic acid and then subjected to fluorography (Bonner and Laskey, 1974).

**Molecular Genetics**

All oligonucleotides were from Midland Certified Reagent Co. (Midland, TX); all restriction enzymes from New England Biolabs (Beverly, MA). To generate GAPDH cDNA, mRNA was isolated from WTB and FDL1.25 using a Fast Track™ mRNA Isolation Kit (Invitrogen Corp., San Diego, CA). Reverse transcription of the mRNA was primed with random hexamers and was carried out using the GeneAmp® RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). PCR (Mullis and Faloona, 1987) was performed with 24-mer oligonucleotides corresponding to the 5' and complementary to the 3' ends of the coding sequence (nucleotides 74-97 and 1049-1072, respectively) published for hamster GAPDH cDNA (Vincen and Fort, 1990) and was carried out for 30 cycles of 95°C, 2 min; 50°C, 1.5 min; 72°C, 2 min. The cDNAs obtained from parent and mutant cell-electrophoresed at 1 kb. These PCR products were cloned directly into Bluescript KS (Stratagene, La Jolla, CA) that had been modified to a T vector following the procedure of Marchuk et al. (1991).

**Analysis of cDNA.** Orientation of insert in the vector was determined by digestion with HindIII. cDNAs were then compared for single-strand conformational polymorphisms (SSCP), using the published procedure (Orita et al., 1989) with the minor modifications noted below. cDNAs were amplified as segments of 300-400 bp using as primers 20-mer oligonucleotides, end-labeled with [γ-32P]ATP (~6000 Ci/mmol) and T4 polynucleotide kinase. 100 ng of plasmid, 100 pmole of each primer (diluted with cold primer to 1 × 106 cpm/100 pmole), 2.6 mM MgCl2, 2.5 U of Taq polymerase, and 0.2 mM of each deoxynucleotide were used in the amplification, run for 12 cycles of 95°C, 2 min; 50°C, 1 min; 72°C, 2 min. Samples were prepared and electrophoresed on 6% polyacrylamide gel containing 10% glycerol as described in Orita et al. (1989), except that electrophoresis was for 10–12 h at 30 W. Sequencing of the plasmid inserts was by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase Version 2.0 (United States Biochemical, Cleveland, OH) and the primers employed above for PCR-SSCP.

Cloning of GAPDH cDNAs into the mammalian expression vector pcDL-SRa296 (Takebe et al., 1988), modified by inclusion of a polynucleotide, is summarized as follows: First, a 5' fragment of the cDNA containing a NotI restriction site and a Kozak translation initiation site (Kozak, 1987), is generated by PCR using as sense primer 5' TTT AAT TAT GCG CCA CGT TGA GGC GCC ACC ATG GTA GTC GGT GTG AAA 3', the last 20 nucleotides of which correspond to the first 20 nucleotides of the coding sequence, and as antisense primer an internal sequence complementary to nucleotides 298-317 of the coding region. Similarly, a 3' fragment containing a termination codon and an XbaI site was generated with a primer corresponding to nucleotides 604-623 of the coding sequence and an antisense primer 5' GTA TTT AAG TAT GCG CCA CGT TGA GGC GCC ACC ATG GTA GTC GGT GTG AAA 3', ligated into XbaI and BstEII sites of pcDL-SRa296, and then cloned into pcDL-SRa296 by sequence digestion of both the ligation product and plasmid with NotI and XbaI. Note that between each of the steps of this procedure, the material was subjected to some form of purification: Magic PCR Clean-Up, Magic DNA Clean-Up (Promega, Madison, WI) or electrophoresis then excision from agarose gels.

Cloned plasmids were tested by double digestions, first with NotI and XbaI, second with SfiI and BstEII. Clones were further checked by sequencing, using for the 5' end an anti-sense primer complementary to nucleotides 81-100 of the coding sequence, and at the 3' end a sense primer corresponding to nucleotides 921-940. This allowed verification of the recombinant cDNA from positions within the 940-bp middle segment of the GAPDH cDNA, through the segments obtained by PCR and on into the vector.

To generate stable transfectants pcDL-SRa296 and pcDL-SRa296-GAPDH, were linearized with SfiI, and pReCMV (Invitrogen Corp.) was linearized with PvuI. Following digestion DNAs were put through Magic DNA Clean-Up. WTb cells (1 × 107/105 flask) were harvested by trypsinization, washed twice with PBS, and then resuspended in “cyto-mix” (formulated as described in van den Hoff et al., 1992) to 1.25 × 106.
cells/ml. Aliquots of 0.35 ml were placed in 4-mm electroporation cuvettes; 12 μg of pcDL-SRe296 or its GAPDH<sub>FD</sub> derivative and 2.5 μg of pRe/CMV were added to the cuvettes; electroporation was performed at 290 V, 960 μF. Cells were diluted and plated for selection and measurement of viability by colony formation (65-80% untreated controls); after 48 h G418 (GIBCO BRL, Gaithersburg, MD) was added to the growth medium at 800 active μg/ml. After 14 d colonies were picked, amplified, tested as described below, and then cloned by limiting dilution.

To determine the presence of GAPDH RNA containing the mutation, cells were grown in duplicate on 12-well trays, and then RNA was extracted using a Micro-scale Total RNA Separator Kit (Clontech Laboratories, Inc., Palo Alto, CA). Yields ranged from 10–16 μg/well. RNA (1 μg) was reverse transcribed for 1 h, and then a segment of the cDNA was amplified using as sense primer an oligonucleotide corresponding to nucleotides 604–623 of the coding sequence and an anti-sense primer complementary to 979–1002. Amplification was for 30 cycles of 95°C, 2 min; 53°C, 1.5 min; 72°C, 2 min. Samples were tested by allele specific hybridization, following the procedure of Myerowitz (1988), using as probes 32P-labeled 15-base oligonucleotides, CCGTGTTCTTACCCC and CCGTGTTTCCTACCCC, corresponding to nucleotides 693–707 of the wild type and mutant sense strands, respectively. Temperatures used for differential separation were 47°C and 45°C for wild type and mutant probes, respectively. Following exposure to film, results were quantitated by cutting the blots (3/4-in squares surrounding the spots) and measuring the Cerenkov radiation; following subtraction of background and correction for any difference in the specific activities of the probes, ratios of GAPDH<sub>FD</sub>/total GAPDH were calculated.

**Results**

The mutant FD1.3.25 was identified because it exhibited greater cell-associated fluorescence than wild type cells following an overnight incubation with FITC-dextran (see Materials and Methods). As compared to its parent, WTB (Fig. 1 a), FD1.3.25 (Fig. 1 b) has an unusual appearance, manifesting an active surface ruffle(s), with many clear vacuoles in the cytoplasm behind the ruffled edge. The nature of these vacuoles is unknown; although they are often closely apposed by endocytic compartments, they do not contain endocytosed tracer at any time (10 min to 20 h) following internalization. They are not acidic, based on lack of staining with acridine orange. These vacuoles are less refractile than the occasional lipid droplet observed in both mutant and parent cells, and they fail to stain with either Oil red O or filipin. Casual observation suggests that the vacuoles move slowly outward to the ruffle, then disappear (data not shown). Cells in Fig. 1 (c-e) will be discussed in a later section.

**Endocytosis**

Various aspects of endocytic activity in WTB and FD1.3.25 are compared in Table I. The increased fluorescence of intracellular FITC-dextran for which the mutant was isolated reflects elevated accumulation of fluid phase tracers: After a 1-h pulse, 2-h chase with FITC-dextran or LY, measurements of individual cells showed ~2.7 times more intracellular tracer in FD1.3.25 than in parental cells. Measurements of cell populations labeled in identical fashion showed a ~2.4-fold increase in the mutant.

In the mutant following the 1-h pulse, 2-h chase fluid phase tracer is located in lysosomes. Fractionation of ho-
Tracer accumulation

Analysis by phase contrast microscopy, to minimize day to day variation, the average of each experiment. Values were calculated as described above, except that means were corrected for background determined as described in Materials and Methods, and then normalized to 100 μg cell protein.


transferrin was used as the endocytic tracer in either continuous pulses or pulse–chase incubations (data not shown).

Table I. Comparison of Endocytic Activities in Parental and Mutant Cells

<table>
<thead>
<tr>
<th></th>
<th>WTB</th>
<th>FD1.325</th>
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</thead>
<tbody>
<tr>
<td>1-h pulse, 2-h chase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FITC-dextran</td>
<td>2.8 (2.4–3.3)</td>
<td>2.7 (1.7–3.7)</td>
</tr>
<tr>
<td>LY</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>HRP internalization</td>
<td></td>
<td></td>
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<tr>
<td>3 min</td>
<td>2.8 ± 0.2 ng</td>
<td>2.7 ± 0.2 ng</td>
</tr>
<tr>
<td>6 min</td>
<td>7.0 ± 0.5</td>
<td>7.2 ± 0.6</td>
</tr>
<tr>
<td>9 min</td>
<td>10.5 ± 1.0</td>
<td>10.3 ± 1.0</td>
</tr>
<tr>
<td>30 min</td>
<td>22.1 ± 0.7</td>
<td>29.0 ± 1.6</td>
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<tr>
<td>Tf recycling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/2</td>
<td>10.0 ± 1 min</td>
<td>10.0 ± 1 min</td>
</tr>
<tr>
<td>LY Recycling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-min pulse</td>
<td>1</td>
<td>1.3 (1.1–1.6)</td>
</tr>
<tr>
<td>1-h chase</td>
<td>0.60 (0.52–0.77)</td>
<td>1.2 (0.9–1.5)</td>
</tr>
<tr>
<td>2-h chase</td>
<td>0.44 (0.26–0.63)</td>
<td>1.1 (0.9–1.4)</td>
</tr>
</tbody>
</table>

Transferrin was measured with HRP, were similar in mutant and parent (Table I), leaving decreased exit of internalized tracer as the remaining possibility. However, measurements of transferrin recycling in parent and mutant gave identical results, indicating that exocytosis from the early portion of the endocytic pathway was unaffected in FD1.3.25. Increased accumulation in the mutant appears to result from decreased exit of fluid phase tracer from compartments beyond the early endosome. As shown in Table I, the levels of intracellular tracer were similar in WTB and FD1.3.25 following a 30-min pulse (1 and 1.3, respectively), but after 1 h of chase the parent had lost ~40% of the tracer and after 2 h, 55%, whereas the mutant retained ~85% over this time.

Distribution and Morphology of Endocytic Compartments

Although WTB and FD1.3.25 contained similar amounts of tracer after the 30-min pulse, false color images generated from these samples indicated a different distribution of labeled compartments in the parent and mutant cells. Tracer appeared to be randomly distributed throughout the cytoplasm of the parent, whereas in the mutant labeled vesicles were concentrated at one or both poles of the cell (data not shown). This polar localization reflects vesicle redistribution subsequent to tracer internalization. After a 10-min pulse and 5-min chase of LY, both parent (Fig. 2 a) and mutant (Fig. 2 c) cells showed labeled vesicles scattered throughout the cytoplasm. After a 15-min chase, most of the tracer in FD1.3.25 was clustered at the poles (Fig. 2 d), whereas little change in distribution was observed in WTB (Fig. 2 b). After 30 and 45 min of chase ~50 and 90%, respectively, of WTB cells exhibited some polar clustering of tracer, albeit to a lesser extent than that observed in FD1.3.25 after 15 min. No polar labeling was observed in either cell type when FITC- or Texas red–transferrin was used as the endocytic tracer in either continuous pulses or pulse–chase incubations (data not shown).

Figure 2. Fluid phase tracer is translocated to the cell poles subsequent to internalization; polar localization requires intact microtubules. After incubation of WTB (a and b) and FD1.3.25 (c and d) with LY for 10 min, tracer was chased for 5 min (a and c), or 15 min (b and d). Following the 5-min chase LY appears randomly distributed in both cell types; after the 15-min chase most of the tracer is clustered at the poles of the mutant, but remains scattered in the parent.
WTB and FD1.3.25 were examined under the electron microscope after a 30-min pulse with HRP (Fig. 3). As was observed by light microscopy, there is a dramatic difference in tracer distribution in parent (Fig. 3 a) versus mutant (Fig. 3 e) cells. A congregation of mainly large (0.8–1.3 μm) endocytic vesicles, including multivesicular bodies, was frequently observed at one end of the mutant cells (Fig. 3, e, g, and j); this distribution was not observed in parental cells. The characteristic vacuoles of FD1.3.25 (Fig. 1 b) do not preserve well under the conditions used for electron microscopy; where found, they are clearly devoid of tracer or any other detectable material (data not shown). Both cell types have small (80–160 nm) labeled vesicles distributed throughout the cytoplasm, with an increased concentration in the vicinity of the Golgi complex (Fig. 3, b and f). Vesicles at the lower end of this size range are sometimes arranged in tandems of up to eight vesicles, resembling tubules. In no case was label observed in the Golgi complex. The morphology of medium-sized (160–800 nm) vesicles also differed in parent and mutant. In the former these round vesicles exhibited a uniform periphery (Fig. 3, c and d), whereas in the latter, vesicles at the higher end of the size range (400–800 nm) often manifested tubular extensions (average length 430 nm), giving them a characteristic comma or swan shape (Fig. 3, h, i, k, l, and m). As shown in Fig. 3 (h and i), these compartments frequently appear to be surrounded by smaller labeled vesicles, which may be additional tubular extensions outside the plane of section. Vesicles of this type were common enough to be found in nearly every section of the mutant cells, whereas examination of as many or more sections of WTB revealed a single example of this structure (Fig. 3 c). In FD1.3.25 following a 10-min pulse HRP also appeared in these odd-shaped vesicles, albeit they were less frequent (data not shown).

In other experiments cells were examined after various times of pulse or pulse–chase with transferrin-HRP (data not shown). In both WTB and FD1.3.25 after a 40-min pulse most of the tracer was found concentrated near the Golgi complex in small vesicles or long tubular compartments similar to those shown in Fig. 3 (b and f). In contrast to HRP, transferrin-HRP was not observed in vesicles with tubular extensions, nor at the cell poles in the mutant. Unlike transferrin, HRP can survive for hours in the late endosomal/lysosomal environment; thus, the absence of transferrin-HRP from these compartments in the mutant seems indicative of lack of movement of transferrin to, rather than degradation of transferrin within those compartments.

Proteins Binding Taxol-polymerized Microtubules

A variety of observations (manuscript in preparation) suggested that microtubule-dependent movement of endocytic vesicles in FD1.3.25 might be altered. Thus, it seemed reasonable to look among microtubule-associated proteins for the affected protein. We compared the proteins present in taxol-polymerized microtubules from parent and mutant. No reproducible difference was observed on examination of initial microtubule pellets by SDS-PAGE, but when these pellets were washed with 0.4 M NaCl or 2 mM ATP, a polypeptide electrophoresing at ~36 kD was observed in preparations of the mutant but not the parent (Fig. 4). A rather prominent polypeptide of 36 kD was present in the initial microtubule pellets from both cell types; analysis of the various pellets by 2-D gel electrophoresis showed that a 36-kD polypeptide of basic pI is lost from the microtubule pellet of WTB cells after incubation with salt (Fig. 5) or ATP (data not shown) but remains in the pellets from FD1.3.25. Microtubule pellets prepared from stable WTB × FD1.3.25 hybrids contained this same polypeptide after washes with salt or ATP (data not shown), suggesting that altered binding of the protein is dominant. Polypeptide purified from the 2-D gels was blocked at the NH₃ terminus, thus was unsuitable for sequencing.

Several studies (Kumagai and Sakai, 1983; Durrieu et al., 1987; Walsh et al., 1989; Launay et al., 1989; Somers et al., 1990) had reported that GAPDH, a homotetramer comprised of 36-kD monomers electrophoresing at basic pI in urea, binds to microtubules. Rabbit muscle GAPDH (Sigma Chemical Co.) was mixed in microgram quantities with radiolabeled microtubule pellets from the CHO cells. The muscle enzyme, detected by staining, and the CHO cell 36-kD polypeptide, detected by fluorography, showed very similar mobilities on 2-D gels (Fig. 5). The relevant spot was excised from the gels, incubated with endoproteinase Glu-C (Calbiochem-Behring Corp.), then electrophoresed. Each of the radiolabeled proteolytic fragments co-electrophoresed with a stained proteolytic fragment from the muscle enzyme (data not shown).

Cloning, Analysis, and Expression of GAPDH cDNA

GAPDH cDNA was prepared from WTB and FD1.3.25 making use of the sequence published for the Syrian hamster enzyme (Vincent and Fort, 1990). The PCR product obtained electrophoresed at 1 kb, the expected length for GAPDH cDNA. As noted above, the 36-kD polypeptide from FD1.3.25 exhibited dominance (or co-dominance) in stable hybrids, raising the possibility that FD1.3.25 might itself be a heterozygote. Thus, prior to undertaking sequencing of GAPDH cDNA from mutant and parent, the cDNA clones were examined for SSCP. Of 19 clones from FD1.3.25, five showed an identical shift in electrophoretic mobility upon SSCP analysis.
Figure 4. SDS-PAGE of taxol-polymerized microtubules from WTB and FD1.3.25. Cells on one (of four) dishes were labeled overnight with 40 \( \mu \)Ci \(^{35}\)Smethionine; label was chased for 20 min prior to harvesting the cells. Microtubules were prepared as described in Materials and Methods; samples corresponding to material obtained from 1/20 of a dish were analyzed on a 4–12% Tris-glycine gel: initial microtuble pellets from WTB (lane 1) and FD1.3.25 (lane 2); microtubule pellets washed in 0.4 M NaCl-WTB (lane 3), FD1.3.25 (lane 4); microtubule pellets washed in 2 mM ATP-WTB (lane 5), and FD1.3.25 (lane 6). The arrow indicates the polypeptide (~36 kD) present in microtubules from the mutant after washing with salt or ATP. Bars denote the positions of molecular weight markers (200, 97.4, 68, 43, 29, and 18.4 kD).

mobility in the 3' one-third of the cDNA; dideoxy-sequencing of these five clones revealed a single nucleotide change, C\rightarrow T, at nucleotide 700 of the coding sequence, which would result in Pro\textsubscript{234}→Ser. Sequencing of three FD1.3.25 clones that appeared identical to WTB by SSCP showed them to have sequences identical to one another, to that of GAPDH cDNA in WTB clones, and to the sequence reported for Syrian hamster GAPDH.

The nucleotide change reflects an actual mutation in FD1.3.25's GAPDH DNA, rather than an error introduced on PCR amplification. Eleven independent PCR amplifications, initiated with eight independent preparations of either mRNA or total RNA from WTB and FD1.3.25 cells, were assessed by allele specific hybridization following RT-PCR; all preparations from WTB hybridized only to the WTB-specific probe, all preparations from FD1.3.25 hybridized to both WTB- and mutant-specific probes. Quantitating results of the hybridization showed that the steady state level of mutant GAPDH RNA in FD1.3.25 is 25 ± 5% of the total GAPDH RNA, in good agreement with our finding that 26% of the cDNA clones initiated with FD1.3.25 mRNA contained the mutation.

To determine whether GAPDH\textsubscript{FD} was responsible for the altered phenotype of FD1.3.25, parental cells were stably transfected with a mammalian expression vector containing the mutated GAPDH cDNA and a second vector conferring resistance to G418. After selection colonies were picked, amplified and tested for mutant GAPDH RNA by allele specific hybridization following RT-PCR; four of the first seven independent transfectants tested were positive, these were cloned by limiting dilution. Mutant/total GAPDH RNA in one of the cloned transfectants was only 5%; the other three ranged from 12–16% and these were used for further studies.

These transfectants all express the mutant protein. High-speed supernatants prepared from homogenates of the transfectants contained a 36-kD polypeptide that remained bound to taxol-polymerized microtubules after washing with high salt (Fig. 6) or ATP (data not shown). Note that the amounts of bound polypeptide are very similar in the transfectants and FD1.3.25, although the fraction of mutant/total GAPDH RNA in the latter is about twice that measured in the transfectants (see Discussion). GAPDH activity in WTB, FD1.3.25 and the transfectants is identical: 0.44 ± 0.02 U/10\(^6\) cells.

The transfectants closely resemble FD1.3.25 in phenotype. Each exhibits an active ruffle and large vacuoles in the cytoplasm behind the ruffled edge (Fig. 1, c–e). As shown in Table II, the transfectants each exhibited increased accumulation of LY. As was observed with FD1.3.25, this appears to result not from increased internalization, but from decreased loss of tracer from late stages of the endocytic pathway. Following a 30-min pulse with LY, labeled endocytic vesicles were found clustered at the poles of the cells (data not shown). Electron microscopic examination of transfectants 1B and 5C following a 30-min pulse with HRP revealed large labeled compartments clustered at one end of the cell as was seen with FD1.3.25 (compare Fig. 7, a and f with Fig. 4 e). As in the mutant, medium-sized endocytic vesicles with tubular projections were commonplace in the transfectants (Fig. 7, c–e, g, and i–m).

### Discussion

The results of this study show that a single amino acid substitution in a minor (15–25%) fraction of CHO cells’

| Table II. Comparison of Endocytic Activities in WTB and WTB-GAPDH\textsubscript{FD} Stable Transfectants |
|----------------------------------|------------------|------------------|------------------|------------------|
|                                 | WTB              | 1B               | 5C               | 9C               |
| HRP internalization*            |                  |                  |                  |                  |
| 6 min                           | 6.8 ± 0.6        | 7.0 ± 0.3        | 6.6 ± 0.6        | 7.1 ± 0.7        |
| 30 min                          | 23.8 ± 1.2       | 29.4 ± 1.6       | 28.9 ± 1.0       | 31.2 ± 2.4       |
| LY Recycling†                   |                  |                  |                  |                  |
| 30-min pulse                    | 1                | 1.3 (1.1–1.5)    | 1.2 (1.0–1.5)    | 1.3 (1.1–1.6)    |
| 90-min chase                    | 0.48 (0.28–0.59) | 1.1 (0.8–1.4)    | 0.9 (0.8–1.2)    | 1.0 (0.7–1.3)    |

*Results presented are from three experiments with duplicate samples for each time point. Values given, corrected for background as described, represent ng HRP/100 \( \mu \)g cell protein.
†Results presented are from three experiments each of which contained WTB and a pair of transfectants; values presented reflect measurements from 88 WTB cells, and from 60 cells of each transfectant and are normalized as described in Table I. Values in parentheses represent the range obtained with individual cells, normalized to the corresponding wild type cell in the pulse sample, as again described in Table I.
GAPDH polypeptides effects several phenotypic changes. In terms of cell morphology, cells expressing GAPDH<sub>F</sub> exhibit a prominent ruffle at one edge with numerous unidentified vacuoles in the cytoplasm behind the ruffle (Fig. 1). The net effect of the mutant enzyme on endocytosis is increased accumulation of fluid phase tracers, apparently due to decreased retroendocytosis of tracers from late endocytic compartments (Tables I and II); no difference was observed between parent and mutant with respect to either initial rates of fluid phase tracer internalization or ki-
...tetrmeric form, any of three models could account for increased accumulation. In each of these models it is assumed that recycling from lysosomes is less than that from earlier compartments. Model 1. The normal pathway is circumvented; i.e., fluid phase tracers bypass early compartments, proceeding directly to lysosomes. Model 2. Forward (toward the lysosome) movement of tracer proceeds normally, but backward movement is inhibited. Model 3. Forward movement is accelerated, so tracer spends less time in compartments that actively recycle.

Regarding model 1, macropinocytosis seemed an attractive possibility given the increased ruffling and large vacuoles consequent to expression of GAPDHFD. Two quite different pictures of macropinocytosis have emerged from studies of two cell types: in A431 cells stimulated with EGF, the macropinocytic pathway appeared totally distinct from the coated pit pathway; tracer internalized via macropinosomes was eliminated from the cells by exocytosis 2 h after internalization (Hewlett et al., 1994). But, in macrophages stimulated with macrophage colony stimulating factor, the coated pit and macropinocytic pathways were shown to converge after the early endosome (Racoosin and Swanson, 1992, 1993). Because cells with GAPDHFD exhibit increased, not decreased, accumulation on long chases, we can eliminate A431-like macropinocytosis as an explanation for our results. On the other hand, a macrophage-like pathway in which fluid phase tracer bypassed earlier compartments en route to lysosomes would be consistent with our findings. However, the vacuoles in cells with GAPDHFD did not contain endocytic tracer, and cells with GAPDHFD did not exhibit increased internalization of HRP (Tables I and II) as would be expected if endocytosis via macropinocytosis were added to that occurring through coated pits. Thus, any macropinocytic uptake would have to be balanced by a decrease in uptake via coated pits. We observed no decrease using either transferrin or lysosomal enzymes (data not shown) as ligands.

Decreased backward movement of tracer (model 2) could reflect either inhibition of budding of recycling vesicles from late endosomes or lysosomes (model 2A), or decreased fusion of those vesicles with their target organelles (model 2B). Inhibition of vesicle budding is an appealing hypothesis, in light of the tubulo-vesicular, HRP-containing 400 nm compartments characteristic of cells expressing GAPDHFD. There are several precedents for inhibition of vesicle budding resulting in tubularization. In temperature-sensitive shibire mutants of Drosophila, pinching off of endocytic vesicles from plasma membrane coated pits is blocked, resulting in elongated tubules extending inward from the membrane (Narita et al., 1989). In brefeldin A–treated cells, budding of vesicles from the Golgi and trans-Golgi network is inhibited, resulting in tubularization of those organelles (Klausner et al., 1992). Interestingly, brefeldin A has been shown to stimulate ADP-ribosylation of two proteins, one of which is GAPDH (De Matteis et al., 1994). Regarding model 2B, GAPDH has been shown to be an...
Effective fusogen in systems employing phospholipid vesicles (López-Vinals et al., 1987). GAPDHFD may be decreased in this activity. A caveat regarding model 2B is that the thwarted recycling vesicles must re-enter the forward-moving endocytic pathway, because on cell fractionation we observed no accumulation of tracer in FD1.3.25 at other than lysosomal densities.

Model 3, invoking increased forward movement of tracer, is similar to that proposed to explain decreased exocytosis of internalized tracer from macrophages manifesting tubular lysosomes (Swanson et al., 1985, 1987a). The tubular structure of lysosomes was hypothesized to promote more efficient transfer of solute from a compartment that rapidly filled and emptied into a compartment that emptied more slowly. Maintenance of tubular lysosomes depends on intact microtubules (Swanson et al., 1987b). That GAPDHFD affects the interaction of late endocytic compartments with microtubules is consistent with sequestration of 0.8–1.3 μm HRP-containing compartments at the cell poles in FD1.3.25 (Figs. 2 and 3) and transfectants (Fig. 7), the altered association of GAPDHFD with microtubules in vitro (Figs. 4–6), the disappearance of the 400 nm tubular endocytic compartments upon microtubule depolymerization and, following shifts in cytoplasmic pH, the increased outward movement of late endocytic compartments in cells with GAPDHFD (manuscript in preparation). This model holds the added attraction of invoking a gain, rather than a loss of function associated with GAPDHFD. The former is easier to reconcile with phenotypic expression in cells that, based on RNA levels, contain only one monomer of GAPDHFD for every five monomers of GAPDHWT.

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