Vol. 186, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1992 Pages 102-113

Novel Glycosylation-Defective Baby Hamster Kidney Cells

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Received May 18, 1992

The plant lectin wheat germ agglutinin (WGA) has previously been used to select more than ten different glycosylation-defective phenotypes in a variety of mammalian somatic cells. Three WGA-resistant phenotypes have now been obtained spontaneously from baby hamster kidney (BHK) cells. These mutant BHK cells exhibit a pattern of cross resistance and sensitivity to multiple plant lectins, suggesting that the cell surface carbohydrates of these cells are altered. Two WGA-resistant BHK phenotypes appear similar to WGA-resistant CHO cells that lack terminal sialic acid and galactose residues on their cell surface carbohydrates. The third WGA-resistant BHK cell phenotype has not previously been seen in WGA-resistant mammalian cells.

The plasma membrane of animal cells contains carbohydrate molecules covalently linked to a variety of proteins and lipids. One way to study the structure and function of these cell surface carbohydrates has been to isolate glycosylation-defective mutant

0006-291X/92 $4.00

cells. These somatic cell mutants have been used to examine the pathways of carbohydrate biosynthesis (1), metastasis (2,3) and the molecular cloning of glycosyltransferases (4,5). Glycosylation-defective cell lines have predominantly been derived by selection for resistance to cytotoxic plant lectins (1).

Multiple glycosylation-defective mammalian somatic cell lines have been selected for resistance to the plant lectin wheat germ agglutinin WGA (1). These WGA-resistant (WGA^R) lines have been particularly useful in studies on natural killer (NK) cell recognition (6), insulin receptor activity (7) and metastasis (8). The possibility of selecting a large number of different glycosylation-defective mutants using a single lectin suggested the utility of WGA as a cytotoxic agent to select novel glycosylation-defective BHK cells.

In this paper, we report the selection of spontaneous glycosylation-defective BHK lines. Three mutant phenotypes were obtained that appear to be affected in cellular glycosylation.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The parental baby hamster kidney (BHK) cell line BHK 21/c13 was a gift from Dr. Clayton Buck. Parental Chinese hamster ovary (CHO) cells were obtained from Dr. Pamela Stanley. Cells were cultured in monolayer in alpha medium containing 10% fetal calf serum and antibiotics (GIBCO, Grand Island, NY) at 37°C in a humidified atmosphere with 5% CO_2.

Lectins. The lectins: WGA (wheat germ agglutinin, from T. vulgaris); Con A (Concanavilin A, from C. ensiformis); Ricin (toxin from R. communis); LCA (from L. culinaris); E-PHA (erythroagglutinin from P. vulgaris); RCA-I (agglutinin from R. communis); PSA (from P. sativum); BSL- I B4 (isolectin from B. simplicifolia); DBA (from D. biflorus); SBA (from G. max); UAE-1 (from U. europaeus); PNA (from

A. hypogaea); BSL-1 (from B. simplicifolia); SJA (from S. japonica); and SuWGA (succinylated WGA) were purchased from Vector Labs, Burlingame, CA; The lectin L-PHA (leukoagglutinin from P. vulgaris) was obtained from Burroughs Wellcome, England; and BSL-II (isolectin from B. simplicifolia) was purchased from Sigma Chemical Co., St. Louis, Missouri. Lyophilized lectins were rehydrolyzed in 0.15M NaCl, 10mM Na_2HPO_4, pH7.2 (PBS), filter sterilized and stored at 4°C.

Selection Protocols. Cells were aliquoted at $5x10^5$/100-mm tissue-culture dish into alpha medium with 10% FCS and antibiotics overnight at 37°C in a humidified atmosphere with 5% CO_2. The next day, the cells had doubled (10^6 cells/100-mm dish) and selective lectin was added. After 8 days at 37°C, the surviving colonies were picked into sterile 12 x 75-mm tubes in nonselective medium. After 6-7 days at 37°C, cells were transferred to T-25 tissue-culture flasks and grown in monolayer.

Determination of Lectin-Resistance. Two rapid screening assays were used to determine the lectin-resistance properties of surviving colonies. Colonies picked into 12 x 75-mm tubes were examined after growth in nonselective media for 4 days at 37°C. A 0.5ml aliquot of cells (10^3 cells) were transferred to a 24-well plate in the presence or absence of selective lectin in a total volume of 1ml. An alternative assay plated 0.1ml cells ($2x10^2$ cells) in 96-well microtiter dishes in the presence or absence of lectin in a final volume of 0.2ml. After 4-5 days at 37°C, plates from both screening assays were stained with 0.2% methylene blue (Fisher Scientific Co., Pittsburgh, PA) in 50% methanol and scored for growth compared to parental BHK cells. Colonies resistant to the selective lectin were then tested by the semiquantitative P test (9) against several lectins.

For the P test, 500 cells were plated in 96-well microtiter plate and grown at 37°C overnight. The presence of a range of

lectin concentrations were then added to individual wells. After 4 days at 37°C, plates were stained with methylene blue. Colonies exhibiting novel lectin-resistant phenotypes were cloned by limiting dilution in 96-well microtiter plates.

Lectin-resistant clones were tested for their quantitative resistance or sensitivity to lectins by P test and D10 analysis (the concentration of lectin that reduces cell survival to 10%). For D10 analysis, 30 or 300 cells were plated in 24-well tissue-culture plates overnight at 37°C. The next day, the presence or absence of a range of lectin concentrations were added to individual wells. Cells were grown for 8 days at 37°C and the plates were then stained with methylene blue. Colony growth was scored and the relative plating efficiency was corrected for the growth of cells in the absence of lectin.

Galactosyltransferase Activity Assay. Cell sonicates were incubated with UDP-[3-^{3}H]-galactose, N-acetyl-D-glucopyranosylamine, ADP, $MnCl_2$ and a trisaccharide acceptor, as previously described (10) for 1 hour at 37°C. Radiolabeled product was isolated and counted by liquid scintillation (11).

RESULTS

Lectin-Resistance Properties of Hamster Cells. At least 10 different wheat germ agglutinin-resistant (WGA^R) glycosylation-defective cell lines have been isolated from CHO cells (1). The lectin-resistance (Lec^R) phenotype of parental CHO and BHK cells were compared in order to determine if the carbohydrates expressed on the cell surfaces of CHO and BHK cells were similar. BHK cells are twofold resistant to WGA compared to CHO cells (Table 1). In addition, BHK cells are also more resistant to the lectins L-PHA, LCA and Ricin (Table 1). The different Lec^R phenotypes of the hamster cells suggested the possibility that WGA might select

TABLE 1

LECTIN-RESITANCE PROPERTIES OF HAMSTER CELL LINES

	D10 (μg/ml)				
Cell Line	WGA	L-PHA	Con A	Ricin	LCA
BHK	4.2	10.2	14	0.072	45
CHO	2.2	2.1	18	0.007	16
	FOLD-RESISTANCE				
BHK	2	5	WT	10	3

The lectin-resistance properties of BHK and CHO cells were examined by D10 analysis as described in Materials and Methods. The fold-resistance of BHK cells to lectin are compared to CHO cells. WT, wild type level of resistance.

novel lectin-resistant BHK cell mutants not previously isolated from CHO cells.

Selection of WGA-resistant BHK Cells. Two WGA^R phenotypes were isolated in selection 1 (Table 2). Colonies survived the selection at a frequency of approximately 10^{-6}. Two colonies were cloned by limiting dilution, and two clones W1.1 and W1.4, were found to be greater than tenfold WGA^R. A slightly WGA^R phenotype was obtained using a decreased concentration of WGA in selection 2 (Table 2). Clone W4.2 showed approximately 2-fold resistance to WGA.

Sensitivity of Lec^R BHK Cells to Plant Lectins. Glycosylation-defective mutants exhibit unique phenotypes of cross-resistance and sensitivity to various plant lectins (1). Parental BHK and the Lec^R BHK clones were rapidly screened for resistance and sensitivity against a panel of sixteen plant lectins using the semiquantitative P test. Mutants cells with increased sensitivity to a lectin, compared to parental cells may indicate a

TABLE 2

SELECTION OF LECTIN-RESISTANT BHK CELLS

Selection Number	Cells	WGA (μg/ml)	Frequency Surviving Colonies	WGA-Resistant Phenotype of Clones WGA	Clone
1	BHK	20	8.9x10-7	R>10	W1.1
				R>10	W1.4
2	BHK	15	7.5x10-6	R2	W4.2
3	BHK	25	<2.7x10-7	ND	None

BHK 5 cells were used to obtain spontaneous lectin-resistant mutants by plating in lectin for eight days. Surviving colonies were picked into nonselective medium, cultured, and tested for resistance to WGA by the P test as described in Materials and Methods. Lectin-resistant colonies were cloned by limiting dilution and the clones were shown to be WGA resistant. R, -fold resistant compared to parental BHK cells; ND, not determined.

carbohydrate structure present on the cell surace of the Lec^R line that is not present on the parent cell surface. On the other hand, resistance to a lectin suggests the absence or reduction of the oligosaccharide binding determinant for that lectin.

Clones W1.1 and W1.4 are WGA- and L-PHA-resistant compared to parental BHK cells. W1.1 was observed to be highly BSL-II-sensitive and E-PHA-resistant, while W1.4 is LCA-sensitive compared to parental BHK (Table 3). Clone W4.2 exhibited approximately twofold WGA-resistance and Ricin- and LCA-sensitivity compared to parental BHK cells. The lectins: PSA, BSL-I B4, DBA, SBA, UAE-I, PNA, BSL-I and SJA exhibited little or no cytotoxicity to parental BHK and the Lec^R BHK cells isolated (Table 3). The patterns of resistance and hypersensitivity to multiple lectins

TABLE 3

SENSITIVITY OF PARENTAL AND WGA-RESISTANT BHK CELLS TO PLANT LECTINS

	Lectin-Sensitivity (μg/ml)			
Lectin	Parental BHK	W1.1	W1.4	W4.2
WGA	3-6	>30	>90	6-9
L-PHA	10-20	>90	>90	10-20
Ricin	0.1-0.3	0.12	0.05-0.25	0.05-0.25
Con A	20-30	30-40	30	10-20
LCA	>160	>160	80	40
BSL-II	>200	1-10	ND	>200
E-PHA	1-10	>200	"	1-10
PSA	50-200	50-200	"	50-200
BSL-I B4	>200	50-200	"	>200
DBA	>200	>200	"	>200
SBA	>200	>200	"	>200
UAE-1	>200	>200	"	>200
PNA	>200	>200	"	>200
BSL-I	>200	>200	"	>200
SJA	>200	>200	"	>200
SuWGA	>200	>200	"	>200

Parental BHK and WGA-resistant BHK clones were tested for sensitivity to plant lectins by the P test as described in Materials and Methods. ND, not determined.

suggest that the Lec^R BHK cells express altered cell surface carbohydrates and are therefore, glycosylation-defective mutants.

The Lec^R BHK cells were examined by D10 analysis against a panel of six lectins in order to quantitate the level of resistance or hypersensitivity. Clones W1.1 and W1.4 are 4-fold L-PHA-resistant, 2-fold Con A-resistant and >200-fold BSL-II-sensitive. W1.1 is slightly LCA-resistant, while W1.4 is

TABLE 4

LECTIN-RESISTANCE PROPERTIES OF PARENTAL AND WGA-RESISTANT BHK CELLS

	D10 (μg/ml)					
Cell Line	WGA	L-PHA	Ricin	Con A	LCA	BSL-II
W1.1	>150	45	0.102	25.0	66	1.5
W1.4	127	44	0.095	23.0	26	1.7
W4.2	6.3	7.2	0.036	13.0	49	ND
Parental BHK	4.2	10.2	0.072	14.0	45	>400

	FOLD-RESISTANCE					
	WGA	L-PHA	Ricin	Con A	LCA	BSL-II
W1.1	R>35	R4	(R)	R2	(R)	S>250
W1.4	R30	R4	-	R2	S2	S>235
W4.2	(R)	(S)	S2	-	-	ND

The lectin-resistance properties of parental BHK and WGA-resistant BHK cells were examined by D10 analysis as described in Materials and Methods. The quantitative D10 value for each lectin is shown (top panel). The fold resistance or sensitivity of each WGA-resistant BHK cell line is compared to parental BHK cells for each lectin (bottom panel). R, -fold resistance; S, -fold sensitivity; ( ), less than twofold resistant or sensitive; -, not significantly different from parental cells; ND, not determined.

2-fold LCA-sensitive. And whereas W1.4 is 30-fold WGA-resistant, W1.1 is greater than 35-fold WGA-resistant (Table 4). The highly WGA^R and $BSL\text{-}II^S$ phenotype of W1.1 and W1.4 appears similar to that of Lec8 CHO cells. Lec8 CHO are 100-fold WGA^R compared to parental CHO (1) and approximately 12-fold $BSL\text{-}II^S$ (J. Ripka and P. Stanley, unpublished observation).

The final Lec^R BHK cell isolate, W4.2, is slightly WGA^R. At the same time W4.2 is 2-fold Ricin-sensitive, slightly L-PHA-sensitive, and exhibits parental sensitivity for both Con A

TABLE 5

GALACTOSYLTRANSFERASE ACTIVITY OF WGA-RESISTANT BHK CELLS

Cell Line	Specific Activity (pmol/mg*h)	Relative Activity (%)
Parental BHK	8.53	100.0
W1.1	10.38	121.7
W1.4	8.19	96.0
W4.2	9.14	107.2

Cell sonicates were incubated with UDP-[3H]-Gal and a synthetic trisaccharide (Ref. 10) acceptor specific for galactosyltransferase activity.

and LCA (Table 4). The Lec^R phenotype of W4.2 does not appear to be similar to any previously isolated Lec^R hamster cell lines.

Galactosyltransferase Activity of Lec^R BHK Lines. The lectin-resistance phenotype of W1.1 and W1.4 suggested these cells terminated in N-acetylgucosamine residues. This could be due to the lack of glactosyltransferase activity. Enzyme assays indicated that W1.1 and W1.4 exhibited *in vitro* galactosyltransferase activity similar to parental BHK cells (Table 5). The W4.2 cell line was also unaffected in galactosyltransferase activity (Table 5).

Revertion of Glycosylation-Defect. The sensitivity of W1.1 and W1.4 cells to BSL-II suggested a possible experiment to isolate revertants of these lines. Parental BHK cells survive selection in BSL-II (Selection 1, Table 6). On the other hand, W1.1 cells do not proliferate in the presence of BSL-II (Selection 2, Table 6). In order to determine if the BSL-II selection is suitable to recover revertants with the parental BHK phenotype, cell mixing experiments were utilized. Small numbers of parental phenotype BHK cells were recovered in the presence of a large quantity of W1.1 cells (Selection 3, Table 6). This suggested that even a single

TABLE 6

REVERSION OF WGA-RESISTANT BHK CELLS

Experiment	W1.1 Cells	BHK Cells	Plates	Colonies	Frequency
1	0	5	2	8	0.8
2	10^6	0	25	0	$<4x10^{-8}$
3	10^6	5	2	10	1.0

WGA-resistant BHK and parental BHK cells were plated separately or together, as noted above, in the presence of 20-40 μg/ml BSL-II for 9 days. The total number of colonies surviving the selection were counted after staining plates with methylene blue as described in Materials and Methods.

revertant in the presence of 10^6 W1.1 would be recovered. No spontaneous revertants of W1.1 were obtained using the BSL-II selection system. The frequency of spontaneous revertion was therefore, determined to be $<4 \times 10^{-8}$ (Table 6).

DISCUSSION

Three new lectin-resistant BHK phenotype were spontaneously isolated by selection with WGA. Two phenotypes are qualitatively similar and highly WGA-resistant, whereas the third is slightly WGA-resistant. Several intragenic glycosylation-defective mutants have been previously isolated. These mutants exhibit qualitatively similar lectin-resistant phenotypes that differ in quantitative resistance and sensitivity to lectins. The similar Lec^R phenotypes of W1.1 and W1.4, suggest these may also be intragenic mutants.

The high level of resistance to WGA (which recognizes terminal sialic acid residues) and the high sensitivity to BSL-II (which recognizes terminal N-acetylglucosamine residues), suggest these cells lack terminal sialic acid and galactose residues on their ASN-linked oligosaccharides and instead are terminated in

N-acetylglucosamine residues. Clones W1.1 and W1.4 have a similar lectin-resistance phenotype to the Lec8 CHO cell line. The enzyme defect in Lec8 CHO cells has been localized to a deficiency in the transport of UDP-Gal into the golgi (12). As a result cell surface strucutures lack terminal galactose and sialic acid residues and terminate in N-acetylglucosamine residues in Lec8 CHO. Terminal N-acetlyglucosamine residues could also result from the absence of galactosyltransferase activity. However, clones W1.1 and W1.4 exhibit in vitro galactosyltransferase activity. Consequently, the enzyme defect in W1.1 and W1.4 may be a deficiency in the transport of UDP-Gal into the golgi, similar to Lec8 CHO or a completely different mutation.

The third WGA^R BHK line, W4.2, exhibits little sensitivity or resistance to plant lectins. The slight WGA^R, and Ric^S phenotype suggest, that these cells may be deficient in a single type of terminal sialic acid residues on either glycoproteins or glycolipids.

The new Lec^R BHK cells will prove particularly valuable in studying the role of cell surface carbohydrates in tumorigenicity and metastasis since they have been isolated without the use of mutagens. These Lec^R BHK cells can be injected into syngeneic hamsters, which can serve as hosts for tumor formation, unlike Lec^R CHO cells which require immunodeficient hosts for tumor formation (13).

These new Lec^R mutants may be used to directly clone the altered glycosylation enzyme activity without the need to purify the enzyme activity first. The low revertion frequency of W1.1 suggests this cell line may be utilized as a recipient of the gene coding for the glycosylation activity affected. DNA-mediated transformation followed by lectin selection with BSL-II may isolate transformants containing the gene of interest as has been accomplished for other glycosyltransferase activities (4,5).

ACKNOWLEDGMENT

This research was supported by National Institutes of Health grant CA 35377.

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