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INVOLVEMENT OF OP18 IN CELL PROLIFERATION

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Summary: Op18 is a highly conserved major cytosolic phosphoprotein that is expressed at high levels in acute leukemia and in neuroblastoma. In this study we present evidence pointing to a role for Op18 in cellular proliferation. Blocking of Op18 mRNA translation using antisense oligonucleotides delayed entrance of mitotically stimulated normal peripheral blood lymphocytes into the S phase. Moreover treatment of HL-60 promyelocytic leukemia cells with DMSO or PMA which induced terminal differentiation resulted in a decrease in the level of Op18 RNA and protein. Inhibition of lymphoid proliferation with cyclosporin also resulted in reduced Op18 levels.

We have previously undertaken studies aimed at identifying proteins that are altered in their expression in acute leukemia cells relative to non-leukemic proliferating cells of corresponding lineage. As a result, we have identified a major novel cytosolic phosphoprotein which we have designated Op18 [1, 2]. Our evidence to date indicates that Op18 is overexpressed and does not get phosphorylated in acute leukemia to the extent it does in non-leukemic proliferating lymphoid cells that we have studied [3]. Furthermore in neuroblastoma, we have observed a statistically significant correlation between diminished levels of Op18 phosphorylation and N-*myc* oncogene copy number, suggesting a functional significance to Op18 phosphorylation [2]. It has been proposed that the Op18 protein, also referred to as stathmin in the mouse plays a role in intracellular signal transduction [4-6].

In previous studies we have reported the cloning and sequencing of Op18 cDNA and genomic DNA and presented evidence that Op18 is conserved and ubiquitous and that its overabundance in leukemia results from increased expression of a structurally unaltered gene [3, 7]. To determine the role of Op18 in cell proliferation, we have examined the effect of inhibiting Op18 mRNA translation using antisense oligodeoxynucleotides on entry of lymphoid cells into the S phase; we have also examined the effect of inhibiting lymphoid proliferation using cyclosporin, and the effect of inducing terminal differentiation of HL-60 leukemia cells using DMSO or PMA on the levels of Op18.

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Material and Methods

All radioactive isotopes: [^{32}P]γ ATP (>3000 Ci/mmol), [^{32}P]αd CTP (>3000 Ci/mmol), [methyl-^{3}H] thymidine (47 Ci/mmol), L-[^{35}S] methionine (>1000 Ci/mmol) and the random primer labeling kit were purchased from Amersham. Magna NT nylon membranes were from Micron Separation Inc. Cell culture media were obtained from Irvine Scientific, GIBCO and Flow Laboratories; Ficoll-Paque was from Pharmacia. Cyclosporin (CSA) was purchased from Sandoz and phytohemagglutinin (PHA) was from Burroughs-Wellcome. Oligonucleotides were synthesized on Applied Biosystems Inc., automated DNA synthesizers employing β-cyanoethyl phosphoramidite chemistry on controlled pore glass support. The oligos were purified by reverse phase HPLC, and quantitated by spectrophotometry. The oligos were also ^{32}P end labeled and analyzed on native 12% acrylamide gel to check their stability.

Effect of Op18 antisense oligos on cell proliferation

Antisense (AS) oligonucleotides (5' ATC AGA AGA AGC CAT) complementary to the first 15 bases of Op18 protein coding sequence were synthesized. As a control, nonsense (NS) oligonucleotides (5' GAC ATA GAC ATA GAC) which have the same base composition as AS-oligos but in a scrambled arrangement were also synthesized. The nonsense oligos sequence did not match any sequence using the PIR databases.

Resting normal lymphocytes were isolated from the venous blood of healthy individuals by Ficoll-Paque according to manufacturer's protocol, and cultured in complete media (RPMI + 10% Fetal bovine serum + 4 mM L-glutamine + 100 u/ml penicillin + 100 μg/μl streptomycin) in a tissue culture flask for 2 hrs. Lymphocytes which remained in suspension were separated from the monocyte layer that adhered to the culture flask. The lymphocytes were then counted and resuspended in complete media at a concentration of 2 x 10^6 cells/ml and aliquoted in a 24 well plate.

Cells were treated with 25 μM of either antisense or nonsense oligos before stimulation with anti-CD3 (OKT3 ascites, 1/1000). Untreated cells or cells stimulated with only OKT3 were used as controls. The culture medium was replenished every 12 hrs, and in the wells where cells were treated with oligos, 25 μM of AS or NS oligos were also added. The rate of proliferation of cells incubated with antisense or nonsense oligos was determined by ^{3}H thymidine incorporation and the DNA content of the cells was determined by flow cytometry. The rate of new protein synthesis in treated cells was determined by incubation of 3 x 10^5 cells with 100 μCi of ^{35}S methionine in 500 μl methionine free media for 4 hrs; equal numbers of TCA precipitable counts were separated by 2-D PAGE and followed by quantitative analysis of autoradiographs.

Flow Cytometry

At each time point, 0.5 x 10^6 cells per well were vigorously suspended and transferred to a plastic tube. The wells were then washed with standard buffer (1% FA buffer, 1% fetal calf serum, 10% NaN3; pH 7.15 - 7.25) which was then added to the cells. 2 ml of standard buffer were then added and the cells were collected by centrifugation for 5 min at 1900 rpm at 4° C. After decanting the supernate, the cell pellets were vortexed and 25 μl of chicken erythrocytes (CRBC-1.3 - 1.6 x 10^6/ml), and 2 ml of standard buffer were added. The cells were then centrifuged as before and the supernate decanted. After vortexing, the cells were stained with 1 ml of propidium iodide (0.01 M Tris, 10 mM NaCl, 0.7 u/ml RNase, 7.5 x 10^{-5} M propidium iodide and 1 μl/ml NP-40) vortexed and incubated on ice for 1 hr. The cells were then in 75 μl of 2% paraformaldehyde and their DNA content determined by flow cytometry [8-10]. The data was analyzed using PARA 1 program [11].

^{3}H thymidine incorporation

For each sample, three 50 x 10^3 cells/well/100 μl were labeled in complete media with 0.8 - 1 μCi ^{3}H thymidine/well for 4 hrs to 16 hrs. The cells were then harvested on glass fiber filters and counted in scintillation fluid.

Effect of cyclosporin on the level of Op18

Resting normal lymphocytes were isolated and cultured in complete media at a concentration of 2 x 10^6 cells/ml. In time course studies, cells were treated for 3 days with 0.5 μg - 1 μg cyclosporin (CsA) at the time of their stimulation with anti-CD3 (OKT3 ascites, 1:200). Resting lymphocytes and lymphocytes stimulated with OKT3 were used as controls. At each time point, the rate of proliferation was determined by ^{3}H thymidine incorporation and the level of Op18 was determined by quantitation of silver stained 2-D gels [12].

Quantification of Op18

Spot detection and quantitation of Op18 were performed as previously described [13]. The Op18 spots (Op18, Op18a, Op18b) as well as 15 other reference spots were quantified by placing

a cursor on each spot, on the graphics monitor and typing the name of the spot. The integrated intensity of each spot was measured in units of optical density times square millimeter. The reference spots were used to adjust the Op18 spots integrated intensity as previously described [13] to compensate for any variability in protein loading or staining between gels.

HL-60 Differentiation Experiments

HL-60 cells were cultured in complete media and treated with 1.5% DMSO for 8 days and with 50 nM PMA for 5 days [14, 15]. For each time point, the rates of cellular proliferation were determined by ^{3}H thymidine uptake and cell aliquots were harvested and frozen at -80° C. Polypeptides of untreated controls and treated cells were analyzed by 2-D PAGE followed by silver staining [12]. Total cellular RNA from untreated and treated cells were prepared by the guanidine thiocyanate method [16] and analyzed by dot-blot hybridization. The filters were hybridized to Op18 cDNA, washed at high stringency and visualized by autoradiography. The membranes were stripped and re-probed with glyceraldehyde phosphate dehydrogenase (GADPH) cDNA as control [17].

Results and Discussion

While Op18 is present in greater amount in acute leukemia cells relative to proliferating non-leukemic lymphoid cells, it still occurs as a major protein constituent in proliferating peripheral blood lymphocytes that have been stimulated with the mitogen PHA [1]. Therefore in order to block the increase in Op18 levels to a significant extent, in stimulated peripheral blood lymphocytes using Op18 antisense oligonucleotides, we have compared the effect of PHA and of anti-CD3 antibody (OKT3) on the proliferative response of peripheral blood lymphocytes and on the level of Op18. While some variability was observed in the proliferative response using either agent, stimulation with anti-CD3 antibody particularly at high dilution (1/1000) generally resulted in a more moderate proliferative response, as determined by ^{3}H thymidine incorporation, and a more moderate increase in Op18 levels, as determined by 2-D PAGE and RNA analysis, than with PHA.

Three experiments were undertaken to determine the effect of Op18 antisense oligos on proliferation in response to OKT3. Inhibition of proliferation in the presence of antisense oligos appeared to be dependent on the extent of stimulation achieved with the anti-CD3 antibody. In two time course experiments in which the proliferative response with anti-CD3 was moderate ^{3}H thymidine uptake in the presence of antisense was significantly lower than with nonsense oligos with the same base composition. Data for one experiment for which flow cytometric analysis of DNA content was also undertaken is presented in Table 1. In a third experiment, a substantially greater proliferative response was observed in the presence of anti-CD3, and ^{3}H thymidine incorporation in the presence of nonsense or antisense oligos was identical.

Quantitative 2-D PAGE analysis of newly synthesized ^{35}S labeled Op18 from the above experiments showed approximately a 50% reduction in antisense relative to nonsense treated cells within 60 hrs under conditions in which aliquots with an equal number of counts were loaded onto gels. This indicated that Op18 antisense oligos blocked Op18 translation, thus causing a decrease in Op18 polypeptide. In the same experiment we observed that the increment in the percentage of cells in the S phase following anti-CD3 treatment was reduced and delayed in cells treated with anti-sense relative to treated nonsense oligos cells. By 72 hours, twice as many cells had progressed to the G2 and M phases in non-sense as in antisense treated cells (23.36 *vs* 12.62 %, respectively). Our findings therefore are that in the presence of mild stimulation, Op18 synthesis was sufficiently blocked to result in a measurable delay in cell proliferation and in ^{3}H thymidine

Table 1. Effect of Op18 antisense oligos on the proliferation of peripheral blood lymphocytes stimulated with OKT3

Time	Oligo	^{3}H thymidine uptake (cpm)	Flow cytometry %G0/G1	%S	%G2+M
12 hrs	Antisense	821.4	94.13	4.73	1.12
	Nonsense	1027.9	89.33	9.15	1.5
24 hrs	Antisense	1338.9	95.60	3.81	0.18
	Nonsense	1495.1	94.65	5.11	0.22
36 hrs	Antisense	2026.7	93.31	6.40	0.28
	Nonsense	2121.0	87.56	10.59	1.84
48 hrs	Antisense	2463.2	93.13	5.58	1.28
	Nonsense	3905.1	89.7	7.74	2.55
60 hrs	Antisense	2427.6	88.93	7.92	3.13
	Nonsense	7001.6	78.24	18.26	3.49
72 hrs	Antisense	5650.0	72.82	14.54	12.62
	Nonsense	9752.7	62.24	14.39	23.36

Resting normal lymphocytes were cultured in complete media and treated with 25 μM antisense or nonsense oligos for 12 hrs before addition of 1:100 OKT3 ascites. Aliquots were removed every 12 hrs to measure the level of proliferation as determined by thymidine incorporation, and the DNA content as determined by flow cytometry (Materials and Methods Section).

uptake. However, with massive stimulation, Op18 synthesis resulting from the activation of the Op18 gene was not sufficiently inhibited to affect cell proliferation.

In other experiments, we determined whether the block in cell proliferation caused by the immunophilin inhibitor cyclosporin A (CsA)is associated with a lack of increase in Op18 protein in OKT3 treated lymphocytes. CsA prevents the transcription of early T cell activation genes, thus inhibiting the signal transduction pathway mediated by the T cell receptor [18]. Results of these experiments showed that the increase in Op18 which occurs beginning on day 2 following stimulation with anti CD-3, did not occur in the presence of cyclosporin. There was also a reduction in ^{3}H thymidine incorporation in the presence of CsA (Table 2). These results indicate that CsA prevents the activation of Op18 gene which is observed in late G1.

HL-60 responds to a variety of inducing agents by ceasing to proliferate and giving rise to cells with some granulocytic or monocytic characteristics [19]. In HL-60, the c-*myc* oncogene which is transcribed at high levels, shows a marked decrease in mRNA expression after treatment of cells with DMSO, retionic acid, vitamin D3 or phorbol esters and also displays characteristics associated with normal cell maturation [19]. We therefore examined the effect of inducers on Op18 levels and proliferation of HL-60 cells. Dot Blot analysis of total cellular RNA from time course experiments showed that Op18 mRNA levels drop sharply after 5 days of treatment with 1.5% DMSO and after 2 days of PMA treatment. This decrease in Op18 mRNA (Figure 1) is paralleled by a decrease in Op18 polypeptide (Figure 2) and a decrease in cellular proliferation as determined by ^{3}H thymidine incorporation (Table 3).

In previous studies we have shown that the Op18 gene has a higher level of expression (RNA and protein) in actively proliferating cells than in resting normal lymphocytes and that the

Table 2. Effect of cyclosporin (CsA) on OKT3 treated peripheral blood lymphocytes

	Day	Treatment	Total Op18 (integrated intensities)	^{3}H Thymidine Incorporation (cpm)
Experiment 1	2	OKT3	1.398	7180
		OKT3 + CsA	0.897	264
	3	OKT3	3.358	8302
		OKT3 + CsA	0.488	181
Experiment 2	2	None	.834	454
		OKT3	2.352	11564
		OKT3 + CsA	.828	433
	3	None	0.505	1397
		OKT3	3.127	14242
		OKT3 + CsA	0.617	1005
Experiment 3	2	None	0.174	228
		OKT3	1.296	10829
		OKT3 + CsA	0.3	568
	3	OKT3	1.63	8080
		OKT3 + CsA	0.222	716

Resting normal lymphocytes were isolated and cultured in complete media. Aliquots from untreated cells (None) or cells treated with anti-CD3 (OKT3) or anti-CD3 and cyclosporin (OKT3 + CsA) were collected. The amount of total Op18 in the different samples was quantitated as described in Materials and Methods. ^{3}H thymidine uptake was used as a measure of cell proliferation.

induction of Op18 mRNA occurs at a time when the cells are entering S-phase. In this paper we present evidence that progression of cells through the cell cycle is dependent on the occurrence of Op18 at high levels and that downregulation or a block in proliferation as in the case of HL-60 cells

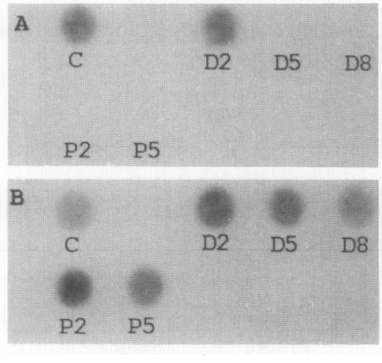

Figure 1. Dot Blot analysis of HL-60 RNA shows a decrease in Op18 mRNA after induction with DMSO or PMA. 5 µg of total cellular RNA from untreated and induced HL-60 cells were hybridized to human Op18 cDNA probe (panel A) or chick GADPH probe (panel B). Ctrl: untreated cells, D2: cells treated with 1.5% DMSO for 2 days, D5: cells treated with DMSO for 5 days, D8: cells treated with DMSO for 8 days, P2: cells treated with 50 nM PMA for 2 days and P5: cells treated with PMA for 5 days.

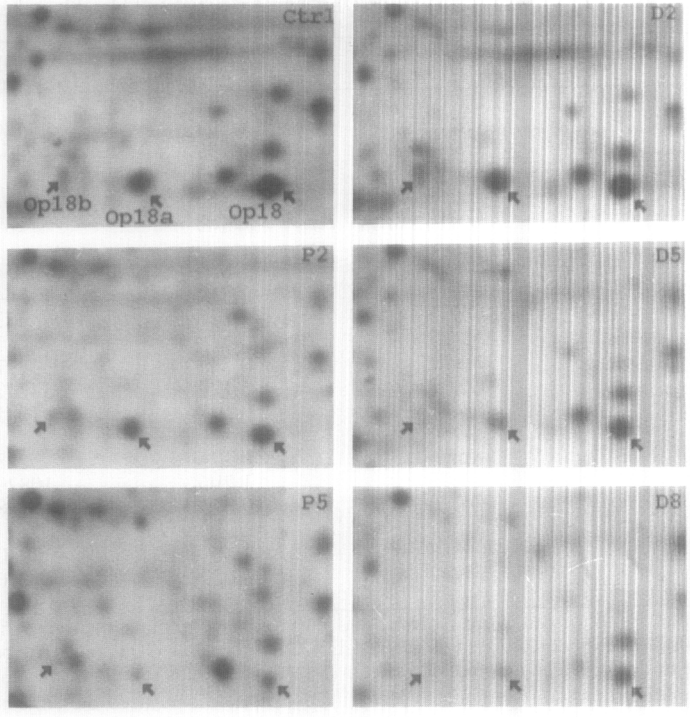

Figure 2. Close-up sections of silver stained 2-D gel pattern of HL-60 polypeptides show a decrease in total Op18 polypeptide after induction of cells with DMSO or PMA. Arrows point to unphosphorylated form of Op18 (Op18) and the two major phosphorylated forms (Op18a, Op18b). Ctrl: untreated cells, D2, D5, D8: cells treated with 1.5% DMSO for 2, 5 and 8 days respectively. P2 and P5 cells treated with 50 nM PMA for 2 and 5 days respectively.

treated with PMA or DMSO, and cyclosporin treatment of lymphocytes stimulated with anti-CD3 is associated with reduced levels of Op18. These findings together with the high degree of conservation of the gene and its high level of expression in leukemia and neuroblastoma which we have demonstrated, point to an important role for Op18 in normal and malignant cell proliferation.

Table 3. Effect of DMSO and PMA treatment in the level of cellular proliferation of HL-60

Time	^{3}H thymidine incorporation (cpm) Treatment		
	None	1.5% DMSO	50 nM PMA
2 days	61,535	45,986	3,964
5 days	47,918	1,649	1,569
8 days	42,415	1,067	

HL-60 cells were untreated or treated with 1.5% DMSO or 50 nM PMA for different time periods. Three aliquots (50 x 10^6 cells) from each sample were labeled with ^{3}H thymidine for 4 hrs, harvested and counted as described in Material and Methods.

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References

[1] Hanash, S. M., Strahler, J. R., Kuick, R., Chu, E. H. Y. and Nichols, D. (1988) J. Biol. Chem. 263, 12813-12815.
[2] Hailat, N., Strahler, J., Melhem, R., Zhu, X. X., Brodeur, G., Seeger, R. C., Reynolds, C. P. and Hanash, S. M. (1990) Oncogene 5, 1615-1618.
[3] Melhem, R. F., Zhu, X. X., Hailat, N., Strahler, J. and Hanash, S. M. (1991) J. Biol. Chem. in press,
[4] Sobel, A., Boutterin, M. C., Beretta, L., Chneiweiss, H., Doye, V. and Peyro-Saint-Paul, H. (1989) J. Biol. Chem. 264, 3765-3772.
[5] Doye, V., Soubrier, F., Bauw, G., Boutterin, M. C., Berretta, L., Koppel, J., Vandekerckhove, J. and Sobel, A. (1989) J. Biol. Chem. 264, 12134-12137.
[6] Doye, V., Boutterin, M. C. and Sobel, A. (1990) J. Biol. Chem. 265, 11650-11655.
[7] Zhu, X. X., Kozarsky, K., Strahler, J. R., Eckerskorn, C., Lottspeich, F., Melhem, R., Lowe, J., Fox, D. A., Hanash, S. M. and Atweh, G. F. (1989) J. Biol. Chem. 264, 14556-14560.
[8] Vindelov, L. L. (1977) Virchow's Arch (Cell Pathol) 24, 227-242.
[9] Vindelov, L. L., Christensen, I. J. and Nissen, N. I. (1983) Cytometry 3, 328-331.
[10] Greenwood, J. H., Kahn, L. E., Cohn, R. J., Wolber, R. A., Lovett, E. J. and Hudson, J. L. Draft Report to Coulter Corporation, Hialeah, FL.
[11] Bagwell, C. B. (1979) Theory and application of DNA histogram analysis (Ph.D. thesis), Univ. of Miami, Coral Gables, FL.
[12] Strahler, J. R., Kuick, R. and Hanash, S. M. (1989) Protein Structure: A Practical Approach (T. Creighton), , pp. 65-92. IRL Press Ltd., Oxford
[13] Kuick, R., Hanash, S. M., Chu, E. H. Y. and Strahler, J. R. (1987) Electrophoresis 8, 199-204.
[14] Collins, S. J., Ruscetti, F. W., Gallagher, R. E. and Gallo, R. C. (1978) Proc. Natl. Acad. Sci. USA 75, 2458-2462.
[15] Okazaki, T., Kato, Y., Mochizuki, T., Tashima, M., Sawada, H. and Uchino, H. (1988) Exp. Hematol. 16, 42-48.
[16] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
[17] Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual. , pp. Cold Spring Harbor Press, Cold Spring Harbor, New York
[18] Schreiber, S. L. (1991) Science 251, 283-287.
[19] Reitsma, P. H., Rothberg, P. G., Astrin, S. M., Trial, J., Bar-Shaivit, Z., Hall, A., Teitelbaum, S. L. and Kahn, A. J. (1983) Nature 306, 492-494.