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The Role of Calmodulin in Cell Transformation

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Two cell lines transformed with temperature sensitive retroviruses were examined for: their ability to grow in low Ca^{2+} medium, their calmodulin levels and changes in calmodulin acceptor proteins. Both cell lines grow in low Ca^{2+} medium at the permissive temperature 34°C while both lines did not replicate at the non-permissive temperature 39°C. The NRKLA23 cells have nearly twice as much calmodulin at the permissive temperature than they do at the non-permissive temperature while the 6M2 cells have an equal amount of calmodulin at both temperatures. Both cell lines exhibit changes in the calmodulin acceptor proteins going from the permissive to the non-permissive temperature. We suspect that the changes in the calmodulin acceptor proteins may be involved in the altered Ca^{2+}-sensitivity of growth in the cells going from the permissive to non-permissive temperature.

The transformation of cultured mammalian cells by carcinogen or virus results in a wide spectrum of changes in morphology and growth control. Some of these changes include: the ability to grow in agar, loss of contact inhibition of growth, increased glucose uptake and the ability to grow in low Ca^{2+} medium. Each of these *in vitro* markers of transformation have proved useful but none is diagnostic. That is, these markers are not universally associated with the ability of a transformed cell to form a tumor. It has been shown that calmodulin levels are elevated 2-4 fold in a wide variety of neoplastic cells (1,2) and recently Wei *et al.* (3) have shown that increased levels of calmodulin correlate directly with tumor growth rate. Calmodulin is a ubiquitous Ca^{2+}-binding protein that mediates nearly all the Ca^{2+}-dependent function in the cell by specifically binding and activating its cellular acceptor proteins (e.g. adenylate cyclase,

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phosphodiesterase, myosin light chain kinase, etc.) (4). The Ca^{2+}-calmodulin complex is critical for the initiation of DNA synthesis and calmodulin may regulate the progression of a cell through the cell cycle from G_1 to S (5) since inhibitors of calmodulin appear to block cells near the G_1/S transition (5,6). Non-transformed cells in low Ca^{2+} medium are also blocked prior to S phase (7). On this basis, we decided to investigate the role of calmodulin and its acceptor proteins in transformation.

Materials and Methods

Cells: NRK49F is a rat kidney fibroblast cell line obtained from American Type Culture Collection. The cells were maintained in Dulbecco's Modified Eagle's medium supplemented with 10% calf serum. NRKLA23 are rat kidney fibroblasts transformed with a temperature sensitive mutant of Rous sarcoma virus. They were maintained in McCoy's medium supplemented with 15% fetal bovine serum. The NRKLA23 cells were a generous gift of Peter Vogt (USC) (8). The 6M2 cells are a rat kidney cell line transformed with a temperature sensitive transformation mutant of Moloney sarcoma virus (ts 110). The 6M2 cells (9) were maintained in McCoy's medium supplemented with 15% fetal bovine serum. Penicillin and streptomycin (1%) were routinely added to all culture media.

Growth Curve: Calcium free Dulbecco's Modified Eagle's Medium was commercially prepared by Grand Island Biological Company. Serum was prepared free of calcium by treatment with Chelex 100 (Bio-Rad) according to the procedure of Tupper *et al.* (10). The medium and serum were assayed by atomic absorption to determine their calcium content. Cells were grown in Costar 35 mm tissue culture dishes, their number determined by Coulter counter and routinely maintained at 34°C under humidified 5% CO_2 in air. Prior to the growth assay, cells were split into culture dishes and half maintained at 34° and half at 39°C in the low calcium medium (20 μM) for 48 hrs, which is considered time zero for the growth curve. This 48 hr pre-incubation allows the change in viral transforming gene expression to occur and also alters the cell Ca^{2+} equilibrium. Before the addition of low Ca^{2+} medium the cells were washed with Ca^{2+} and Mg^{2+} free phosphate buffered saline.

Calmodulin Acceptor Protein Isolation and Analysis: The 6M2 and NRKLA23 cells were radiolabeled at 34 and 39° with ^3H-leucine (specific activity 40-60 Ci/mol) at a concentration 100 μCi/ml of medium for 12 hours. Cells were washed with Dulbecco's phosphate buffered saline (DPBS) scraped and washed twice with DPBS by pelleting at 1000 rpm for 5 mins with gentle resuspension. The final cell pellet was lysed by addition of two ml of distilled water (4°C) containing 10-4 M PMSF, 1% aprotinin and 3 mM EDTA (pH 7.4) followed by homogenization in a Dounce homogenizer using the B pestle (six strokes). Lysis was confirmed by phase microscopic observation. The lysed cells were mixed with 1/5 th volume 500 mM NaCl, 40 mM Tris-HCl, 2 mM EDTA, pH 7.3 and the mixture centrifuged at 10,000 x g for 15 min. The supernatant was recovered and 1 M $CaCl_2$ added to bring the final calcium concentration to 2 mM above the EDTA level. The supernatant was centrifuged at 15,000 x g for 15 min. and applied to a calmodulin

coupled Sepharose 4BCL affinity column. The column was washed extensively with 500 mM NaCl, 40 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.3. The column was then washed with 75 mM NaCl, 40 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.3 followed by elution of calcium dependent binding proteins with 75 mM NaCl, 40 mM Tris-HCl, 2 mM EGTA, pH 7.3. Radioactivity of fraction aliquots in Aquasol was determined in a Beckman LS 7500 liquid scintillation spectrometer and those fractions containing cpm in excess of 500 cpm were pooled for further analysis. The purified protein samples were then separated on an 11% SDS-polyacrylamide gel. The gel was fixed 2 hours in 10% isopropyl alcohol and 7.5% acetic acid (11). The fixed gel was treated with autoflour (National Diagnostic) according to their recommended procedure, vacuum dried and exposed to Kodak X-OMAT RP film for analysis.

Results and Discussion

Two viral temperature-sensitive (ts) mutants with defects in transformation were selected for study. One is a Rous sarcoma virus mutant (LA23) with a temperature sensitive defect in the *src* gene (8). The *src* gene product is a phosphoprotein of about 60,000 (60K) daltons termed pp^{60} *src*; it has been shown to possess a protein kinase activity as an intrinsic property of the protein (12). The other is a Moloney mouse sarcoma virus mutant (ts 110, 9). It encodes a *gag-mos* hybrid protein that is responsible for the transformed phenotype (13,14,15,16). Recent evidence indicates that a protein kinase activity is associated with the virus-encoded $P85^{gag\text{-}mos}$ (17). The use of cell lines temperature sensitive for the transformed phenotype reduces the phenotype variation that occurs when the normal parental cells are cultured separately from their transformed counterpart. Both cell lines used in this study express transformed properties at 34°C but revert to a normal phenotype at 39°C. Clear morphologic differences in cellular appearance occur at the two temperatures (8,13).

The cells were assayed by radioimmunoassay (RIA) for calmodulin content at 34° and 39°C (Figure 1). The NRKLA23 cells show nearly a two-fold increase in calmodulin content at 34°C vs 39°C, which is consistent with previous observations (18). The 6M2 cells on the other hand do not show any significant difference in calmodulin content at 34° or 39°C. This observation is the first report of a transformed cell line that does not show an increase in calmodulin levels. While

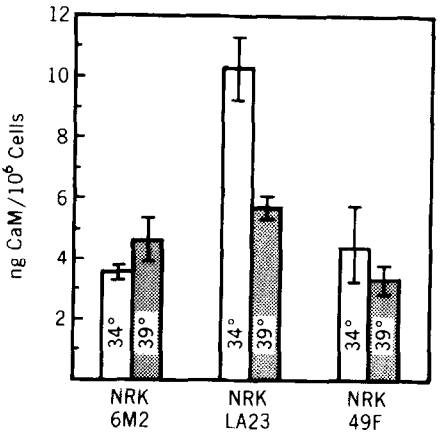

Figure 1. The calmodulin content of the 6M2, NRKLA23 and NRK49F cells when grown at either 34 or 39°C. The cells were assayed for calmodulin content by radioimmunoassay (20).

the results in Figure 1 express levels of calmodulin based on cell number, the results are the same when calmodulin levels are expressed per mg protein. As a further control we tested the parental cell line (NRK49F) (not virally transformed) and found no difference in calmodulin level whether the cells were grown at 34° or 39°C.

The ability of transformed cells to grow in low Ca^{2+} medium may be due to elevated levels of calmodulin in these cells (when compared to their normal counterparts). We then examined the ability of 6M2 cells to grow in a Ca^{2+}-deficient medium. Figure 2 shows that 6M2 cells have clearly retained this property of transformation. They clearly grow as well as the NRKLA23 cells at 34°C, and are growth inhibited at 39°C. Ca^{2+} repletion of the medium at 39°C promotes growth. These findings suggests that 6M2 cells are capable of growth in low Ca^{2+} medium without an elevation in calmodulin levels.

The present data would suggest it is not the levels of calmodulin per se that are critical in maintaining the transformed state but rather the distribution of calmodulin and its interaction with the key regulatory enzymes that the Ca^{2+}-calmodulin complex activates. These enzymes are referred to as CAPs or calmodulin acceptor proteins. To test this hypothesis, the 6M2 cells at 34° and 39°C were examined for

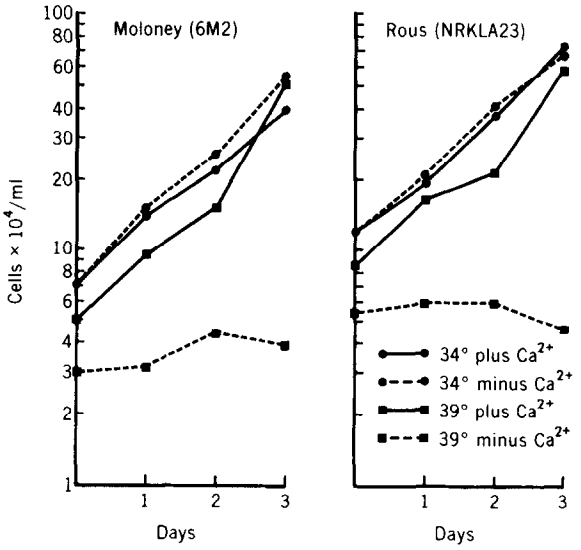

Figure 2. The ability of 6M2 and NRKLA23 cells to proliferate in low Ca^{2+} medium (20 μM) at the permissive temperature for transformation at 34°C and at the non-permissive temperature 39°C. Each point on the growth curve was performed in duplicate.

changes in their CAPs. Figure 3 is a fluorogram of labeled proteins EGTA eluted from a calmodulin-affinity column. The two different ts cell lines show a striking qualitative similarity in CAPs, especially at 68 and 80K daltons where the level of these proteins is nearly the same. This probably reflects the fact that both cell lines are derived from parental NRK cells. Despite the fact the cells share a common origin there are some qualitative as well as quantitative differences between the cell lines. The 6M2 cells have more of CAPs at 35 and 43K while the LA23 cells have CAP proteins at 72 and 84K that are drastically reduced or absent from 6M2 cells. A 90K protein is absent from LA23 but not from 6M2 cells. These changes in CAP pattern between LA23 and 6M2 could reflect the influence of different transforming viruses in the maintenance of the transformed phenotype especially as far as Ca^{2+}-regulation is concerned. The most important change that occurs in both 6M2 cells and LA23 cells is the lack of a band of apparent molecular weight 70,000 at 34°C (the transformed or permissive temperature) that is present at 39°C. This could reflect a transformation-specific change in cells transformed with oncogenic

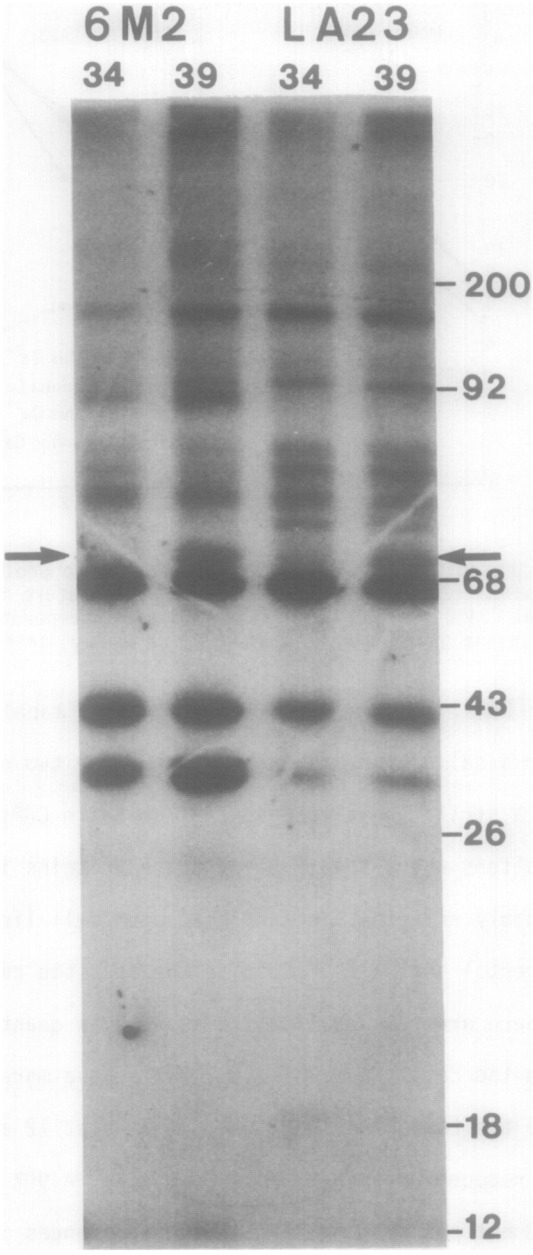

Figure 3. The fluorogram of calmodulin acceptor proteins isolated from 6M2 and NRKLA23 cells when grown at either 34 or 39°C. The proteins were isolated by calmodulin-Sepharose chromatography and were eluted from the column with EGTA. Arrow indicates 70 K protein.

viruses and may be involved in the loss of Ca^{2+}-regulation by these ts cells at 34°C.

The 6M2 cells were valuable in this study because they clearly show that elevated levels of calmodulin are not required for a cell to

express the transformed phenotype, or specifically, grow in low Ca^{2+} medium. This observation is consistent with the study by Durkin et al. (19), showing that ts cells did not require elevated levels of calmodulin to enter S phase of the cell cycle but calmodulin function was required. This implies that while calmodulin function is vital to cellular regulation, an elevation in calmodulin level is not as critical. Elevated levels of calmodulin cannot explain the loss of Ca^{2+}-regulation that occurs in 6M2 cells at 34°C but a possible explanation may rest with the calmodulin acceptor proteins. It is the CAPs which execute the cell response to a Ca^{2+}-signal for which calmodulin acts as the mediator. The 6M2 as well as NRKLA23 cells have lost CAP_{70} at 34°C (the Ca^{2+}-deregulated temperature) but regain this protein at 39°C. We speculate that in transformed cells the activity or distribution of cellular CAPs are altered and the response of the cell to a regulatory Ca^{2+} signal is modified.

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References

1. Watterson, D.M., Van Eldik, L.J., Smith, R.E. and Vanaman, T.C. (1976) Proc. Natl. Acad. Sci. USA 73: 2711-2715.
2. Chafouleas, J.G., Pardue, R.L., Brinkley, B.R., Dedman, J.R. and Means, A.R. (1981) Proc. Natl. Acad. Sci. USA 78: 996-1000.
3. Wei, J.-W., Morris, H.P. and Hickie, R.A. (1982) Cancer Res. 42: 2571-2574.
4. Means, A.R. and Dedman, J.R. (1980) Nature 285: 73-77.
5. Chafouleas, J.G., Bolton, W.E., Hidaka, H., Boyd, A.E. and Means, A.R. (1982) Cell 28: 41-50.
6. Hidaka, H., Sasaki, Y., Tanaka, T., Endo, T., Ohno, S., Fuju, Y. and Nagata, T. (1981) Proc. Natl. Acad. Sci. USA 78: 4354-4357.
7. Durkin, J.P., Boynton, A.L. and Whitfield, J.F. (1981) Biochem. Biophys. Res. Commun. 103: 233-239.
8. Ash, J.F., Vogt, P.K. and Singer, S.J. (1976) Proc. Natl. Acad. Sci. USA 73: 3603-3607.
9. Horn, J.P., Wood, T.G., Blair, D.G. and Arlinghaus, R.B. (1980) Virol. 105: 516-525.
10. Tupper, J.T., Kaufman, L. and Bodine, P. (1980) J. Cell Physiol. 104: 97-104.
11. Laemmli, U.K. (1970) Nature 227: 680-685.
12. Gilmer, T.M., Parson, J.T. and Erikson, R.L. (1982) Proc. Natl. Acad. Sci. USA 79: 2152-2156.

13. Brown, R., Horn, J.P., Wieble, L., Arlinghaus, R.B. and Brinkley, B.R. (1981) Proc. Natl. Acad. Sci. USA 78: 5593-5597.
14. Horn, J.P., Wood, T.G., Murphy, E.C., Blair, D.G. and Arlinghaus, R.B. (1981) Cell 25: 37-46.
15. Stanker, L.H., Gallick, G.E., Kloetzer, W.S., Murphy, E.C. and Arlinghaus, R.B. (1983) J. Virol. 45: 1183-1189.
16. Murphy, E.C. and Arlinghaus, R.B. (1982) Virol. 121: 372-383.
17. Kloetzer, W.S., Maxwell, S.A. and Arlinghaus, R.B. (1983) Proc. Natl. Acad. Sci. USA 80: 412-416.
18. Durkin, J.P., Boynton, A.L. and Whitfield, J.F. (1981) J. Cell Biol. 91: 2a.
19. Durkin, J.P., Whitfield, J.F. and MacManus, J.P. (1982) J. Cell Biol. 95: 19a.
20. Chafouleas, J.G., Dedman, J.R., Munjaal, R.P. and Means, A.R. (1979) J. Biol. Chem. 10262-10267.