Gene Inactivation in Lec35.1 (Mannosylation-defective) Chinese Hamster Ovary Cells

A CAUTIONARY NOTE*

(Received for publication, August 26, 1992)

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The most extensively characterized Lec35 line has been termed Lec35.1 (18). In this report, we examined the ability of these cells to express GPI-anchored proteins by transfection with a cDNA encoding the human folate-binding protein (FBP) (4), which has a well characterized GPI anchor (5), using the eukaryotic expression vector pBJ20. In contrast to normal cells, stable Lec35.1 transfectants failed to express FBP. However, this was not due to an inability to produce GPI anchors; in fact, the Lec35 mutation was found to be sufficiently leaky to produce small amounts of these structures (18). Rather, lack of FBP activity was caused by an unexpected inactivation of the FBP cDNA in every Lec35.1 transfectant that was examined. Genetic analysis of the Lec35.1 transfectants revealed that inactivation was time-dependent and associated with disruption of the transfected cDNA. The implications of these results for transfection experiments with other glycosylation mutants, as well as the similarity between the Lec35.1 mutation and the mutation causing the human disease paroxysmal nocturnal hemoglobinuria, are discussed.

EXPERIMENTAL PROCEDURES

Reagents—Sera, nutrients, antibiotics, and G418 sulfate (Geneticin) used to prepare culture medium were from Gibco. Hygromycin B was from Calbiochem. Bacillus cereus PI-specific phospholipase C was from Boehringer-Mannheim. Folic acid-free M199 medium and [H]folic acid (40 Ci/mmol) were gifts from B. Kamen, University of Texas Southwestern Medical Center at Dallas (UT-Southwestern).

Plasmids—pBJ20 (6.2 kb) is a member of the cytomegalovirus family (6) of eukaryotic expression vectors and was derived from the initial vector of this series, pCMV1. Starting with pTZ18R (Phar- macia LKB Biotechnology Inc.), pCMV1 was created in the laboratory of D. Russell (UT-Southwestern) by adding (in relative order as they exist in the plasmid) a fragment containing nucleotides -760 to +3 of the promoter/enhancer of the human cytomegalovirus major immediate early gene, a synthetic polynucleotidic acid, a fragment containing the transcription terminator and polyadenylation signal (nucleotides 1535-2157) of the human growth hormone gene, and a fragment from pUCD-X containing the SV40 origin of replication and early promoter/ enhancer (6). pCMV1 was further modified (C. Brewer and M. Roth, UT-Southwestern) by splicing a 1.3-kb fragment containing a neo- mycin resistance gene downstream of the SV40 early promoter, pBJ20 was created by P. Beck (UT-Southwestern) by altering a BglII site in the polynucleotide to form a unique EcoRI site and by inserting a 0.35-kb HindIII-BamHI fragment into the polynucleotide (downstream of the EcoRI site) containing the SV40 small T intron of pMSG (Pharmacia LKB Biotechnology Inc.).

p535 was constructed in S. Lacey’s laboratory (UT-Southwestern) by ligating a 0.99-kb EcoRI fragment encoding the human folate acid-binding protein cDNA (4) into the EcoRI site of pBJ20 in the sense orientation.

Cell Lines—Normal CHO cells (CHO-K1) were from ATCC (CCL 61). Lec35.1 cells were previously termed PIR and SwR-100 and are described in the accompanying paper (18) and in prior publications.
Wash and rinse were pooled, mixed with 10 ml of liquid scintillation and blotting were performed digestion, agarose gel electrophoresis, transfer to Pall A membranes, subjected to digestion with 30 units/ml PI-specific phospholipase C, ['H]folic acid-labeled monolayers were suspended by scraping and liquid scintillation counting. Supernatants obtained after digestion were collected and analyzed by.

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Cell Culture and Cell Fusion—Cells were normally maintained in Ham's F-12 medium buffered at pH 7.2 with 15 mM NaH2PO4 and containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2% fetal bovine serum, and 8% calf serum as described (1). This medium was used for transfection experiments as well. For experiments involving selection with 10 μg/ml concanavalin A, 10% fetal bovine serum was used in place of the fetal bovine/calf mixture. Cell fusion was performed exactly as described (2, 3) by selecting for resistance to G418 and hygromycin.

Transfection—The Polybrene method (7) was used to create stable G418-resistant transfectants. Normal CHO cells and Lec35.1 cells were transfected with either pJB2O or pF53. Transfectants were selected with 1 μg/ml G418. After 14 days, well resolved colonies were isolated with glass cloning cylinders and designated by the parental line, plasmid, and a letter to indicate the order picked, e.g. CHO-F53B. Several were further subcloned by limiting dilution and labeled with the number of the subclone, e.g. CHO-F53B2. As indicated in the text, pools of G418-resistant transfectants were sometimes taken. G418 was used during the first 3 weeks of selection after transfection and then omitted from the medium. During the course of these studies, pJB2O and pF53 transfectants were periodically re-screened with G418 and concanavalin A/swainsonine (1) to ensure that they retained the expected phenotype.

Assay and PI-specific Phospholipase C Digestion of Cell Surface Human Folic Acid-binding Protein—Assays were typically performed with 24-well plates as described (4), but they were also performed with larger culture vessels by increasing the volumes of the various wash and incubation solutions. Approximately 4 x 106 cells were plated in 24-well plates and allowed to grow 3–4 days until confluent. Plates were chilled on ice and all steps were performed with ice-cold media. Monolayers were washed twice with 1.5 ml of PBS and then treated for 30 s with 1.5 ml of acid saline (0.15 M sodium chloride adjusted to pH 3 with acetic acid) to displace bound folic acid that originated from the F-12 medium. After washing twice with 1.5 ml of PBS, monolayers were incubated with 1.2 ml of folic acid-free M199 medium containing 0.5 μM ['H]folic acid for 1 h with gentle rocking. The medium was removed and the monolayers washed again with 1.5 ml portions of PBS. At this point, some samples were used in PI-specific phosphohexose C assays (see below). Bound ['H]folic acid was recovered by incubating the monolayers with 1.5 ml of acid saline for 30 s and rinsing the wells with 1 ml of PBS. The acid saline wash and rinse were pooled, mixed with 10 ml of liquid scintillation fluid and counted in a liquid scintillation counter. Nonspecific background was determined by omitting the first acid-saline wash and was subtracted out. The cellular FBP gene does not express detectable amounts of FBP.

To determine the specificity of FBP to PI-specific phospholipase C, ['H]folic acid-labeled monolayers were suspended by scraping and subjected to digestion with 30 units/ml PI-specific phosphohexose C at 37°C in the presence of leupeptin and phenylmethylsulfonyl fluoride essentially as described earlier (5). Both the cell pellets and supernatants obtained after digestion were collected and analyzed by liquid scintillation counting.

Southern Blotting—Isolation of genomic DNA, restriction enzyme digestion, agarose gel electrophoresis, transfer to Pall A membranes, and blotting were performed as described earlier (8).

RESULTS

FBP cDNA Is Stably Expressed in Normal CHO Transfectants but Not in Lec35.1 Transfectants—In order to test for GPI anchor biosynthesis in the Lec35.1 mutant, we chose to express the human FBP (4) by ligating FBP cDNA into the expression vector pJB2O. The noteworthy features of this vector include a cytomegalovirus promoter/enhancer to drive expression of the cloned cDNA (6), a SV40 small T-antigen intron to enhance RNA expression, a marker for G418 resistance, and a polylinker including an EcoRI site. The resulting construct, pF53, was transfected into normal or Lec35.1 CHO cells, and individual transfected colonies were isolated by selection for G418 resistance to ensure that each contained the transfected cDNA. Well resolved transfectants were picked and in some cases subcloned by limiting dilution. Throughout these studies, transfectants were periodically re-screened both for G418 resistance to ensure that they contained the plasmid and for concanavalin A/swainsonine resistance (1) to ensure that they had the expected phenotype. Each transfectant was then assayed for binding of [3H]folic acid as described under "Experimental Procedures." Fig. 1 shows the results obtained with normal and Lec35.1 cells transfected with pF53. Two observations can be made. First, the expression levels among normal transfected cells varied greatly, over a range of 30-fold. In addition to the three normal transfected cells, 10 others were assayed, and all were found to have FBP activities within this range. This variation of expression was not surprising; in our hands, stable expression of N-acetylglucosamine-1-phosphate transferase subcloned into pJB2O and transfected in CHO cells varied 40-fold (9).

Second, the expression of FBP activity in all Lec35.1 transfected cells was much lower than in normal cells and was generally insignificant (less than 2% of the positive control value), as compared with the background binding in normal and Lec35.1 vector-transfected controls. This includes the eight Lec35-F53 transfected cells shown in Fig. 1 and six others (data not shown).

To determine whether or not the absence of FBP activity in Lec35.1 transfectants was due to the phenotypic effects of the Lec35 mutation, presumably by preventing GPI lipid biosynthesis, several FBP-transfected Lec35.1 lines were fused to normal hygromycin-resistant CHO cells (followed by selection with G418 and hygromycin) to correct the Lec35 defect. It has been shown that fusion of Lec35 cells with normal CHO cells efficiently corrects the Lec35 mutation by complementation (2, 3). As shown in Table I, none of six independent Lec35.1 FBP transfectants exhibited increased FBP activity after fusion with normal CHO cells. As a control, a normal CHO FBP-transflectant that had high activity yielded a hybrid that was active after fusion with normal CHO.

Fig. 1. Binding of [3H]folic acid by normal and Lec35.1 transfectants. Binding assays were performed in duplicate as described under "Experimental Procedures." Data are presented as the log of percent binding with respect to binding by the CHO-F53B2 transfectant. Left panel, for normal cells, one vector transfectant (CHO-JB20A2) and three FBP transfectants (CHO-F53B2, G, in order of decreasing binding) were assayed. Right panel, for Lec35.1 cells, one vector transfectant (Lec35-JB20A1) and eight FBP transfectants (Lec35-F53A1, B1, C1, J, K, M, N, O) were assayed. The limit of sensitivity was estimated to be 2% of the binding by the CHO-F53B2 transfectant, which was 1592 cpm.

2 P. Stanley, personal communication.
were plated in duplicate wells of a 24-well plate and the values were given. The 100% value was 24,000 cpm, and the limit of sensitivity of these assays was estimated to be 0.5% of this value.

Transfectant hybridized Relative FBP activity %
CHO-F53B2 100
CHO-JB20 <0.5
Lec35-F53A1 <0.5
Lec35-F53B1 <0.5
Lec35-F53C1 <0.5
Lec35-F53p <0.5
Lec35-F53K <0.5
Lec35-F53N <0.5
Lec35-F53O <0.5
Lec35-JB20 <0.5

* One transfectant, Lec35-F53J, was unusual in that it initially had a low level of activity (not shown) and rise to a hybrid that also had measurable activity (shown above). After additional time in culture, Lec35-F53J lost its activity (see Fig. 1). Therefore, Lec35-F53F appears to have been a Lec35 transfectant with an unusually slow rate of FBP inactivation.

cells. This result suggested that the absence of FBP activity in Lec35.1 transfectants was not due to a post-translational defect affecting the synthesis of GPI anchor structures. In fact, as will be shown in the next section, under the appropriate conditions, transfected Lec35.1 cells were able to express PI-specific phospholipase C-sensitive FBP.

Lec35.1 FBP Transfectants Express FBP Activity Soon after Transfection—The results of the previous section could be explained by one of two possibilities. It was possible that during the transfection process and chromosomal integration, the FBP cDNA was destroyed in each of the Lec35.1 transfectants. Alternatively, the FBP cDNA in Lec35.1 transfectants could have been initially active after transfection, followed by a loss of activity later on.

To determine whether the FBP cDNA in Lec35.1 cells was functional at early times after transfection, pooled colonies of stable Lec35.1 transfectants were assayed 3 weeks after transfection. In the experiments described in Fig. 1, individual transfected colonies were analyzed, and in general, they were not assayed until at least 2 months after transfection. As indicated in Table I, the Lec35.1 transfectant pool expressed significant amounts of FBP activity 3 weeks after transfection, although the activity diminished with time. The FBP activity was susceptible to PI-specific phospholipase C digestion, indicating the presence of a GPI anchor structure. Thus, as indicated in the accompanying paper (18), the Lec35.1 allele was sufficiently leaky to allow production of some GPI-anchored FBP. The basis for the fraction of FBP that was releasable without PI-specific phospholipase C (about 18% from normal transfectants, 33–42% from the Lec35.1 transfectant pool) is not clear, but may represent FBP that failed to acquire a GPI anchor and was therefore easily displaced from the cell surface.

From the data in Table II and other similar experiments (data not shown), we estimated that the half-time for inactivation was somewhere between 1 and 2 weeks. Unfortunately, it was not feasible to obtain a more accurate rate because of several sources of error. For example, it must be assumed that the relative fractions of individual transfectants that make up the pool fluctuate with time. We also found that the FBP activities of transfected cells can vary up to 3-fold, depending on the cell densities and the length of time cells are maintained after reaching confluence. This was particularly difficult to control because different transfectants would be expected to plate and grow at different rates.

Loss of FBP Activity in Lec35.1 Transfectants Is Associated with Rearrangement of the Transfected FBP cDNA—The loss of FBP activity could be explained by either a structural mutation of the transfected FBP cDNA or a transcriptional suppression such as that caused by imprinting. The results of Fig. 2 demonstrate that in every Lec35.1 transfectant, the FBP cDNA underwent extensive rearrangement. Genomic DNA was isolated from 13 transfectants (including three CHO-F53 transfectants and eight Lec35-F53 transfectants), digested with EcoRI, and probed with [32P]FBP cDNA fragment (17) encompassing the entire coding region. The positions of the 1.0-kb EcoRI-EcoRI fragment that resulted from transfected, unarranged FBP cDNA and the fragment of the cellular FBP gene are indicated by the open and closed arrowheads, respectively.

Fig. 2. Southern blot analysis of genomic DNA from normal and Lec35.1 transfectants. Genomic DNA was isolated from various transfectants as indicated and digested with EcoRI. 10-μg samples were fractionated on a 1% agarose gel, transferred to Pall A membrane, and probed with [32P]FBP cDNA fragment (17) encompassing the entire coding region. The positions of the 1.0-kb EcoRI-EcoRI fragment that resulted from transfected, unarranged FBP cDNA and the fragment of the cellular FBP gene are indicated by the open and closed arrowheads, respectively.

| Cell          | Time after transfection | Relative FBP activity | Released by PI-specific phospholipase C |
|---------------|-------------------------|-----------------------|----------------------------------------|
| Normal        | 55 days                 | 100                   | 18                                     | 96                                      |
| Lec35.1       | 22 days                 | 34                    | 33                                     | 100                                     |
| Lec35.1       | 31 days                 | 8                     | 42                                     | 100                                     |
| Lec35.1       | 55 days                 | <2                    | ND                                     | ND                                      |

TABLE II
Effects of time in culture and PI-specific phospholipase C treatment on FBP activities in normal and Lec35.1 transfectants

A stable normal FBP transfectant (CHO-F53B2) and a pool of Lec35.1 FBP transfectants (a mixture of approximately 25 colonies) were assayed for [3H]folic acid binding at various times after transfection of the Lec35.1 cells as described under "Experimental Procedures." Where indicated, release of receptor-bound [3H]folic acid after treatment with (+) or without (-) PI-specific phospholipase C was assayed. The binding by the CHO-F53B2 transfectant was arbitrarily defined to be 100% at each time point. The values shown are the results of duplicate assays. ND, not determined.
It is possible that the apparently intact copy in Lec35-F530 is actually an abnormal fragment that comigrates with the normal fragment. Interestingly, two pairs of Lec35.1 transfectants (Lec35-F53A1, C1; Lec35-F53J, M) had abnormal fragments of the same size, suggesting that "hot spots" for rearrangement may exist. Since each of these lines remained resistant to G418, they all retained some portion of the vector.

Compared with the transscripts in CHO-F53B2 and CHO-F53F, which were of normal size, RNA blot analysis of five Lec35.1 transfectants revealed either the absence of detectable FBP mRNA (Lec35-F53B, J, K) or an abnormally truncated mRNA (Lec35-F53A1, C1) (data not shown), giving further support to the conclusion that a structural alteration of the FBP cDNA had occurred.

**DISCUSSION**

This study was initiated to assess GPI anchor production in Lec35.1 cells. As shown in the accompanying paper (18), these cells are defective for MPD-dependent mannose transferase reactions of GPI lipid biosynthesis and dolichol-linked oligosaccharide biosynthesis. The initial experiments with cloned cDNA for FBP, a GPI-linked protein, appeared to support this conclusion because stable Lec35.1 transfectants did not express FBP activity. However, subsequent experiments showed that this was due to inactivation of the FBP cDNA. In fact, it was shown that prior to cDNA inactivation, the Lec35 mutation was leaky enough to allow GPI-anchored FBP to be produced in Lec35.1 cells.

It should be stressed that the choices of the pJB20 expression vector and FBP cDNA were based only on technical considerations, without regard to their potential for gene rearrangement. In cases involving normal CHO cells, either human FBP (Fig. 1) or hamster GlcNAc-1-P transferase cDNA (9), pJB20 proved to be an effective expression vector with no indication of rearrangement. Additional studies will be required to determine which features of the vector and/or cDNA are required for the observed rearrangements in Lec35.1 cells. This would clearly include a survey of several different cDNAs and vectors. It will also be necessary to map the rearrangements and "reconstruct" the event, as has been done for gene deletions involving Alu-repetitive elements (8).

The obvious implication of these results is that caution must be employed when expression of proteins from transfected cDNA is used to assess post-transcriptional or post-translational events in mutant cell lines. This also includes expression cloning strategies because it may not be possible to rescue an intact plasmid. Since it is not clear to what extent the mannosylation defect is responsible for gene inactivation, investigators studying glycosylation mutants should be particularly cautious.

The gene inactivation phenotype that has been described here is quite distinct from mutator phenotypes that have been described previously in CHO cells (10, 11). In these cases, the phenotypes were due to alterations in nucleotide synthesis (10) or DNA synthesis (11), leading to mutations in the DNA. In these mutator strains, the mutation frequencies for marker genes generally increased 10–100-fold, as compared with normal cells (10, 11), to reach mutation frequencies in the range of $10^{-4}$ (10). However, this does not compare with the mutation frequency for FBP cDNA in Lec35.1 cells because an absence of FBP activity was observed in every Lec35.1 transfectant.

The actual basis for the observed gene inactivation is unclear, and at least two possibilities should be explored. In one model, a MPD-dependent reaction may be required for maintenance of certain genes. Nuclear glycoproteins have been described (12), and thus it is possible that a nuclear protein required for repair synthesis of DNA must be mannosylated for function. The MPD could be synthesized in the endoplasmic reticulum and be transported to the nuclear envelope by contiguous membranes. Such a reaction would be prevented in a Lec35 mutant. Another model would require inactivation of a "primary" gene that is required for maintenance of a group of "secondary" genes, including the Lec35 gene and genes with sequences similar to those found in pF53.

It is worth noting that Lec35.1 was isolated by a gradual acquisition of phenotype (Fig. 3, Ref. 1) on a time scale that was similar to the inactivation of the FBP cDNA. Thus, the original selection that gave rise to the Lec35.1 cells may have indirectly selected for the loss of a primary maintenance gene.

The effect of the gradual selection with diploid CHO cells would have been to allow the sequential mutation of both copies of the primary gene, followed by inactivation of the secondary genes. It should be possible to distinguish between these two models by repeating this study with Lec35.2 cells (18), which were not isolated by gradual selection, and Lec15 cells, which fail to make MPD. Although attempts have been made, we have never been able to isolate a revertant of Lec35.1, so we were not able to determine if the Lec35.1 phenotype strictly correlated with gene inactivation.

One unexpected consequence of this work is to bring to light the similarity between the phenotype of the Lec35.1 mutant and the defect in the human disease paroxysmal nocturnal hemoglobinuria (PNH) (13). This similarity exists at both the biochemical and genetic levels. Biochemically, it has been shown that hematopoietic cells from individuals with PNH are deficient for GPI-anchored proteins. This includes erythrocytes, platelets, lymphocytes, and granulocytes, and clinical manifestations appear to result from the extreme sensitivity of the PNH red cells to complement-mediated hemolysis, due to a deficiency of GPI-anchored decay accelerating factor (13). As a result, PNH patients suffer from anemia and frequent venous thrombotic events and are generally expected to survive less than 10 years. Biochemical studies indicate that synthesis of GlcNAc-Pi and GlcN-Pi is normal in PNH polymorphonuclear cells (14, 15), but that intact PNH granulocytes fail to make the mature GPI anchor precursor lipid, even though they appear to synthesize MPD (15, 16). Thus, one or more of the mannosylation reactions required for the GPI anchor precursor are suspect.

This biochemical phenotype is consistent with the one in Lec35 cells, in which the first mannosylation reaction is
defective in vivo. Clearly, additional biochemical studies on PNH cells will be necessary to test this hypothesis. Since the Lec35 mutation may affect all three MPD-dependent mannosylation reactions in GPI lipid biosynthesis, it might be expected that cells from patients with differing extents of the disease would accumulate different GPI intermediates that vary by the number of mannose residues.

The genetic defect in Lec35.1 cells may also help to explain the unusual genetics of PNH. PNH is most likely caused by a somatic mutation in a clone of a totipotent stem cell, giving rise to abnormalities in all hematopoietic cells that arise from this clone (13). PNH patients have mixtures of normal (PNH1), moderately affected (PNHII), and severely affected (PNHIII) cells, which presumably arise from different stem cell clones. The proportions of these populations change with time. Interestingly, the PNHII population has been noted as particularly unstable, and appears to give rise to PNHIII cells (for example, see Fig. 31.6 in Ref. 13). This change in severity of the mutation is reminiscent of that observed in the Lec35.1 cells. Thus, PNH1, PNHII, and PNHIII clonal cells could differ by the extent of inactivation of the required gene (possibly the Lec35 homologue).

Acknowledgments—We thank Dr. Barton Kamen, Dr. Stephen Lacey, Dr. Pamela Beck, and Dr. Richard Lark, all of this institution, for [3H]folic acid and binding medium, for pF53, for pJB20, and for performing RNA blot analyses, respectively.

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