BioFeedback

Migration in Polyacrylamide Gels of the 80K Protein Substrate for Protein Kinase C in Mouse Fibroblasts is Dependent on the Choice of Crosslinker

A number of recent papers have described a protein of 80K apparent molecular weight in fibroblasts and other cell types which undergoes rapid and pronounced phosphorylation in response to phorbol esters, certain growth factors, and other agents which activate protein kinase C (2,5,6). The evidence linking phosphorylation of the 80K protein to activation of protein kinase C has stimulated a great deal of interest, because monitoring the phosphorylation of this protein is potentially a very useful tool in studies of tumor promotion and signal transduction. However, as we attempted to replicate some of the work in the literature in the course of our own studies, we found an apparent discrepancy. In our hands, stimulation of quiescent Swiss 3T3 cells with phorbol myristate acetate (PMA), dioctanoylglycerol, phospholipase C, or serum led to significant phosphorylation of a protein with an approximate molecular weight of 90K, rather than 80K. Despite the fact that most of the published work dealing with the 80K protein was obtained with Swiss 3T3 cells, we were initially unable to detect any significant phorbol ester-dependent phosphorylation occurring in the region of our polyacrylamide gels corresponding to 80K. Representative autoradiograms of our early results are shown in Figure 1(a)-(c). In all of these experiments, the procedures were essentially those of Rodriguez-Pena and Rozengurt (5). In short, quiescent cultures of Swiss 3T3 cells were labeled with ^{32}P, exposed to either 100 ng/ml PMA (lanes indicated by +) or to the equivalent concentration of the DMSO solvent (lanes indicated by -) for 15 min. The cultures were washed and the phosphorylated trichloroacetic acid-precipitated proteins were resolved by polyacrylamide gel electrophoresis and visualized by fluorography. Gel (a) is a 5-15% gradient gel prepared on a GelBond support with AcrylAide (an olefinic derivative of agarose from FMC BioProducts) used as the crosslinker in place of bisacrylamide. Gels (b) and (c) are 8% and 10% linear gels, respectively, both on GelBond with AcrylAide. In all cases, prominent phosphorylation (arrowheads in + lanes) in the region of 90K rather than 80K accompanied conditions in which we expected to see phosphorylation in the 80K region.

In the process of attempting to reconcile our data with that in the literature, we decided to omit the GelBond support in some experiments. The effect of this is shown in Figure 1(d), which is an autoradiogram of a conventional 10% linear acrylamide-bisacrylamide gel on which aliquots of the same samples that had been run on the gel of Figure 1(c) were run. In the absence of GelBond and AcrylAide, we found that the major phorbol ester-stimulated phosphorylation migrated in the region of 80K (arrowhead, Figure 1(d)), exactly as expected from the literature. To determine whether the GelBond or AcrylAide was responsible for the retarded migration of this band in our earlier experiments, a 10% gel was prepared on a GelBond support with bisacrylamide as the crosslinker instead of AcrylAide. The result is shown in gel (e). As in gel (d), prominent phosphorylation appeared in the 80K region rather than at 90K. The 80K protein appeared to be the only phosphoprotein in these samples which ex-

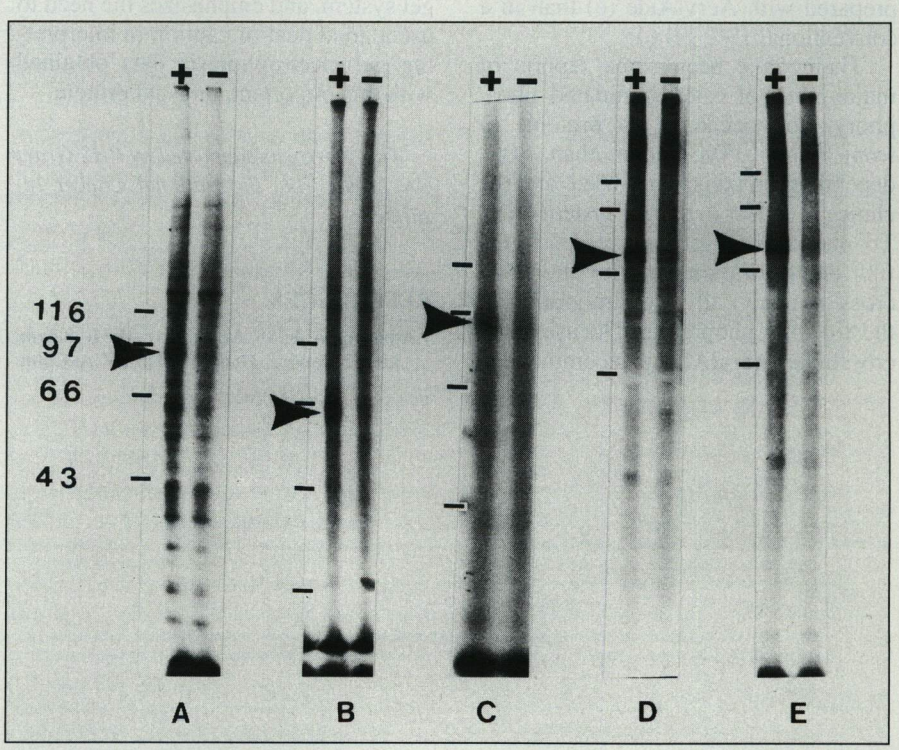

Figure 1. Autoradiograms of five SDS-polyacrylamide gels (Laemmli buffer (4)). The trichloroacetic acid-precipitated phosphorylated proteins of quiescent Swiss 3T3 fibroblasts were resolved following exposure to either serum-free culture medium containing 0.02% DMSO solvent (-) or 100 ng/ml phorbol myristate acetate plus 0.02% DMSO (+) for 15 min essentially as described in Reference 5. (a) A 5-15% gradient gel containing AcrylAide on GelBond; (b) an 8% gel containing AcrylAide on GelBond; (c) a 10% gel containing AcrylAide on GelBond; (d) a 10% gel without either GelBond or AcrylAide; (e) a 10% gel on GelBond but with the omission of AcrylAide. Gels were poured from stock solutions containing 32% acrylamide, 1% AcrylAide (gels a-c) and 30% acrylamide, 0.8% N,N′-methylene bisacrylamide (gels d-e). Aliquots of the same sample were run on gels (c-e). The molecular weights of the standards used (β-galactosidase, 116,250; phosphorylase b, 97,400; bovine serum albumin, 66,200; and ovalbumin, 42,700) are indicated and the position of the 80K phosphoprotein is shown by arrowheads. The relative migration of the standards, with respect to each other, remained unchanged, whereas the relative position of the 80K protein is dependent on the presence of AcrylAide.

hibited this behavior. Identical results were obtained when the experiments were repeated using Balb/c 3T3 cells in place of the Swiss (not shown).

It is apparent from Figure 1 that the choice of crosslinker (AcrylAide compared with bisacrylamide) has a significant effect on the migration of the 80K protein relative to the molecular weight markers on polyacrylamide gels. This is not due to a simple effect of the degree of crosslinking in the gel, as the 80K protein migrates reproducibly at 90K in 5-15% gradient gels as well as 8 and 10% linear gels (Figure 1(a)-(c)). This is also not due to a higher degree of crosslinking induced when AcrylAide is substituted for bisacrylamide, as a comparison of Figure 1(c) and (d) shows the reverse to be true: The standards migrate farther in a 10% gel prepared with AcrylAide (c) than in a conventional 10% gel (d).

There have been some reports of major phorbol ester stimulated phosphorylation occurring in proteins of from 68 to 90K rather than 80K, depending on the species used, and the choice of buffer in the gel system (1,3). To our knowledge, this report is the first demonstration that the choice of crosslinker can affect the migration of the 80K phosphoprotein. The use of the crosslinker AcrylAide is recommended by the manufacturer of GelBond because of the resiliency it imparts to gels, making it particularly effective with plastic support films. There are major advantages to using gel supports such as GelBond: (1)They are especially useful when working with gels with highly radioactive samples because they minimize direct contact with the gel; (2) there is little risk of tearing the gel (even with low percentage acrylamide gels); and (3) gels attached to supports can be air-dried, eliminating the risk of contamination of a gel dryer apparatus. Since it is likely that others in the field will be using gel supports in similar applications, we feel it is important to communicate our results. In addition, our report provides another example of the sensitivity of the 80K protein to different components of the gel system, and emphasizes the need to use a good deal of caution in interpreting gel electrophoresis data obtained with this important, unusual protein.

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REFERENCES

1. **Aderem, A.A., K.A. Albert, M.M. Keum, J.K.T. Wang, P. Greengard and Z.A. Cohn.** 1988. Stimulation-dependent myristoylation of a major substrate for protein kinase C. Nature *332*:362-364.
2. **Blackshear, P.J., L. Wen, B.P. Glynn and L.A. Witters.** 1986. Protein kinase C-stimulated phosphorylation *in vitro* of a M_r 80,000 protein phosphorylated in response to phorbol esters and growth factors in intact fibroblasts. J. Biol. Chem. *261*:1459-1469.
3. **Chida, K., H. Hashiba, K. Sasaki and T. Kuroki.** 1986. Activation of protein kinase C and specific phosphorylation of a M_r 90,000 protein of promotable balb/3T3 and C3H/10T1/2 cells by tumor promoters. Cancer Res. 46:1055-1062.
4. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*:680-685.
5. **Rodriguez-Pena, A. and E. Rozengurt.** 1986. Phosphorylation of an acidic mol. wt. 80,000 cellular protein in a cell-free system and intact Swiss 3T3 cells: a specific marker of protein kinase C activity. EMBO J. *5*:77-83.
6. **Rozengurt, E., M. Rodriguez-Pena and K.A. Smith.** 1983. Phorbol esters, phospholipase C, and growth factors rapidly stimulate the phosphorylation of a M_r 80,000 protein in intact quiescent 3T3 cells. Proc. Natl. Acad. Sci. USA *80*:7244-7248.

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