A link between ferritin gene expression and ribonucleotide reductase R2 protein, as demonstrated by retroviral vector mediated stable expression of R2 cDNA

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Abstract **We have constructed a retroviral expression vector for the mammalian ribonucleotide reductase R2 component. Stable infectants, which express a *myc* epitope tagged R2 protein from the vector cDNA were obtained and described for the first time. Cells containing the recombinant protein exhibited increased ribonucleotide reductase activity, and were resistant to the antitumour agent hydroxyurea, which targets the R2 component of ribonucleotide reductase. Furthermore, a direct link between ferritin gene expression and R2 protein was observed, since cells containing vector expressed recombinant R2 protein exhibited increased H-chain and L-chain ferritin gene expression.**

Key words: Ferritin; DNA synthesis; Ribonucleotide reductase; Gene expression

1. Introduction

Iron is required for the activities of proteins involved in DNA synthesis, electron transport and oxygen metabolism [1]. However, iron is insoluble under physiological conditions so that specialized proteins, the ferritins, are needed to maintain it in an available form [1,2]. Ferritin is composed of 24 subunits that are the products of ferritin (H)-chain and ferritin (L)-chain genes, and they can combine in different proportions to produce families of isoferritins that exhibit differences in synthesis and turnover rates, and in the ability to accept or release iron [1,2]. Ribonucleotide reductase consists of two non-identical subunits called R1 and R2 in mammalian cells, and since this enzyme converts ribonucleotides to deoxyribonucleotides, it is essential for the synthesis of DNA [3,4]. A requirement of iron for DNA synthesis is due to an iron centre in the R2 protein. Treatment of cells with the antitumour agent, hydroxyurea, destabilizes the iron centre, and destroys a tyrosyl free radical in the R2 protein, resulting in a loss of enyzme activity and an inhibition of DNA synthesis and cell proliferation [5]. We have shown that cells selected for resistance to the cytotoxic effects of hydroxyurea contain elevated levels of the R2 protein of ribonucleotide reductase, which is rate-limiting for enzyme activity [3,4]. Interestingly, these drug resistant cells also showed increased ferritin gene expression, suggesting that ferritin plays an important role as a storage protein for detoxifying excess iron released from inactive R2, and/or as an intracellular reservoir for iron that is required for activation of the R2 protein [5,6]. However, hydroxyurea resistant cells contain changes in a variety of enzyme activities in addition to ribonucleotide reductase (the target for the drug) and ferritin, as we have demonstrated in previous studies [3,4,6,7]. The R2 gene is frequently amplified in mammalian cells resistant to hydroxyurea, and the elevated expression of genes like ornithine decarboxylase and a gene coding for a disulphide isomerase activity in these cells appears to be due to the co-amplification of these genes, which are located on the same chromosome close to the R2 gene [6–9]. Furthermore, the selection of drug resistant cell lines in culture over several months can lead to additional non-specific changes associated with these cell lines [7]. If there is a connection between ribonucleotide reductase and ferritin gene expression as previous studies suggest, then the expression of a recombinant R2 in mouse cells should elicit an accompanying elevation in ferritin gene expression in these cells. In the present study, we have taken this direct approach to test this important question, which does not suffer from the problems associated with selection of drug resistant mutant cell lines. We have constructed a retroviral expression vector for R2, and we have used it to investigate gene expression in cells soon after infection.

2. Materials and methods

2.1. Cell culture conditions

NIH 3T3 mouse cells and a H-*ras* transfected cell line called CIRAS-1 (or C-1) derived from mouse 10T1/2 cells, were used in this study [10]. Cells were cultured in α-minimal essential medium (Gibco, Grand Island, NY) supplemented with 10% calf serum (Fetalclone III, Hyclone, Logan, UT) as previously reported [10]. The retroviral vector package lines ψ2, PA317, and their derivatives were maintained in Dulbecco's modified Eagle's medium (Gibco) containing 10% calf serum and 0.45% glucose. Determinations of cell division times, and sensitivities to hydroxyurea cytotoxicity by estimating relative colony forming efficiencies, were carried out as described previously [10,11].

2.2. Construction of the R2 expression vector

The c-*myc* epitope-tagged mouse R2 cDNA was obtained by polymerase chain reaction (PCR) using the 5'-primer CGCGGATCC*GCCACC*ATGGAACAAAAGCTTATTTCTGAAGAAGACTTG**ATG**CTCTCCGTCCGCACC (where the Kozak sequence, a possible ribosomal binding signal [12], is in italics; the sequence encoding the human *myc* epitope is underlined; and the natural ATG initiation codon is in bold), and the 3'-primer CGCGGATCCTTAGAAGTCAGCATCCAAGG (where the termination codon is in bold). The PCR reaction contained, in a final volume of 100 μl, 200 μM of each dNTP, 0.3 μM of each primer, 100 ng of template DNA pcD-M2 [13], 1.0 unit of *Taq* DNA polymerase and 1×amplification buffer (Pharmacia Biotech, Uppsala, Sweden). Amplification was achieved by initial denaturation of the template DNA at 94°C for 2 min, and then 25 temperature cycles each consisting of 10 s denaturing at 94°C, 30 s annealing at 55°C and 1 min extension at 70°C. The PCR product was phenol:chloroform-extracted and ethanol-precipitated. The DNA was redissolved in 100 μl of 1×TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM of EDTA) containing 5 μg of protease K (Boehringer-Mannheim, Laval, Quebec) and incubated at 37°C for 30 min. The protease

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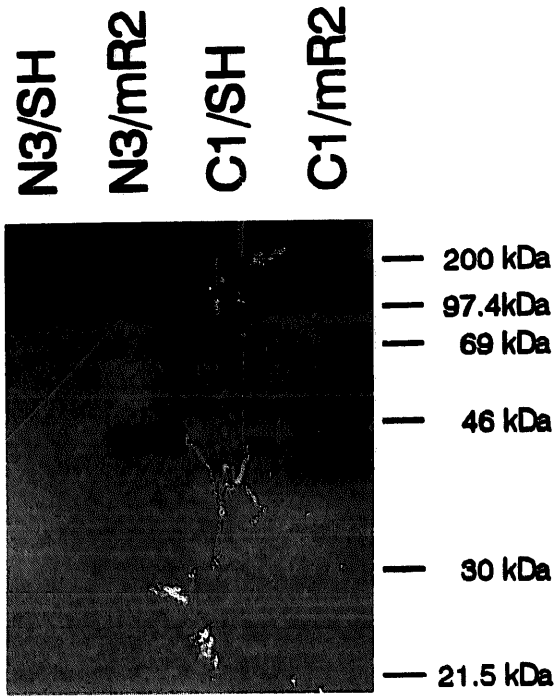

Fig. 1. Western blot analysis of *myc* epitope-tagged R2 protein. NIH 3T3 and C1 cells were infected with *myc*-R2 expression vector virus or pLXSH control vector virus, and selected for stable infectants to give the N3/mR2 and C1/mR2, or N3/SH and C1/SH cell lines, respectively. The positions and molecular weights of protein markers are shown on the right.

K-treated DNA was again extracted with phenol:chloride and precipitated with ethanol. The DNA was digested with *Xho*I, agarose gel-purified and finally ligated into *Xho*I-digested and dephosphorylated pLXSH (kindly provided by A.D. Miller, Fred Hutchinson Cancer Center, Seattle) to generate the retroviral vector pSH/mR2. The ligation product was electroporated into competent *Escherichia coli* DH5α cells. Recombinant vector from transformed bacteria was screened by PCR using the primers described above. The authenticity and orientation of the vector R2 cDNA insert were confirmed by sequence analysis, using a sequencing kit (Gibco BRL, Burlington, Ontario).

2.3. Packaging of retroviral vector and preparation of stock

Packaging was carried out using published procedures [13,14]. Briefly, column-purified vector DNA was initially transfected into ψ2 cells by calcium phosphate precipitation [10]. Culture medium was changed 24 h later and collected after another 24 h incubation period. Following centrifugation to remove cells, 1 ml of the supernatant was used as viral stock to infect a second package line PA317. Permanent packaging cells were selected from uninfected cells by growing the cells in medium containing 400 μg/ml hygromycin (Boehringer-Mannheim). To prepare viral stock, cells from fresh monolayers were split at a ratio of 1:10, and were added to medium minus hygromycin when they reached about 50% confluency. After 3 days incubation the medium was collected, centrifuged to remove cells and was used as viral stock. The infectivity of viral stock was determined by titration on NIH 3T3 cells in the presence of hygromycin [13,14].

2.4. Western blot analysis of cell expressed epitopic R2

Western analysis was performed as previously described [5,6], using the anti-*myc* mouse monoclonal antibody, 9E10 (ATCC, Rockville, MD), and horseradish peroxidase-conjugated goat anti-mouse antibody (Amersham, Mississauga, Ontario). Blotting was visualized with an enhanced chemiluminescence (ECL) Western blotting detection system (Amersham) following the manufacturer's directions.

2.5. Northern blot analysis

Total cellular RNA was extracted from logarithmically growing cells using a rapid RNA extraction kit (Stratagene, LaJolla, CA), by following the manufacturer's instructions. Electrophoresis, ^{32}P-labelling of cDNA probes [5,6] and blotting procedures have been described [5,6,10]. Radioactivity on filters was detected by a phophorimager (Molecular Dynamics Inc., Sunnyvale, CA), and quantitation was performed using Image Quant software (Molecular Dynamics, Inc.).

2.6. Ribonucleotide reductase assay

Logarithmically growing cells were added to culture plates at about 3×10^6 cells/150 mm plate, cultured for 24 h, removed with trypsin, collected by centrifugation, washed with cold buffer, and lysed in 100 μl phosphate-buffered saline, pH 7.2 containing 1 mM dithiothreitol and 1 mM of the protease inhibitor AEBSF (Calbiochem, San Francisco, CA), by three cycles of freeze-thawing. Following centrifugation, the supernatant was used for enzyme activity assays using [^{14}C]CDP (Moravek Biomedical, Brea, CA) as we have previously described [6,15].

3. Results and discussion

3.1. Recombinant R2 expression

A retroviral vector-mediated expression system was used to obtain high infection efficiency and to achieve stable expression of the R2 protein in mammalian cells. To distinguish the vector gene product from the endogenous R2, we added a human c-*myc* epitope coding for 10 amino acids plus methionine to the 5'-end of the R2 cDNA. In addition, to enhance binding of the recombinant R2 mRNA to ribosomes and to increase the efficiency of protein synthesis, we placed a typical Kozak sequence upstream of the coding region [12]. This fusion cDNA was inserted into the retroviral vector pLXSH to generate the R2 viral expression vector pSH/mR2. NIH 3T3 and C1 cells were infected with this vector, or a control vector which is identical to pSH/mR2 but lacks the *myc*-R2 fusion cDNA, and stable infectants were selected in the presence of hygromycin. The *myc*-R2 vector infected cells were called N3/mR2 (NIH 3T3 cells) and C1/mR2 (C1 cells), and the corresponding control vector infected populations were called N3/SH (NIH 3T3 cells) and C1/SH (C1 cells). As shown in Fig. 1, the anti-*myc* monoclonal antibody detected a protein band of 45 kDa corresponding to the R2 protein in *myc*-R2 vector infected cells [4–6], providing an example of stably expressed R2 cDNA in mammalian cells.

Table 1
Sensitivity to hydroxyurea

Hydroxyurea (μM)	Relative colony formation efficiency			
	N3/SH	N3/mR2	C1/SH	C1/mR2
200	1.0×10^{-3}	3.8×10^{-2}	2.1×10^{-3}	1.4×10^{-2}
300	$<5\times10^{-4}$	2.2×10^{-3}	5.6×10^{-4}	3.9×10^{-3}
400	$<1\times10^{-5}$	7.0×10^{-4}	4.0×10^{-5}	1.1×10^{-3}
600	ND	ND	$<1\times10^{-5}$	1.0×10^{-4}

All analyses were performed on duplicate dishes, with values varying by less than 15%. ND = not determined.

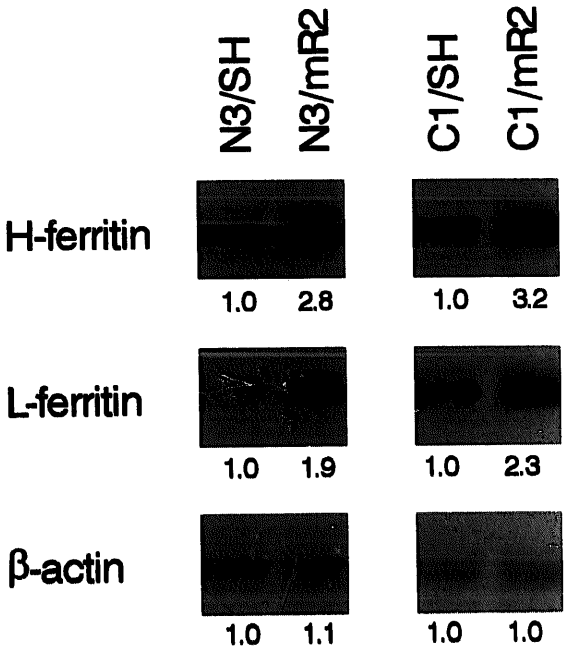

Fig. 2. Northern blot analysis of ferritin gene expression. Total cellular RNA was extracted from logarithmically growing cells. H- and L-ferritin probes [5,6] were prepared by nick-translation using [α-^{32}P]dCTP. The β-actin cDNA fragment was labelled with [α-^{32}P]dCTP by random primer labelling and used as a loading control. Numbers show the relative amounts of mRNA from each pair of test and control lines as analyzed with a phosphorimager.

To determine if the *myc*-R2 protein is biologically active, the sensitivity of cells to the cytotoxic effects of hydroxyurea was evaluated in colony forming assays. In drug resistance studies, it has been shown that increased levels of active R2 leads to reduced sensitivity to hydroxyurea, which works by destabilizing the iron centre in the R2 protein [5,6]. Table 1 shows that the drug sensitivities of N3/mR2 and C1/mR2 cells were markedly reduced when compared to control cells infected with vector alone. Since R2 is limiting for enzyme activity in proliferating cells [4], an increase in active R2 protein should also result in a modest but consistent elevation in ribonucleotide reductase activity in *myc*-R2 expressing cells when compared to cells infected with the control vector. Table 2 shows that this is correct, since both N3/mR2 and C1/mR2 cells exhibited 2–3 fold increases in ribonucleotide reductase activity when compared to N3/SH and C1/SH cells. This increase in enzyme activity did not appear to affect the proliferative abilities of the N3/mR2 or C1/mR2 cells since their cell doubling times were about the same as N3/SH or C1/SH cells (Table 1).

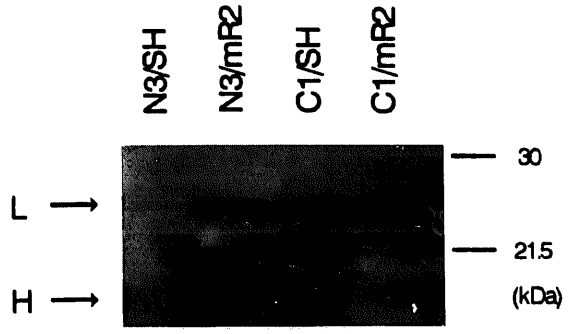

Fig. 3. Western blot analysis of the ferritin proteins. Rabbit polyclonal antibody, which recognizes the 18 and 24.5 kDa ferritin subunits (Boehringer Mannheim, Laval, Quebec), was used as previously described [5,6]. The positions of the subunits are indicated on the left.

3.2. Ferritin gene expression

By using the R2 viral expression vector that we have constructed we have obtained cells that have increased ribonucleotide reductase activity due to elevated levels of active R2 protein, and this was achieved without selecting for resistance to drugs like hydroxyurea, that target the R2 subunit of the enzyme. We were interested in determining whether the elevated levels of R2 would be accompanied by increased expression of the ferritin genes, as has been observed with cell lines selected for resistance to hydroxyurea [4–6]. Fig. 2 shows that both H-chain and L-chain ferritin mRNAs are elevated in cells expressing the recombinant R2. Quantitated analysis showed that H-chain ferritin gene expression in N3/mR2 and C1/mR2 cells was 2.8 and 3.2 times greater than the expression observed in N3/SH and C1/SH cells, respectively. Also, expression of the L-chain ferritin gene was increased in N3/mR2 and C1/mR2 cells by 1.9 and 2.3 times when compared to the results obtained with N3/SH and C1/SH cells, respectively. The increases in ferritin H and L mRNA levels in N3/mR2 and C1/mR2 cells were also accompanied by increases in H and L ferritin protein levels in these cells, when compared to their respective control populations (Fig. 3).

In total, the results of this study demonstrate that ferritin gene expression is tightly linked to the presence of R2 protein. This is likely due to the iron centre of the protein which is required for enzyme activity, since a loss of iron destroys ribonucleotide reductase activity preventing DNA synthesis and cell proliferation [4]. Free iron is extremely toxic to cells [1,2]. Therefore, during the synthesis of R2 protein, it is reasonable to expect a requirement for ferritin-associated iron to generate an active R2 protein, and during R2 protein turnover it would be essential to sequester excess iron released during this process. These findings also indicate an important role for

Table 2
Ribonucleotide reductase activity and cell doubling times

Cell line	CDP reduction[a] (fold increase)	Cell doubling time[b]
N3/SH	0.83 ± 0.08	16.7
N3/mR2	1.71 ± 0.11 (2.06)[c]	17.3
C1/SH	0.92 ± 0.11	12.9
C1/mR2	2.70 ± 0.22 (2.93)[c]	12.2

[a]Enzyme activity is expressed as nmol of CDP reduced/mg of protein per h. Results in this column represent average ± SE of three independent experiments.
[b]Cell doubling times are expressed as the average values obtained from two independent experiments.
[c]Number in parentheses indicates the fold increase in enzyme activity compared to the control values.

ferritin in the regulation of DNA synthesis, which occurs through its link to a key rate-limiting step in the synthesis of DNA, ribonucleotide reductase [3,4].

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