Transport of Axl2p Depends on Erv14p, an ER–Vesicle Protein Related to the Drosophila cornichon Gene Product

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Abstract. COPII-coated ER-derived transport vesicles from Saccharomyces cerevisiae contain a distinct set of membrane-bound polypeptides. One of these polypeptides, termed Erv14p (ER–vesicle protein of 14 kD), corresponds to an open reading frame on yeast chromosome VII that is predicted to encode an integral membrane protein and shares sequence identity with the Drosophila cornichon gene product. Experiments with an epitope-tagged version of Erv14p indicate that this protein localizes to the ER and is selectively packaged into COPII-coated vesicles. Haploid cells that lack Erv14p are viable but display a modest defect in bud site selection because a transmembrane secretory protein, Axl2p, is not efficiently delivered to the cell surface. Axl2p is required for selection of axial growth sites and normally localizes to nascent bud tips or the mother bud neck. In erv14Δ strains, Axl2p accumulates in the ER while other secretory proteins are transported at wild-type rates. We propose that Erv14p is required for the export of specific secretory cargo from the ER. The polarity defect of erv14Δ yeast cells is reminiscent of cornichon mutants, in which egg chambers fail to establish proper asymmetry during early stages of oogenesis. These results suggest an unforeseen conservation in mechanisms producing cell polarity shared between yeast and Drosophila.

Key words: ER • Golgi • vesicles • coat proteins • cell polarity

Secretory proteins destined for intracellular organelles or the plasma membrane are first synthesized and processed at the ER of eukaryotic cells. Fully folded secretory proteins are then packaged into ER-derived transport vesicles for export to the Golgi complex and beyond. Several lines of experimental evidence indicate mechanisms of protein retention and retrieval operate during ER–Golgi transport to maintain distinct organelle identity (Sato et al., 1996; Kaiser et al., 1997). In addition, secretory proteins that must move forward are concentrated into ER-derived vesicles during export from this compartment (Quinn et al., 1984; Balch et al., 1994; Rexach et al., 1994); however, the mechanisms of this selection procedure remain obscure. One component of this selective export process is a protein complex, termed COPII, that forms ER-derived transport vesicles and selects secretory proteins by direct or indirect interaction (Barlowe et al., 1994; Kuehn et al., 1998). In the yeast Saccharomyces cerevisiae, formation of ER-derived vesicles has been reconstituted in a cell-free reaction with ER membranes and purified COPII proteins (Salama et al., 1993). We have proposed that additional components of this selection machinery are contained on ER-derived vesicles, and we have undertaken a molecular analysis of protein constituents on purified COPII-coated vesicles (Belden and Barlowe, 1996).

An uncoated form of ER-derived vesicles may be isolated after centrifugation on density gradients. These gradient-purified vesicles contain a set of tightly associated polypeptides that are solubilized by detergents but not by an elevated pH treatment. NH2-terminal polypeptide sequences have been determined from several of the abundant species contained on ER vesicles (Ervs),1 starting with the lowest molecular weight species moving upward (Belden and Barlowe, 1996). In this report, we characterize Erv14p, an integral membrane protein that localizes to the ER and Golgi compartments of yeast cells. Strains that lack Erv14p (erv14Δ) are viable but display novel phenotypes in both haploid and diploid stages of growth. First, haploid erv14Δ strains are defective in selecting the proper bud site. Wild-type budding yeast are highly polarized during vegetative growth, orienting cytoskeletal elements and the secretory pathway toward the emerging bud tip (Drubin et al., 1991). Haploids select bud sites such that mother and daughter cells bud toward each other and

1. Abbreviations used in this paper: CPY, carboxypeptidase Y; Endo H, endoglycosidase H; Erv, ER vesicle; HA, hemagglutinin; PMA, plasma membrane ATPase.
therefore exhibit an “axial” budding pattern. Genetic analyses in yeast have led to the identification of several genes that are required for selection of axial growth sites (Chant, 1996). One of these gene products, Axl2p, is an integral membrane secretary protein that must be delivered to the plasma membrane for establishment of the axial bud site (Halme et al., 1996; Roemer et al., 1996). In erv14Δ strains, Axl2p is largely retained in the ER and the yeast bud in a nonaxial manner. The accumulation of Axl2p in the ER appears to be selective since other secretory proteins examined were transported at wild-type rates. Second, diploid erv14Δ strains display nor-
asal diploid budding patterns but do not sporulate when deprived of nutrients. This sporulation defect does not appear to be related to transport of Axl2p and suggests that Erv14p participates in the transport of additional secretory proteins.

Erv14p shares a high degree of amino acid identity (36%) with the cornichon gene product from Drosophila melanogaster. Mutations in the cornichon gene are known to disrupt anterior–posterior pattern formation during early stages of Drosophila oogenesis and ultimately lead to misorientation of the oocyte cytoskeleton (Roth et al., 1995). This phenotype is in some respects similar to the erv14Δ phenotype. Therefore, experiments with the homologue of cornichon may provide insight on the molecular mechanisms underlying polarity in the Drosophila oocyte and perhaps in a variety of cell types.

Materials and Methods

Yeast Strains and Media

The strains used in this study are listed in Table I. Cultures were grown in either rich medium (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose [YPD]) or minimal medium (0.67% nitrogen base without amino acids, 2% dextrose [YMD]) containing appropriate supplements. Cultures were grown at 30°C unless indicated differently in figure legends. Standard yeast genetic methods used in these studies have been previously described (Sherman, 1991). Manipulation of recombinant DNA was performed as described (Ausubel et al., 1987), and Escherichia coli strain DH5α (Woodcock et al., 1989) was used for these procedures.

Plasmid Construction

The sequence of ERV14 and its upstream and downstream regions were obtained from the yeast genome database for YGL054c (Feuermann et al., 1997). ERV14 was isolated by PCR amplification of genomic DNA prepared from S. cerevisiae strain FY23 (Winston et al., 1995) using the primers GP3 (5’-CCGCGGTCCAACAAGACATTGAAATCC-3’) and GP4 (5’-CGCGGGATCTCTTATCCGATATTACCCG-3’). The restriction sites EcoRI and BamHI were used to subclone ERV14 (including 250 bp upstream and downstream of the open reading frame) into the EcoRI and BamHI sites of pRS316 to produce pRS316-ERV14. This construct was sequenced to confirm proper synthesis during amplification. An epitope tag corresponding to the hemagglutinin (HA) peptide sequence YPYDVPDYA (Wilson et al., 1984) was added on the COOH terminus of Erv14p and was synthesized using the primers GP3 and JP1 (5’-TCC-CGGGGTTAAGCTGTATGGTTAGGTAAGTATCATACCACTTTCAGC-3’). This primer anneals to the 3’-coding sequence of ERV14 and inserts the HA epitope followed by a stop codon and a SacII restriction site. The resulting ~700-bp PCR product was treated with EcoRI and SacII and then ligated into the EcoRI/SacII site of pRS316. The 3’ untranslated region for ERV14 was restored by inserting the 340-bp PCR product synthesized using the primers JP1 (5’-TCC-CGGGGTTAAGCTGTATGGTTAGGTAAGTATCATACCACTTTCAGC-3’) and JP3 (5’-AGGCCGGAGTTCAAGATCATGTCGTATGGTTAGGTAAGTATCATACCACTTTCAGC-3’) into the SacII site. This construct (pRS316-ERV14HA) was sequenced to confirm proper synthesis during amplification.

Strain Construction

The ERV14 locus was targeted for disruption with the HIS3 gene (Baudin et al., 1993). A PCR method was used to amplify an erv14::HIS3 disruption fragment using the primers GP1 (5’-TGCAATTTAAAGTAAAG-
Antibodies and Immunoblotting

Antibodies directed against Sec61p (Stirling et al., 1992), Sec22p (Bednar et al., 1995), Erv5p (Belden and Barlowe, 1996), Emp47p (Schröder et al., 1995), Sec23p (Hicke and Schekman, 1991), CPY (Krohn et al., 1989), Gas1p (Frankhauser and Conzelmann, 1991), Vpl1 (Kane et al., 1992), and plasma membrane ATPase (Carolyn Slayman, Yale University, New Haven, CT) were used in these studies. Anti-HA monoclonal antibody (12CA5; American Optical, Buffalo, NY) for 2.5 h at 4°C was used to detect Erv14p-HA or Axl2-HA. The membrane fraction was resolved on 12.5% polyacrylamide gel and immunoblotted for Sec61p, Erv25p, Sec12p, and anti-HA for Erv14p-HA detection.

In Vitro Vesicle Budding

Microsomes were isolated from strain CYB409 and incubated in the presence or absence of proteins required for reconstitution of vesicle formation as described (Barlowe et al., 1994). A 15-μM portion of the total reaction and 150 μl of a supernatant containing vesicles released from 200-μl budding reactions were centrifuged at 100,000 g (model TLA100.3 rotor; Beckman Instruments) for 15 min. The pellet fractions were dissolved in 30 μl of SDS-PAGE buffer, and 7–10 μl of this material was resolved on 12.5% polyacrylamide gel and immunoblotted for Sec22p, Erv25p, Sec12p, and anti-HA for Erv14p-HA detection.

Subcellular Fractionation

Subcellular fractionation was performed as described by Antebi and Fink (1992) with modifications by Schimmöller et al. (1995). Strains were grown to exponential phase and converted to spheroplasts by lyticase treatment (Baker et al., 1988). Spheroplasts were centrifuged and resuspended in a sucrose solution (10 mM Hepes, pH 7.5, 12.5% sucrose, 1 mM EDTA, 1 mM PMSF) and subjected to 10 strokes in a Dounce homogenizer. Two clearing spins were performed, and the resulting supernatant fluid was placed on a sucrose gradient consisting of nine steps from 22 to 60% (wt/vol) in 10 mM Hepes, pH 7.4, 1 mM MgCl2. The gradients were centrifuged at 55,000 rpm (model SW40 rotor; Beckman Instruments, Palo Alto, CA) for 2.5 h at 4°C. 15 fractions of 0.77 ml each were then taken sequen- tially from the top of the gradient to the bottom. Fractions were diluted in SDS-PAGE sample buffer, and proteins were resolved on polyacrylamide gels and immunoblotted for Sec61p (ER marker), Emp47p (Golgi marker), plasma membrane ATPase (PMA), Vpl1 (vacular marker), and anti-HA monoclonal antibody to detect Erv14p-HA or Asx2-HA. Relative levels of specific proteins in each fraction were quantified by densitometry of immunoblots. GDase activity was determined as de- scribed (Yanagisawa et al., 1990) using CDP to subtract nonspecific phosphatase activity. Sucrose concentrations of individual fractions were de- termined by measuring the refractive index with an Abbé Refractometer (American Optical, Buffalo, NY).

For subcellular fractionation under conditions of divalent cation chela- tion, the method of Kolling and Hollenberg (1994) was followed. Cells were grown and spheroplasted as described above; however, lysates were prepared by agitation with glass beads in buffer containing 10 mM Hepes, pH 7.5, 12.5% sucrose, 10 mM EDTA, 1 mM PMSF. The cleared lysate was loaded onto a similar 22–60% sucrose gradient as described above, except gradients contained 10 mM EDTA, and samples were centrifuged at 30,000 rpm (model SW40 rotor; Beckman Instruments) for 14 h at 4°C. Fractions were collected and analyzed as described above for magnesium-containing gradients.

To characterize the membrane association of Erv14p, yeast cell mem- branes were isolated and treated with various agents as follows. Spheroplasts were prepared as indicated above and resuspended in buffer 88 (20 mM Hepes, pH 6.8, 250 mM sorbitol, 150 mM KOAc, 5 mM MgOAc) containing 1 mM DTT and 1 mM PMSF. After Dounce homogenization, a low-speed supernatant fraction was prepared by centrifugation at 5,000 rpm (model SS34 rotor; Sorvall, Newtown, CT). Aliquots from the supernatant fraction were treated with buffer 88, 0.5 M NaCl, 2.5 M urea, 0.1 M sodium carbonate, or 1% Triton-X 100 in buffer 88. These samples were mixed and incubated 20 min on ice followed by centrifugation at 60,000 rpm (model TLA 100.3 rotor; Beckman Instruments) for 15 min. Equivalent amounts of supernatant and pellet fractions were diluted in SDS- PAGE buffer and resolved on a 12.5% polyacrylamide gel. Blots were probed with anti-Emp47p (integral membrane protein), anti-Sec23p se- rum (peripheral membrane protein), or anti-HA monoclonal antibody to detect Erv14p-HA.

Calcifluor Staining of Bud Scars

Yeast strains in a logarithmic stage of growth were diluted into YPD me- dium to an OD600 of 0.002. When cultures reached an OD600 of 0.5–0.8, an aliquot (1.5 ml) of cells was harvested and resuspended in calcifluor stain at a concentration of 0.1 mg/ml, as described (Pringle, 1991). After incuba- tion for 5–10 min at room temperature, the cells were washed three times in distilled water and resuspended in a final volume of 50 μl. Stained yeast were viewed under a fluorescence microscope, and cells possessing six or more bud scars were examined for an axial or nonaxial budding pattern. Haploid cells that displayed one or more bud scars at opposite poles were scored as nonaxial.

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Sporulation and Cell Survival Experiments

Sporulation efficiency and cell survival studies were performed as follows. Diploid strains were cultured for 8 h at 30°C in YPD to an exponential phase of growth. Cells were then harvested, washed twice with water, and resuspended to an OD600 of 1 in spmd, a nitrogen-deficient medium composed of 1% potassium acetate, 0.1% yeast extract, and 0.05% dextrose. To assess sporulation efficiency, samples from these cultures were microscopically examined daily to determine the number of tetrads per 300 cells. Cell viability from these cultures was assessed over a 7-d period by spreading an equivalent amount of OD600 units on YPD plates. After 4 d of growth on YPD plates, the colonies were counted to determine cell viability.

Indirect Immunofluorescence

Yeast strains were grown in 25 ml of YPD to an OD600 of 0.3 and fixed with 5% formaldehyde for 1 h. Fixed cells were then centrifuged for 3 min in a clinical centrifuge, washed three times in PBSB (PBS, pH 7.4, and 0.7 M sorbitol) and resuspended in a final volume of 400 μl PBSB. β-Mercaptoethanol (20 mM final) and lyticase were added and incubated at room temperature for 30 min to digest cell walls. The fixed spheroplasts were washed twice and resuspended in 400 μl of PBSB, and 15 μl was applied to polyesine-coated multiwell slides (Pringle et al., 1991). Cells were adhered for 10 min, washed, and incubated with blocking buffer (PBSB with 1% BSA and 0.5% Triton X-100) for 10 min. Wells were washed twice with PBSB followed by the addition of primary antibodies (anti-Kar2p at 1:500 and anti-HA at 1:200) diluted in blocking buffer. After incubation at room temperature for 90 min, cells were washed five times with PBSB and then incubated with secondary antibodies that had been diluted in blocking buffer at 1:500 (anti–rabbit IgG conjugated to fluorescein or antia–mouse IgG conjugated to Texas red). After 90 min, cells were washed four times with PBSB followed by a 1-min incubation in PBSB containing 1 μg/ml diaminophenylindolone. Cells were then washed four times with PBSB, and mounting medium (FITC guard; Testog, Inc., Chicago, IL) was layered on wells before sealing with coverslips. For all fluorescence microscopy, images were obtained using a cooled CCD camera, and composites were prepared with Adobe Photoshop (San Jose, CA) software.

In Vivo Labeling

Pulse-chase experiments were performed as previously described (Belden and Barlowe, 1996). In brief, cells were grown at 30°C (25°C for the sec12-4 strain) in selective medium containing 2% dextrose to an OD600 of 0.5. Cultures were harvested, washed, and resuspended at one-tenth the original volume in selective medium lacking sulfate. After culturing for 10 min at 30°C (37°C for sec12-4 strain), cultures were pulsed for 10 min by the addition of [35S]Express label (NEN™ Life Science Products, Boston, MA) and chased by the addition of excess methionine and cysteine. Cell samples were taken at the end of the pulse period and after 10 and 20 min of chase. Cell lysates were prepared by bead-beat lysis, and labeled species were precipitated from a common extract with specific antibodies for CPY, Gas1p, or anti-HA monoclonal antibody that recognizes Axl2p-HA. For endoglycosidase H (Endo H) experiments, 20-min chase time points were taken, and Axl2p-HA was immunoprecipitated in duplicate from indicated strains. Washed immunoprecipitates were equilibrated with 100 mM sodium citrate, pH 5.5, and one tube of each duplicate was incubated with 5 μU of Endo H (Sigma Chemical Co., St. Louis, MO) for 12 h at 37°C. Samples were resolved on 7.5 or 10% polyacrylamide gels, and labeled species were visualized by fluorography.

Results

Identification of Erv14p

Uncoated ER-derived vesicles were isolated, and vesicle proteins were resolved on a 15% polyacrylamide gel as previously described (Belden and Barlowe, 1996). Approximately 0.02 mg of the 14-kD protein was purified and subjected to automated NH2-terminal sequencing. The sequence GAWFLILAVVXNLFG was obtained from the polypeptide corresponding to Erv14p (X represents a residue that could not be unambiguously determined).
produced an ~15-kD immunoreactive species that was absent in untransformed strains (Fig. 2 A). Experiments described in later sections (for example Table I) demonstrate that Erv14p-HA complements the phenotypes displayed in later sections (for example Table I) demonstrate that Erv14p-HA is shown to indicate equivalent amounts of protein were loaded in lanes 1 and 2. (B) In vitro budding reactions with microsomes prepared from CBY409. One tenth of a total reaction (T) and budded vesicles isolated after incubation with COPII proteins (+) or a mock incubation (−). Samples were resolved on a 12.5% polyacrylamide gel and immunoblotted with antisera specific for Sec12p (ER resident), Sec22p (v-SNARE), and Erv25p (abundant vesicle protein) as controls or anti-HA antibody to detect Erv14p-HA.

First, we isolated COPII vesicles from in vitro reactions using ER membranes prepared from the Erv14p-HA-tagged strain CBY409. If Erv14p is an authentic ER protein, it should be selectively packaged into vesicles only when COPII proteins are included in the incubation. Under reconstituted budding conditions (Fig. 2 B), ER-derived vesicles incorporated Erv14p-HA at a level (~12%) comparable to other characterized vesicle proteins, such as Sec22p (Barlowe et al., 1994; Rexach et al., 1994) and Erv25p (Belden and Barlowe, 1996). Resident ER proteins, such as Sec12p and Sec61p (not shown), were not packaged into COPII-coated vesicles under these conditions. In this experiment, [35S]glycopro-0-factor was released at an efficiency of 23% in the presence of COPII proteins and 3% in their absence. Thus, Erv14p satisfies the initial criteria of an ER protein such that this species is selectively exported from ER membranes under conditions that reconstitute vesicle budding.

Next, we investigated the nature of Erv14p association with membranes. The predicted amino acid sequence for Erv14p suggests this protein spans the lipid bilayer three times and is consistent with the result that several of the Erv proteins (including an ~14-kD species) partition to a carbonate inextractable pellet fraction (Rexach et al., 1994). The fractionation behavior of Erv14p-HA was monitored (Fig. 3) under conditions that extract peripherally bound membrane proteins (2 M urea), release luminal proteins (0.1 M Na2CO3, pH 11), or solubilize integral membrane proteins (1% Triton X-100). The fractionation profile of Erv14p-HA was identical to an integral membrane protein such as Emp47p (Schröder et al., 1995) and not the peripheral membrane protein Sec23p (Hicke and Schekman, 1989). We conclude that Erv14p is an integral membrane protein. Application of the “positive inside” rule (von Heijne and Gavel, 1988) suggests a topology such that the NH2 terminus is oriented toward the cytoplasm and the COOH terminus is oriented toward the luminal compartment.

Previous experiments demonstrated that cycloheximide pretreatment does not diminish the level of abundant Erv proteins contained on COPII-coated vesicles (Yeung et al., 1995). This condition depletes ER membranes of known secretory proteins but does not inhibit in vitro synthesis of COPII-coated vesicles. Based on this observation, we hypothesize that the abundant Erv proteins, including Erv14p, are not newly made secretory proteins en route to the Golgi complex. Instead, we propose that many of the Erv proteins are structural components of COPII-coated vesicles and cycle between the ER and Golgi, functioning in aspects of vesicle budding or fusion (Belden and Barlowe, 1996). The following experiments indicate Erv14p localizes to the ER and Golgi compartments and suggest that Erv14p is not an abundant secretory protein. First, whole-cell immunofluorescence (Fig. 4) with an Erv14p-HA strain (CBY409) revealed a distinct perinuclear staining pattern for Erv14p-HA, much of which was coincident with that observed for the ER-resident protein Kar2p/BiP (Rose et al., 1989). A distinct staining pattern was not detected using the anti-HA antibody in strains lacking the Erv14p-HA construct, indicating a specific signal from the HA epitope (data not shown). Second, fractionation of membranes isolated from CBY409 on sucrose gradients (Antebi et al., 1992) showed that ~70% of Erv14p-HA cosediments with the ER marker Sec61p and ~30% cosedimented with the Golgi marker Emp47p (Fig. 5). This subcellular distribution is similar to other vesicle proteins (Emp24p and Erv25p) that appear to cycle between the ER and Golgi compartments (Schimöller et al., 1996; Belden, W.J., and C. Barlowe, manuscript in preparation).
To characterize the function of Erv14p, we analyzed a yeast strain bearing a null allele at the **ERV14** chromosomal locus. Oligonucleotides corresponding to 3' and 5' regions of **ERV14** and **HIS3** were used to direct **HIS3** to the **ERV14** locus, thereby replacing the open reading frame of Erv14p from start to stop codons. A heterozygous diploid (**ERV14/erv14**D) was obtained and dissected. Dissection of individual ascis produced four viable spores. Analysis of these spores by growth on different selective media and PCR amplification to confirm disruption indicated that **ERV14** was dispensable for vegetative growth (data not shown). Strains carrying the null allele grew at rates identical to wild-type strains and were not thermosensitive. Although the logarithmic phase growth rate of **erv14**Δ strains was wild-type, we found that **erv14**Δ strains exhibited a prolonged lag phase when diluted from stationary phase cultures into standard rich media such as YPD (see Materials and Methods). The lag phase delay was further exacerbated when diluted into yeast minimal media such as YMD. This growth phenotype was linked to the **HIS3** disruption of **ERV14** and was corrected when transformed with a plasmid containing the **ERV14** gene.

**Erv14p Is Required for an Axial Budding Pattern in Haploid Cells**

*S. cerevisiae* undergoes polarized cell growth such that the plane of cell division is determined by the site of bud formation and is dependent on cell type. Haploid yeast bud in an axial manner, placing each new bud adjacent to the previous bud site. Genetic analysis of bud site selection has revealed a group of **bud** and **axl** mutants defective in the haploid axial budding pattern. The phenotypes of these...
mutants fall into distinct categories, with one group that displays a random bud site selection pattern and a second group that exhibits bipolar patterns instead of the normal axial pattern (Bender and Pringle, 1989; Chant and Herskowitz, 1991; Chant et al., 1991; Fugita et al., 1994; Chant and Pringle, 1995; Halme et al., 1996; Roemer et al., 1996; Sanders and Herskowitz, 1996). A history of bud site selection in yeast may be visualized by staining with calcofluor, a fluorophore that binds to chitin and reveals previous bud scars as chitin-rich rings (Hayashibe and Katohda, 1973). Logarithmic-stage cultures of wild-type and erv14 mutants were grown in rich medium and stained with calcofluor. Strikingly, erv14Δ haploid strains displayed a non-axial budding phenotype that was not observed in an isogenic wild-type strain (Fig. 6). In a quantitative analysis of bud site selection, the penetrance of this phenotype was incomplete, but a significant fraction of mothers exhibited nonaxial budding patterns (see Table II). Transformation of erv14Δ strains with a CEN-based plasmid expressing Erv14p or Erv14p-HA reversed the nonaxial budding phenotype and confirmed that this defect was caused by deletion of Erv14p (Table II). Furthermore, these results indicated ERV14-HA is functional and complements as effectively as unmodified ERV14.

We also examined the influence of disrupting YBR210w on bud site selection. This predicted open reading frame on chromosome II shares 63% amino acid identity with Erv14p and could be functionally redundant. Disruption of this gene (ERV15) produced viable haploids (CBY347 and CBY353) that displayed no apparent phenotypes. Furthermore, combination of erv14Δ with erv15Δ (CBY354) did not exacerbate the nonaxial budding pattern displayed by a single erv14Δ strain (not shown). We also designed a construct to express an epitope tag on the COOH terminus of Erv15p, an approach that had proven successful for detection of Erv14p. Although DNA sequencing indicated correct synthesis of this construct, we failed to detect a tagged species under our standard growth conditions (data not shown). To rationalize these observations, we speculate that ERV15 is not functionally redundant with ERV14 when cells are grown in rich medium and that ERV15 expression may be restricted to specific stages of the yeast life cycle.

Wild-type a/a diploids exhibit a bipolar budding pattern such that a mother cell may produce a new bud at either pole from the previous bud site. We examined the budding pattern in homozygous diploid strains lacking Erv14p (CBY410) and in strains lacking both Erv14p and Erv15p (CBY411). These strains did not exhibit any alterations in the bipolar positioning of the bud site (data not shown). Therefore, erv14Δ strains possess haploid-specific defects in establishing cell polarity, as reported for a subset of the axl and bud mutants (Chant et al., 1995; Roemer et al., 1996).

### Axl2p Accumulates in the ER of erv14 Mutants

Because Erv14p is localized to the early compartments of the secretory pathway, and this pathway is responsible for the delivery of proteins to the plasma membrane, we considered the possibility that Erv14p was required for transport of factors to the cell surface for establishment of cell polarity. Combined molecular/genetic approaches have provided a wealth of information on the proteins involved in bud site selection in yeast (for review see Chant, 1996).

Axl2p is one of the characterized proteins that is required for an axial budding pattern and displays a bipolar phenotype when disrupted in haploid cells. Axl2p is also an integral membrane glycoprotein with a predicted type I topology and is known to traverse the secretory pathway en route to the cell surface (Roemer et al., 1996). During the biogenesis of Axl2p, N-linked core oligosaccharides are added in the ER and are further extended during passage.
Axl2p-HA detectable in wild-type strains was apparent. In contrast, wild-type strains expressing Axl2p-3HA (Fig. 7). In an initial experiment, we immunoblotted whole-cell extracts from wild-type and mutant strains expressing Axl2p-3HA (Fig. 7). In an env14Δ null strain, an ~150-kD HA-tagged species that was not detected in wild-type strains was apparent. In contrast, wild-type strains expressed an ~220-kD form that was a very minor species in env14Δ null strains. We reasoned that the 150-kD form of Axl2p-3HA that was unique to the env14Δ strain represented a core-glycosylated form of this secretory protein that may accumulate in the ER. The next series of experiments are focused on this hypothesis.

The temperature-sensitive sec12-4 allele blocks ER to Golgi transport when shifted to a restrictive temperature, and ER forms of secretory proteins accumulate (Novick et al., 1980; Stevens et al., 1984). Sec12p is an integral membrane glycoprotein localized to the ER, and it is required for the formation of transport vesicles from this compartment (Nakano et al., 1988; d’Enfert et al., 1991). We transformed a sec12-4 strain with the Axl2p-HA expression construct to monitor the ER form of Axl2p-3HA that accumulates when shifted to a restrictive temperature. Under steady-state conditions (Fig. 7, lane 3), the 150-kD form of Axl2p-3HA was not detected; however, after a shift to 37°C for 2 h, a small amount of this species became apparent in a sec12-4 strain (Fig. 7, lane 4).

The above experiments suggest that Axl2p-3HA accumulates in the ER as a 150-kD core-glycosylated form in env14Δ and sec12-4 strains. The immunoblot experiments provide a steady-state view of Axl2p-3HA in these strains. However, we sought to characterize the kinetics of Axl2p-3HA transport in wild-type and env14Δ strains through a pulse-chase analysis. In these experiments, cells were grown in minimal medium and pulsed with [35S]methionine and cysteine for 10 min to label newly synthesized proteins. Excess cold methionine and cysteine were then added to initiate the chase phase, and the fate of specific secretory proteins (Axl2p-3HA, CPY, and Gas1p) were monitored by selective immunoprecipitation of these species from whole-cell lysates (Fig. 8). CPY is first detected in the ER as the P1 precursor (67 kD), then modified upon arrival to the Golgi complex producing the P2 form (69 kD), and finally processed to the mature form (67 kD) in the vacuole (Stevens et al., 1984). As shown in Fig. 8, wild-type strains and the env14Δ strain exhibited similar kinetics for CPY transport to the vacuole, whereas the sec12-4 temperature-sensitive mutation accumulated the ER form (P1) of CPY when shifted to a restrictive temperature. Similar results were observed for transport of the glycosylphosphatidylinositol-anchored plasma membrane protein Gas1p. Newly synthesized Gas1p appeared in the ER as a 105-kD glycosylphosphatidylinositol-anchored precursor that carries N- and O-linked oligosaccharides. As Gas1p traverses the Golgi complex, outer chain glycosylation residues are added, generating the 125-kD mature form (Nuoffer et al., 1991). Gas1p was transported out of the ER in wild-type and env14Δ strains; however, we see a subtle delay in Gas1p transport (note the ratio of ER form to mature form after a 10-min pulse in the Gas1p panel in Fig. 8). This delay was not as severe as seen in strains bearing deletions of other Erv proteins, such as Emp24p and Erv25p (Belden and Barlowe, 1996). In sec12-4 cells, the ER form of Gas1p accumulated but displayed a reduced electrophoretic mobility during the chase period, as has been previously observed (Schimmoller et al., 1995; Belden and Barlowe, 1996). Based on these results, we...
conclude that the ERV14 gene is not required for transport of CPY and Gas1p from the ER to the Golgi complex, in contrast to sec mutant strains such as sec12-4 (Novick et al., 1980; Stevens et al., 1984). This result is also consistent with the wild-type growth rate for erv14Δ since cell surface expansion (i.e., growth rate) was not impeded in these strains, again in contrast to typical sec mutations that are lethal and prevent expansion of the plasma membrane.

Transport of Axl2p was also examined in these strains. Strikingly, the transport block observed in an erv14Δ strain was as complete as seen in a sec12-4 strain (Fig. 8). We could not detect the mature form of Axl2p in the erv14Δ strain even after the 20-min chase period. This result is consistent with the steady-state analysis in an erv14Δ strain conveyed through immunoblotting in Fig. 7, where the 150-kD form accumulated and the level of mature species was depleted. Furthermore, the 150-kD form of Axl2p that accumulated in an erv14Δ strain was relatively constant during the 20-min chase period, indicating that this species is not rapidly degraded. The transport of Axl2p from the ER to the Golgi appeared to occur rapidly. Several attempts were made to detect earlier secretory forms of Axl2p-HA using shorter pulse times in larger-scale reactions, but we failed to detect ER and Golgi forms of this protein under these conditions. We speculate that a short transitory time coupled with the heterogeneity of modifications on Axl2p-HA as it is transported through the secretory pathway prevents detection of these intermediates.

We proposed that Axl2p is not exported from the ER in erv14Δ strains and sought additional lines of evidence in support of this proposal. Axl2p acquires N-linked oligosaccharide in the ER of yeast cells (Roemer et al., 1996), and if the form that accumulates in an erv14Δ strain is trapped in the ER, treatment with Endo H should liberate this covalently linked carbohydrate and produce a faster migrating polypeptide on polyacrylamide gels (Orlean et al., 1991). After immunoprecipitation of Axl2p-HA from various strains, treatment with Endo H produced an alteration in the mobility of the labeled polypeptide (Fig. 9). The forms of Axl2p-HA that accumulated in an erv14Δ and a sec12-4 strain displayed identical properties upon treatment with Endo H, shifting the ~150-kD ER-form to an ~125-kD species. Based on an approximation of each core oligosaccharide contributing 2 kD in mass (Orlean et al., 1991), we estimate the attachment of ~13 N-linked chains on Axl2p, which was predicted to contain 16 potential N-linked glycosylation sites (Roemer et al., 1996). Treatment of the mature form of Axl2p-HA with Endo H also produced a distinct shift in size from ~220 to ~145 kD, but clearly not to the 125-kD species that appears after removal of N-linked residues from the ER form. We speculate that Axl2p acquires O-linked oligosaccharides in the ER that are then extended upon transport through the Golgi (Tanner and Lehle, 1987). Indeed, preliminary data indicate that Axl2p contains O-linked oligosaccharide (Sanders, S., and M. Gentzsch, personal communication), which would also explain the difference between the predicted size (~90 kD) and the Endo H–treated ER form (~125 kD). Regardless, the form of Axl2p-HA that accumulates in an erv14Δ strain behaves identically to the ER form that accumulates in a sec12-4 strain, a well-characterized mutant representative of mutants blocked in export from the ER (Novick et al., 1980; Stevens et al., 1984; Nakano et al., 1988). This observation, coupled with the fact that CPY and Gas1p are transported and modified correctly, make it unlikely that the erv14Δ mutation alters the glycosylation machinery producing modification defects on Axl2p.

As an independent method to evaluate Axl2p location in erv14Δ strains, we performed immunofluorescence experiments to visualize Axl2p-HA in wild-type and erv14Δ strains. Axl2p is reported to localize to discrete sites at the cell periphery depending on the phase of the cell cycle and

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**Figure 9.** Endo H treatment of Axl2p. Immunoprecipitates of Axl2p-HA from sec12 (CBY471), wild-type (CBY461), and erv14Δ (CBY463) were incubated in the absence (−) or presence (+) of Endo-H. Samples were resolved on a 7.5% polyacrylamide gel, and labeled species were visualized by fluorography.

**Figure 10.** Axl2p is mislocalized in erv14Δ strains. Double-label immunofluorescence microscopy of wild-type (CBY461) and erv14Δ (CBY463) strains with Kar2p antiserum and anti-HA monoclonal antibody for Axl2p-HA detection. Note the distinct staining pattern for Axl2p-HA observed in wild-type cells that is absent in erv14Δ strains.
first appears in nascent buds as a crescent-shaped patch that then diffuses and appears as a ring at the mother–daughter bud neck (Roemer et al., 1996). Immunofluorescence images generated from double staining wild-type and erv14Δ strains with anti-HA and anti-Kar2p revealed the expected localization pattern for Axl2p-3HA in wild-type strains (Fig. 10). In contrast, we did not detect this staining pattern for Axl2p-3HA in erv14Δ strains after examination of several hundred stained cells. Axl2p-3HA appeared to colocalize with Kar2p in some images, but a definitive ER localization for Axl2p remained equivocal through this approach. The accumulation of Axl2p-3HA in the ER may produce a stain that is too diffuse for detection in this compartment. The validity of our immunofluorescence procedure for erv14Δ cells was confirmed by observation of a characteristic ER-staining pattern for Kar2p (Fig. 10). We can conclude that the normal localization pattern of Axl2p-3HA is blocked in an erv14Δ strain, and this result is entirely consistent with a block in export from the ER observed in the pulse-chase experiments.

Because immunofluorescence did not document the subcellular location of Axl2p-3HA in an erv14Δ strain, we performed cell fractionation experiments to determine where Axl2p accumulates in this strain. A standard magnesium-containing sucrose gradient method (Antebi and Fink, 1992) that resolves several yeast organelles was used to monitor the subcellular distribution of Axl2p in a wild-type and an erv14Δ strain. In a wild-type strain (Fig. 11, A and B), the mature form of Axl2p-3HA comigrates with the PMA and is resolved from Golgi (GDPase) and vacuolar (Vph1) membranes. The 150-kD form of Axl2p-3HA that accumulates in an erv14Δ strain (Fig. 11, C and D) comigrates with ER membranes (Sec61p) and is resolved from Golgi and vacuolar membranes. However, the conditions of this gradient do not cleanly separate ER membranes (Sec61p) from plasma membranes (PMA), and we chose another sucrose gradient fractionation procedure to resolve these compartments. This method relies on a more vigorous lysis procedure in the presence of EDTA, a condition that strips ribosomes from the ER and produces a corresponding shift of ER membranes up in the gradient to a lower buoyant density (Kölling and Hollenberg, 1994; Roberg et al., 1997). As seen in Fig. 12, the 150-kD form of Axl2p-3HA observed in an erv14Δ strain coincides with the peak of Sec61p (Fig. 12, C and D) and is resolved from the PMA. Based on the fractionation patterns observed in Figs. 11 and 12, we conclude that Axl2p-3HA in erv14Δ strains is localized to the ER, a result that is entirely consistent with our pulse-chase and immunofluorescence experiments. Furthermore, these sucrose gradient analyses indicate that additional secretory proteins (PMA, GDPase, and Vph1) are synthesized and localized correctly in an erv14Δ strain.

Plieotropic Effects of erv14Δ Mutations

To determine if ERV14 deletion produced altered budding patterns in diploid cells, we constructed the homozygous disruptant strain CBY410. This diploid strain does not display a bud site selection defect (normal bipolar pattern), which is consistent with the observation that axl2Δ homozygous diploids are phenotypically bipolar (Roemer et al., 1996). However, we found that the homozygous erv14Δ disrupted strain would not sporulate under standard sporulation conditions (Sherman, 1991). We tested if erv14Δ/erv14Δ diploids became inviable under these con-
ditions and found that the cell viability of wild-type (CBY453) and mutant (CBY410) cultures were similar after several days in sporulation media. Further, the mutant strains grew on nonfermentable carbon sources as efficiently as wild-type strains, indicating the carbon source contained in sporulation media is adequate. Finally, transformation of CBY410 with the Erv14p-HA plasmid (strain CBY433) restored sporulation competency to wild-type levels (Table III). These results indicate Erv14p is required for some aspect of sporulation.

Previous studies have shown that Axl2p is expressed at equal levels in both haploid and diploid cells, although this protein does not appear to participate in bud site selection in diploids (Roemer et al., 1996). We considered the possibility that delivery of Axl2p to the cell surface could also be required for efficient sporulation. Our previous results (Table II) demonstrated that overproduction of Axl2p partially suppressed the nonaxial budding phenotype of haploid erv14Δ strains. Therefore, we tested if overproduction of Axl2p could rescue the sporulation defect. However, the sporulation efficiency of homozygous erv14Δ disruptants was not affected by transformation with a multicopy version of AXL2 (Table III). In summary, deletion of ERV14 leads to defects in haploid bud site selection, in recovery from stationary phase growth and in sporulation of diploid cells. The nonaxial budding phenotype may be suppressed by overproduction of Axl2p, whereas recovery from stationary phase growth and sporulation were not affected by the expression level of Axl2p. These pleiotropic effects suggest Erv14p functions in other cellular processes besides transport of Axl2p and that perhaps gene products involved in sporulation rely on Erv14p for efficient transport.

### Discussion

Organelles of the eukaryotic secretory pathway remain distinct in spite of dynamic anterograde and retrograde transport processes. Specific coat proteins that catalyze transport between the ER and Golgi are thought to contribute to this organization (Schekman and Orci, 1996), although the molecular mechanisms that link coat proteins with cellular sorting are not understood. Here we characterize a novel protein, termed Erv14p, that was discovered on ER-derived transport vesicles and appears to participate in sorting during ER to Golgi transport. Erv14p is an integral membrane protein that shares significant sequence identity (36%) and similarity (63%) over the entire length of the *Drosophila cornichon* gene product. Localization studies indicate that Erv14p resides in the ER and Golgi compartments of cells and is selectively packaged into ER-derived vesicles in a COPII-dependent manner. Disruption of the ERV14 gene produces viable haploid cells that exhibit defects in axial bud site selection.

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**Table III. Sporulation of Diploid Cells**

| Strain                  | Number of tetrads | n  |
|-------------------------|-------------------|----|
| CBY453 (WT)             | 39                | 200|
| CBY410 (erv14Δ/erv14Δ)  | 0                 | 200|
| CBY432 (erv14Δ/erv14Δ with pRS316) | 0 | 200|
| CBY433 (erv14Δ/erv14Δ with pRS-ERV14HA) | 34 | 200|
| CBY464 (erv14Δ/erv14Δ with p2μm-AXL2) | 0 | 200|

The results shown here are representative of three separate experiments. Sporulation was scored visually by looking for tetrads after 4 d in spmD medium. After 7 d, strains deficient for ERV14 still had not sporulated.
This phenotype correlates with the failure of erv14Δ strains to transport a bud site selection protein (Axl2p) to the cell surface. We propose that Erv14p regulates export of Axl2p and possibly other secretory proteins from the ER in COPII-coated vesicles as depicted in Fig. 13.

Erv14p is composed largely of hydrophobic amino acids and possesses three linear segments of adequate hydrophobicity to span a lipid bilayer (Kyte and Doolittle, 1982). Application of the “positive inside” rule (von Heijne and Gavel, 1988) predicts a topology such that the NH2 terminus is oriented toward the cytoplasm and the COOH terminus is oriented toward the lumenal compartment. The first putative loop (amino acids 29–51) displays a net negative charge, whereas positively charged residues are enriched in loop two (amino acids 81–111). This charge distribution is highly conserved between Erv14p and cornichon, lending some credence to this topology prediction, but there are exceptions to the positive inside rule, and it is not possible to draw a firm conclusion about the transmembrane arrangement of Erv14p at this time. Because Erv14p is detected in the ER, ER-derived vesicles, and Golgi membranes, it appears that this molecule cycles between early compartments of the secretory pathway, and we speculate that amino acid residues in this protein impart selective packaging into COPII- and/or COPI-coated vesicles. Characterized sequence motifs proposed to mediate incorporation into COPII-coated vesicles (Nishimura and Balch, 1997; Dominguez et al., 1998) or COPI-coated vesicles (Nilsson et al., 1989; Cosson and Letourneur, 1994) are not found in Erv14p. Therefore, selective incorporation of Erv14p into ER-derived vesicles could be through direct interaction of COPII with novel signal(s) contained on the cytoplasmic surface(s) of Erv14p or through association of Erv14p with other proteins coupled to the COPII budding machinery.

Surprisingly, deletion of ERV14 did not interfere with bulk secretory function but instead produced defects in yeast cell polarity. To comprehend this phenotype, we reviewed the literature concerning bud site selection in yeast. Haploid yeast cells exhibit an axial budding pattern whereby each new bud forms directly adjacent to the previous bud site. The selection of an appropriate bud site is thought to rely on a series of integrated events: first, the selected site for budding must be marked; second, components required for bud formation are assembled at this site; and third, the actin cytoskeleton and secretory apparatus are directed toward the selected site for growth of the emerging bud (Drubin, 1991). For the axial budding pattern, it has been proposed that this first stage is accomplished through a landmark that persists after cytokinesis and provides the cell with an assembly site for the next round of budding (Chant and Herskowitz, 1991; Snyder et al., 1991). Axl2p is unique among the characterized bud site selection proteins because it must be delivered to the plasma membrane via the secretory pathway. Once anchored in the plasma membrane, Axl2p may recruit additional components to the incipient bud site. In haploid axl2Δ strains, the axial budding pattern is lost, and a bipolar phenotype is observed such that 50% of the newly formed buds are placed at the opposite pole from the previous bud site (Halme et al., 1996; Roemer et al., 1996).

When we examined erv14Δ strains, we observed a nonaxial budding pattern and found that Axl2p accumulated in the ER. The accumulation of Axl2p in the ER provides a rational explanation for the nonaxial phenotype displayed by erv14Δ strains. Based on the following observations, however, we suspect that some Axl2p is slowly transported to the cell surface in erv14Δ strains. First, immunoblot analysis indicated that a low level of the mature form of Axl2p was detected in erv14Δ strains, although this species appears to be synthesized at a slow rate since it was not detected in pulse-chase experiments. Second, only 16% of the erv14Δ cells exhibit budding from both poles in contrast to a more typical bipolar pattern observed in axl2Δ strains (Halme et al., 1996; Roemer et al., 1996). This may be explained if erv14Δ cells deliver some Axl2p to incipient bud sites, but with suboptimal levels of plasma membrane Axl2p, a nonaxial phenotype is displayed intermittently. Third, we find that overexpression of Axl2p partially suppressed the nonaxial budding phenotype in erv14Δ strains (Table II). Again, this observation may be explained if some Axl2p trickles through the secretory pathway in an erv14Δ strain, and overexpression of Axl2p increases the level of this flow.

Could the role of Erv14p in yeast trafficking provide insights on the function of cornichon? The cornichon mutants display phenotypes that are similar to torpedo (EGF receptor) and gurken (TGFα homologue and putative torpedo ligand) mutants. These three genes are components of an intercellular signaling process between germ-line cells and surrounding follicle cells that establishes anterior–posterior polarity during early stages of oogenesis. Both gurken and cornichon are required in germ-line cells, whereas torpedo function is required in surrounding follicle cells of the developing egg chamber. The localized delivery of gurken protein to the plasma membrane of the oocyte presumably activates the EGF receptor torpedo in a subset of follicle cells and specifies their posterior fate. In turn, the polarized arrangement of the surrounding follicle cells provides spatial information to the oocyte and is

Figure 13. Model for Erv14p function in intracellular transport. In a wild-type strain (WT), Axl2p is efficiently packaged into ER-derived vesicles, and axial bud sites are established. In an erv14Δ strain, ER-derived vesicles are produced, but Axl2p fails to enter vesicles, and cells bud in a nonaxial manner.
critical for the reorganization of the microtubule network at mid-oogenesis. In cornichon, gurken, and torpedo mutants, intercellular signaling between the oocyte and surrounding follicle cells fails. Anterior markers are abnormally expressed in the posterior follicle cells, producing egg chambers that have the normal arrangement of oocyte and nurse cells but have duplicated anterior follicle cells at both poles. It is postulated that the gurken signal emanating from the oocyte represses anterior fates in adjacent follicle cells and directs them to a posterior fate (Gonzáles-Reyes and St. Johnston, 1994; Roth et al., 1995). In cornichon mutants, gurken mRNA expression and perinuclear localization is normal. Examination of gurken protein reveals that the expression level is normal, but cornichon egg chambers display a diffuse staining pattern for gurken protein at the oocyte membrane, unlike the tight stripe-like distribution at the dorsal–anterior corner of the oocyte observed in wild-type. Indeed, one explanation offered for this phenotype was that an altered polarity in cornichon egg chambers would cause less efficient membrane targeting of vesicles transporting gurken protein (Roth et al., 1995).

In light of our findings with erv14Δ strains, it is plausible that in cornichon mutants, a landmark on the oocyte cell surface is not established to correctly orient the secretory pathway. Much as the yeast cell uses Erv14p to export Axl2p from the ER and mark the axial bud site, we speculate that cornichon is required for export of a polarity establishment factor from the oocyte ER in Drosophila. Although there are no candidates for this factor at present, the high degree of homology shared between Erv14p and the cornichon gene product suggests this molecule may be related to Axl2p.

The mechanism by which Erv14p catalyzes Axl2p export from the ER remains to be determined, and we can envision several possibilities to explain this transport block. First, Erv14p could directly bind to Axl2p and escort this protein out of the ER, acting as an adaptor for incorporation of Axl2p into COPII-coated vesicles. Direct binding could also be involved in the folding of Axl2p or assembly of Axl2p into an oligomeric complex before exit from the ER, although we do not favor this possibility because proteins involved in these processes (e.g., BiP) appear to remain in the ER and are not selectively packaged into ER-derived vesicles (Salama et al., 1993; Barlowe et al., 1994; Rexach et al., 1994). Furthermore, the forms of Axl2p that accumulate in the ER of erv14Δ and sec12 strains are indistinguishable and are not rapidly degraded, suggesting that Axl2p is stably folded but unable to depart the ER. The erv14Δ phenotype resembles yeast strains that lack Shr3p, a resident ER protein that is required for the export of amino acid permease molecules in COPII-coated vesicles (Ljungdahl et al., 1992; Kuehn et al., 1996, 1998). However, the activities of Erv14p and Shr3p appear to be distinct because Erv14p is selectively packaged into COPII-coated vesicles, whereas Shr3p does not appear to enter these vesicles (Kuehn et al., 1996). Another set of ER proteins that produce selective transport defects (Vma12p, Vma21p, and Vma22p) are required for export of integral membrane subunits of the vacuolar ATPase (Hill and Stevens, 1994, 1995; Jackson and Stevens, 1997). It remains to be determined if these Vma proteins are selected for incorporation into COPII-coated vesicles. However, it has been proposed that Vma12p, Vma21p, and Vma22p function in the assembly of V-ATPase subunits in the ER and that assembly is a prerequisite for export from the ER (Jackson and Stevens, 1997). At present, we are aware of only three proteins that when deleted produce selective ER to Golgi transport defects and are enriched on COPII-coated vesicles: Emp24p (Schmöller et al., 1995), Erv25p (Belden and Barlowe, 1996), and Erv14p. We continue to explore the protein–protein interactions and mechanisms by which these molecules influence sorting during transport from the ER.

We thank Bill Belden for help with strain construction, Terry Roemer and Michael Snyder for generously providing AXL2 constructs, Hannele Ruohola and Trudi Schüpbach for helpful discussions on Drosophila development, and Sylvia Sanders for advice and sharing her unpublished observations.

This work was supported by grants from the National Institute of General Medical Sciences and the Pew Scholars Program in the Biomedical Sciences.

Received for publication 29 April 1998 and in revised form 9 July 1998.

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