Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 238-244

HIGH LEVEL EXPRESSION OF p55, A THYROID HORMONE BINDING PROTEIN WHICH IS HOMOLOGOUS TO PROTEIN DISULFIDE ISOMERASE IN A RETROVIRAL VECTOR

Hirokazu Kato, Thierry Velu* and Sheue-yann Cheng+

Laboratory of Molecular Biology and *Laboratory of Cellular Oncology
National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Received August 22, 1989

Summary To develop an efficient system for a high level expression of a human cellular thyroid hormone binding protein (p55) in eukaryotic cells, a full-length p55 cDNA was inserted into a Harvey murine sarcoma virus-derived vector (pHTBr) and transfected into mouse NIH 3T3 cells. The expressed p55 has a molecular weight of 55,000 and is recognized by the human specific anti-p55 monoclonal antibody. Similar to the endogenous p55, the expressed p55 is localized on endoplasmic reticulum and nuclear envelope. Moreover, p55 was specifically labeled by N-bromoacetyl-3,3',5-triiodo-L-thyronine. Thus, the expressed p55 is structurally indistinguishable from the endogeneous p55. pHTBr was packaged into a virus with the aide of an amphotropic virus. Infection by pHTBr-containing virus yielded a 2-11 fold higher expression than the endogeneous p55 in NIH3T3, rat GH_3, human HepG2 cells and a mouse monoclonal antibody secreting hybridoma.

Introduction We have previously purified an ER-associated T_3 binding protein (p55) to homogeneity from a human carcinoma cell line (1). This protein is nonglycosylated and located in the lumenal face of ER and nuclear envelope (1,2). Recently, the cDNA for p55 was isolated and sequenced (3). Sequence analysis has indicated that the coding region of p55 cDNA has an 85%, 98% and 88% sequence homology to the rat protein disulfide isomerase (4), the ß-subunit of human proly-4-hydroxylase (5) and the chicken glycosylation site binding protein of oligosacchary transferase (6). Immunocytochemical localization of p55 in human tissues showed that its distribution is widespread and is particularly concentrated in cells which are active in protein synthesis and secretion (7). The tissue distribution pattern and the sequence homology of p55 to the above three enzymes suggested that p55 may play a key role in the protein synthesis and secretion. As a step toward understanding the possible involvement of p55 in protein synthesis/secretion, we hope to evaluate the effect of expression of humar p55 in a heterologous system. However, up to the present time, no expression

+To whom correspondence should be sent.

Abbreviations: T_3, 3,3',5-triiodo-L-thyronine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; BrAc[^{125}I]T_3, N-bromoacetyl-T_3; Ha-MuSV, Harvey murine sarcoma virus. PBS, phosphate-buffered saline.

0006-291X/89 $1.50

system has ever been reported for p55. We first asked the question whether p55 could be stably expressed with its native characteristics retained. The present work reported the high level expression of a functional p55 in cultured cell lines from different species.

Materials

[^{125}I]T_3 (2200 Ci/mmol) was obtained from DuPont New England Nuclear. Dulbecco's modified Eagle's and neomycin (G-418 sulfate) were from Gibco Laboratories. BrAc[^{125}I]T_3 was synthesized as described (8).

Retroviral vector construction Figure 1 shows that the retroviral vector pHTBr. It contains a BamHI 1.8 kb fragment of which 1.6 kb is the coding sequence for p55 and 0.2 kb is the 3' untranslated region immediately down stream of the termination codon. The 1.8 kb fragment (HTB) was inserted in place of the viral oncogene v-ras^H in pCO6a-HX, a plasmid containing the full-length Harvey murine sarcoma virus (Ha-MuSV) genome cloned in PBR322. In pCO6a-HX, the v-ras^H gene is flanked by the long terminal repeat (LTR) and by 5' and 3' untranslated regions. The viral Sac II and Pst I sites at nucleotides 940 and 1759 (9), respectively, were both replaced with a Xho I linker. The vector and insert were made compatible by filling in their ends with the DNA polymerase I Klenow fragment. In the resulting pHTBr plasmid, HTB is the only functional gene.

Transfection and selection of cells pHTBr (7 µg) was co-transfected into cells with pRSVneo (1 µg) by calcium phosphate co-precipitation method as described (10) After culturing cells for 10-14 days in the presence of 0.8 mg/ml G418, G-418 resistant colonies were isolated, cultured and screened by indirect immunofluorescence using human specific monoclonal antibody against p55, J9 (2).

Virus infection To rescue p55 containing virus, G418 resistant NIH 3T3 cells (1 x 10^5/60 mm dish) obtained following transfection with pHTBr were infected with an amphotropic helper virus (11). Adsorption was performed for 1 hr at 37°C in the presence of 4 µg/ml polybrene (Sigma). Culture fluid from the infected cells was harvested 6-10 days later, stored at -70°C and used as virus stocks. NIH 3T3 cells were infected by diluting stock virus (1 x 10^{-1}) which were filtered through a cellulose acetate filter (0.45 µm) with a 4 µg of Polybrene per ml as described for virus rescue.

Metabolic labeling and immunoprecipitation Cells (3 x 10^6 cells/100-mm dish) were plated in selection medium containing 0.8 mg/ml of G418. Cells were labeled with [^{35}S]methionine (1 mCi/dish), extracted with 3 mM CHAPS and immunoprecipitated with the human specific monoclonal antibody J9 as described (2).

Affinity labeling of p55 with BrAc[^{125}I]T_3 A431 cells and transfectants containing p55 gene (1 x 10^6 cells/35-mm dish) were washed with 2 ml of PBS (3 times) containing 1 mg/ml glucose at 4°C. Cells were incubated with 0.5 nM BrAc[^{125}I]T_3 in the absence or presence of 1 µM unlabeled T_3 for 1 hr at 15°C. At the end of incubation, cells were washed with PBS followed by incubating with 10 µl of 100 µM freshly prepared dithiothretol in 0.5 ml PBS containing 1 mg/ml glucose for 15 min at 4°C. Extraction of cells with 3 mM CHAPS and immunoprecipitation with J9 were carried out as described (2).

RNA isolation and blot hybridization A431 cells and transfectants containing p55 gene were plated in culture super dishes (600 cm^2). Poly(A$^+$) RNA were prepared as described (3). The blots were probed with a human specific ^{32}P-labeled 29-bp synthetic oligomer with the sequence corresponding to nucleotides 1678-1706 in the 3' untranslated region of p5A5 (3).

Results

Isolation of cell lines expressing p55 The coding sequence of p55 cDNA was cloned into a retroviral vector (Fig. 1). The pHTBr was co-transfected into mouse NIH 3T3 cells with pRSVneo as a selectable marker. The G418 resistant cells were screened for the presence of the p55 gene product by indirect immunofluorescence.

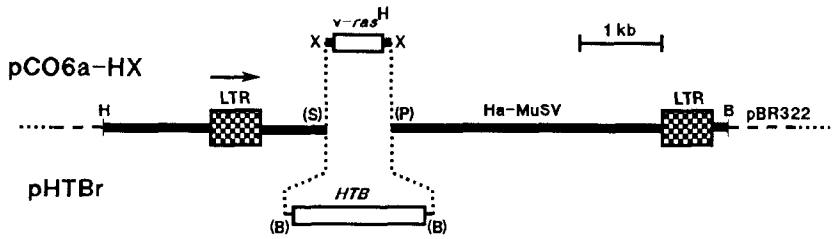

Figure 1: Construction of the human p55 retroviral genome.
The vector was constructed as described in Methods. Ha-MuSV sequences are shown in bold lines, and pBR322 in broken lines. Restriction endonuclease sites: X, Xho I; H, Hind III; S, Sac II; P, Pst I; B, Bam HI. Open boxes: coding sequences of v-ras^{H} and HTB; cross-hatched boxes: Ha-MuSV long terminal repeat. The arrow indicate the direction of transcription.

To distinguish the expressed human p55 from the endogeneous mouse p55, we used a human specific monoclonal anti-p55 antibody, J9 (2). A typical example of cells expressing p55 is shown in Fig. 2C. Typical nuclear envelope-endoplasmic reticulum patterns were seen. This pattern is indistinguishable from that observed in A431 cells (Fig. 2A), in which the localization of p55 was shown previously to be in the ER and nuclear envelope (1,2). By contrast, the control G418 resistant cells which were only transfected with pRSVneo did not show any J9 reactivity (Fig. 2B). Using the immunofluorescence method, positive clonal cell lines were isolated from G418 resistant colonies. One line (G2) was selected for further characterization. As controls, cell lines designated as GO were randomly selected from resistant cells receiving only pSV2neo. Cell lines containing p55 gene have grown for over nine months without any apparent change in p55 expression.

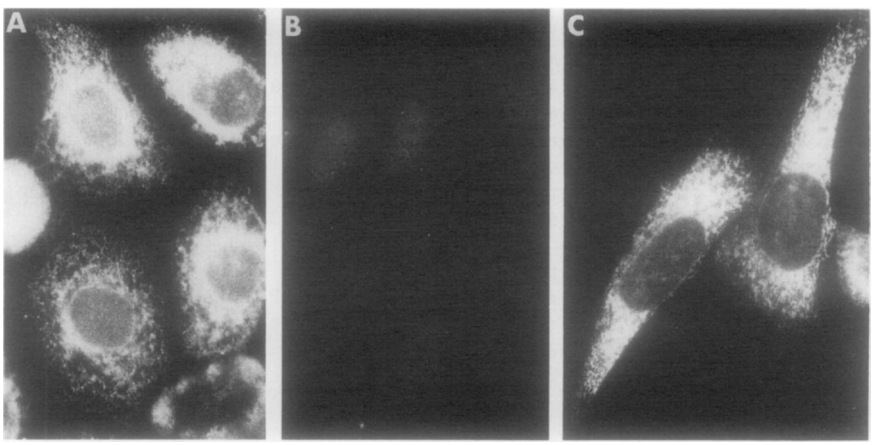

Figure 2: Immunofluorescence localization of the expressed p55 using the human specific monoclonal antibody, J9.
A431 human carcinoma cells (A), GO (B) and G2 (C) were fixed with formaldehyde and incubated with J9 in the presence of saponin as described (2). The immunofluorescence images show that G2 which have been transfected with p55 gene react with the human specific antibody J9, giving nuclear envelope and endoplasmic reticulum pattern.

Extracts from human A431 cells were also immunoprecipitated by J9 (lane 6) or the antibody #3632 (lane 7) for comparison. Identical amounts of p55 were immunoprecipitated. The intensity of p55 band from G2 (lane 1) is ~80% of that in A431 cells (lane 7). These results show that p55 is expressed at a relatively high level in G2.

mRNA analysis To determine the nature of the RNA species encoding the human p55 protein, Northern analysis was carried out by using a human specific 29-bp synthetic oligomer as a probe. The oligomer corresponds to nucleotides 1678-1706 in the 3' untranslated region of human cDNA (3) and only has 20% of sequence homology to that in mouse (12). In G2 cells, a mRNA species with a size ~7.3 kb was detected; whereas in GO, no hybridizable mRNA was detectable by the human specific oligomer (data not shown). The 7.3 kb species corresponds to the expected size of the full length RNA p55 transcript in the vector.

Affinity labeling of the expressed p55 with BrAc[^{125}I]T_3 Previously, it has been shown that p55 in A431 and other cultured cells was specifically labeled by BrAc[^{125}I]T_3, an affinity labeling analog of T_3 (8,13). To see whether the expressed p55 can specifically react with BrAc[^{125}I]T_3, A431, G2 and GO were affinity-labeled with BrAc[^{125}I]T_3 followed by immunoprecipitation with J9. As shown in lanes 1 and 3 (Figure 4), p55 in A431 and G2 cells were labeled by BrAc[^{125}I]T_3, respectively. However, no immunoprecipitable band was detectable in GO (lane 5). Furthermore, the labeling of p55 in G2 is specific as in the presence of 1 μM unlabeled T_3, the labeling of p55 was reduced by 70% (lane 4). The degree of inhibition is similar to that of the endogenous p55 present in A431 cells (75% inhibition) (lane 2). These results indicate that the structure of the expressed p55 is similar to that of the endogenous p55.

Table I. Expression of human p55 using virus

	Cell line	p55 Expression[a] J9	#3632	Infected/control
NIH 3T3	Infection by Virus	+	410	
	Control	-	69	5.9
GH_3	Infection by Virus	+	1067	
	Control	-	98	10.9
J11	Infection by Virus	+	65	
	Control	-	30	2.2
HepG2	Infection by Virus	++	330	
	Control	+	173	1.9

[a] Expression of p55 in cells was evaluated quantitatively by immunoprecipitation using the human specific monoclonal antibody J9 and polyclonal antibody #3632. The intensity of the immunoprecipitated bands were quantified by densiometry. Identical amounts of protein (60 μg) were used for immunoprecipitation. The unit is arbitrary.

Immunoprecipitation of the expressed p55 To determine the size and the amounts of p55 expressed in G2 cells, the cultures were labeled with [^{35}S]methionine and cellular extracts were immunoprecipitated with the human specific monoclonal antibody J9. To see the endogeneous p55, [^{35}S]methionine-labeled extracts of GO and A431 cells were also immunoprecipitated by the rabbit polyclonal antibody #3632 (1,2) which recognizes p55 from both human and mouse. Lane 1 in Figure 3 show that a protein with the expected molecular weight of 55,000 was immunoprecipitated by J9 in G2; whereas no p55 was detectable in GO (lane 2). The endogeneous p55 in GO however, was immunoprecipitated by the antibody #3632 (lane 4). The intensity of the p55 bands was quantified by a densitometer. It is of interest to note that the p55 in G2 immunoprecipitated by #3632 (lane 3) is two times more than that in GO immunoprecipitated by the same antibody (lane 4) or that in G2 immunoprecipitated by J9 (lane 1). Clearly, the expressed and endogeneous p55 in G2 were recognized by the antibody #3632 as expected. These results suggest that the expressed and the endogeneous p55 are under separate transcriptional control.

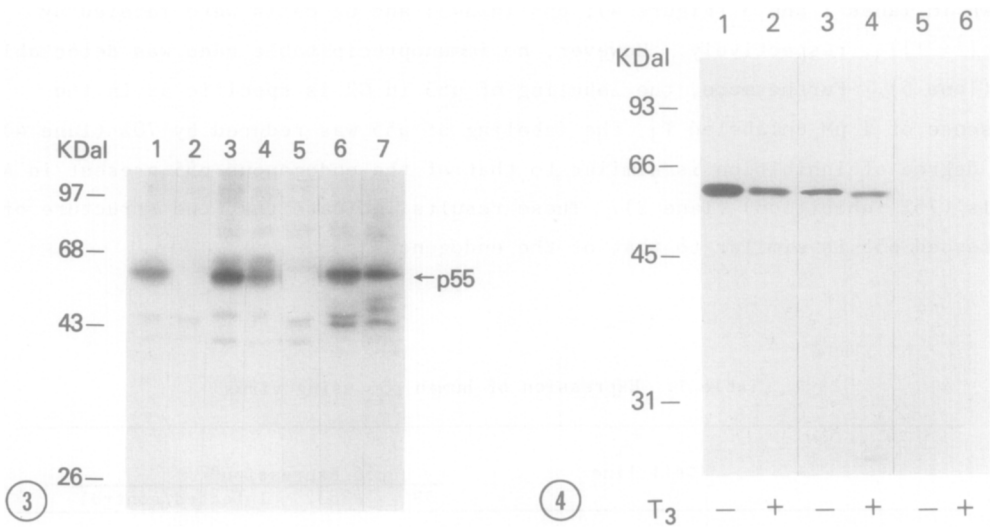

Figure 3: Autoradiogram of the immunoprecipitates from [^{35}S]methionine-labeled extracts of cells.

NIH 3T3 cells (3 x 10^6/100-mm dish) and A431 cells (4 x 10^6/100-mm dish) were labeled with [^{35}S]methionine (1 mCi/dish) for 20 hrs. Cells were extracted with 3 mM CHAPS and an equal amount of protein (30 µg) were immunoprecipitated with J9 (lanes 1, 2 and 6), HB21 (a monoclonal antibody against human transferrin receptor) (lane 5) or #3632 (lanes 3, 4 and 7) as described in "Methods". Lanes 1, 3 and 5, G2 cells; lanes 2 and 4, GO cells; lanes 6 and 7, A431 cells.

Figure 4: Autoradiogram of BrAc[^{125}I]T_3-labeled p55.

NIH 3T3 (1.5 x 10^6 cells/60-mm dish) and A431 cells (1.4 x 10^6 cells/60-mm dish) were washed with PBS containing 1 mg/ml glucose. Cells were cooled to 15°C and 0.5 nM of BrAc[^{125}I]T_3 was added in the absence (lanes 1,3 and 5) and presence (lanes 2,4 and 6) of 1 µM unlabeled T_3. After incubation for 1 hr at 15°C cells were washed, extracted with 3 mM CHAPS, immunoprecipitated with J9 and electrophoresed on a 10% SDS-PAGE as described in "Methods". Lanes 1-2, A431 cells; lanes 3-4, G2 cells; lanes 5-6, GO cells.

p55-containing virus infection in other cultured cells

To show the versatility of this expression vector p55 gene was expressed in other cultured cells of various species. Rat GH_3, human HepG2, NIH 3T3 and mouse J11 (a hybridoma cell line secreting monoclonal antibody against a human cytosolic thyroid binding protein) were each infected with the p55 containing virus or the amphotropic virus as a control. The expression of p55 was evaluated by immunoprecipitation using J9 or #3632. As shown in Table I, p55 expressed was 5.9, 10.9, 2.2 and 1.9 fold more than the endogeneous p55 in NIH 3T3, GH_3, J11 and HepG2, respectively. These results indicate that the retroviral infection is an efficient way to introduce p55 gene into cultured cells of various species.

Discussion

The present study shows that human p55 cDNA can be continuously expressed at a high level in NIH 3T3 fibroblasts transfected with a Harvey murine sarcoma virus derived vector. The molecular size, immunoreactivity and the interaction of the expressed p55 with BrAc[^{125}I]T_3 are indistinguishable from those of the endogenous p55. To facilitate the entry of p55 gene and increase the level of expression in cells, pHTBr was packaged into a virus. NIH 3T3 cells infected by the pHTBr-containing viruses expressed nearly 3 times more p55 than the cells transfected with pHTBr from which the viruses had been rescued. Furthermore, a high level expression of p55 was seen in GH_3, HepG2 and J11 followed virus infection, indicating that the retroviral infection is an efficient way to introduce p55 into cells of various species.

p55 is homologous to protein disulfide isomerase, the β-subunit of prolyl-4-hydroxylase, and the glycosylation site binding component of oligosaccharyl transferase. The present versatile and efficient expression system should be useful in evaluating the roles these enzymes play in protein synthesis/secretion and the structure and function of endoplasmic reticulum.

References

1. Cheng, S-y., Hasumura, S., Willingham, M.C., Pastan, I. (1986) Proc. Natl. Acad. Sci. USA 83, 947.
2. Hasumura, S., Kitagawa, S., Lovelace, E., Willingham, M.C., Pastan, I., Cheng, S-y. (1986) Biochemistry 25, 7881.
3. Cheng, S-y., Gong, Q.H., Parkison, C., Robinson, E., Appella, E., Merlino, G., Pastan, I. (1987) J. Biol. Chem. 262, 11221.
4. Edman, J.C., Ellis, L., Blacher, R.W., Roth, R.A., Rutter, W.J. (1985) Nature 317, 267.
5. Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllyla, R., Huhtala, M-L., Kovu, J., Kivlrikko, K. (1987) EMBO J. 6, 643.
6. Geetha-Habib, M., Noiva, R., Kaplan, H.A., Lennarz, W.J. (1988) Cell 54, 1053.
7. Willingham, M.C., Rutherford, A.V., Cheng, S-y. (1987) J. Histochem. Cytochem. 35, 1043.
8. Horiuchi, R., Johnson, M.L., Willingham, M.C., Pastan, I., Cheng, S-y. Proc. Natl. Acad. Sci. USA 79, 5527.

9. Soeda, E., Yasuda, S. (1985) In Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (ed) 2 suppl. New York: Cold Spring Harbor, pp. 928-939.
10. Shen, D-w., Fojo, A., Roninson, I.B., Chin, J.E., Soffir, R., Pastan, I., Gottesman, M.M. (1986) Mol. Cell. Biol. 6, 4039.
11. Chattopadhyag, S.K., Oliff, A.I., Leneneyer, D.L., Lander, M.R., Lowy, D.R. (1981) J. Virol. 39, 777.
12. Gong, Q.H., Fukuda, T., Parkison, C., Cheng, S-y. (1988) Nucl. Acids. Res. 16, 1203.
13. Cheng, S-y. (1983) Endocrinology 113, 1155.