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VISUALIZATION OF PROTEIN KINASES IN LYMPHOCYTES STIMULATED TO PROLIFERATE WITH CONCANAVALIN A OR INHIBITED WITH A PHORBOL ESTER

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Stimulation of lymphocytes with a mitogenic lectin such as concanavalin A (ConA) results in differentiation and cell division. Among the changes which occur after stimulation are increases in phosphorylation of proteins and in protein kinase activity. We used a high-resolution, nondenaturing gel system to separate and visualize protein kinases in situ. We have clearly identified both autophosphorylating and substrate-dependent kinases. One band of cyclic AMP-dependent kinase activity was significantly enhanced in lectin-stimulated cells. In contrast, treatment of the cells with phorbol ester under conditions which depress stimulation caused a decrease in the activity of one kinase.

Stimulation of lymphocytes with a mitogenic lectin such as ConA leads to cell differentiation and division (1). DNA synthesis, which begins about 24 h after stimulation, is preceded by a number of biochemical changes including phosphorylation of proteins (2-4). Thus far, limitations caused by small sample size as well as the inability of ion-exchange chromatography to resolve many classes of kinases (5-7) have prevented a clear identification of the protein kinases responsible for phosphorylation and their role in stimulation. In order to compare classes of protein kinases and to determine changes in numbers or activities during stimulation, we developed a high-resolution, nondenaturing gel system to visualize the kinase activity in situ. We examined kinases from ConA-stimulated lymphocytes as well as from lymphocytes treated with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) under conditions where response to lectins is depressed. We found six distinct bands of substrate-dependent kinase activity. One of these was

ABBREVIATIONS: ConA, Concanavalin A; kDa, kilodalton; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; TPA, 12-O-tetradecanoyl phorbol-13-acetate.

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stimulated in ConA-treated cells. A second was inhibited in TPA-treated cells. In addition, we have been able to visualize autophosphorylating components.

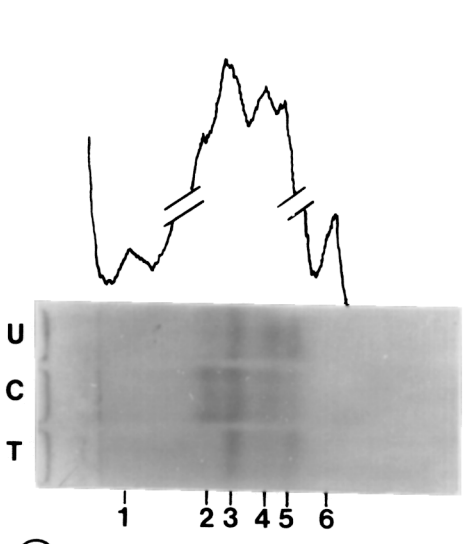
MATERIALS AND METHODS

Lymphocytes were isolated from bovine lymph nodes and cultured at 1×10^7 cells/ml (8). ConA was added at a final concentration of 7.4 μg/ml. TPA was 10^{-7} M. Twenty-four hours later the cells (50-150 ml) were collected on ice and centrifuged from the medium at 300 × g for 10 min. They were washed three times in phosphate-buffered saline (PBS) and resuspended in 2.0 ml of buffer A (9) plus 0.1% Triton X-100, or a solution of 50 mM Tris-HCl, pH 7.4; 50 mM β-mercaptoethanol; 2 mM EGTA; 2 mM EDTA; 1 mM phenylmethylsulfonyl fluoride (PMSF); 10 mM NaF; and 0.1% Triton X-100. The lymphocytes were sonicated (Fisher Sonic Dismembrator Model 300) with four bursts of 15 sec each with a 15-sec rest between bursts. Cells were >95% broken as observed microscopically. The sonicated samples were held on ice for 30 min with occasional shaking before being centrifuged at 100,000 × g for 1 h (Beckman L565, SW 50.1 rotor). The supernatants were collected, aliquoted and frozen at -80°C. The day before electrophoresis, samples were dialyzed against 0.1 M Tris-Cl (pH 6.8), 2 mM PMSF, aliquoted, and stored at -80°C overnight.

Samples, 100 μg protein, were run on Laemmli gels (10) without SDS. A 5% acrylamide running gel (9 cm length) containing 10% glycerol, and a 3% acrylamide stacking gel with 6% glycerol (1 cm) were polymerized onto a gel support film (Gel Bond, FMC Corp., Rockland, Maine). Samples were electrophoresed at 50V through the stacking gel and 200V through the running gel in the cold for approximately 4½ h. Following electrophoresis the gels were soaked for 1 h each in two changes of 50 ml ice-cold 50 mM Tris-HCl (pH 7.5). Slabs were cut in half or thirds depending on the experiment. For phosphorylation each section was incubated in a small plastic box for 30 min at 37°C in 25 ml of a solution of 0.3 M Tris-acetate (pH 7.4), 0.04 M Mg-acetate, 0.15 M NaF, 1 mM $NaPO_4$ (pH 7.4), 0.3 mM EGTA, plus or minus histone type II-AS (Sigma) at 4 mg/ml. The reaction was initiated by the addition of [γ^{32}P]ATP (Amersham) diluted to 2-3 Ci/mM and used at 2 μCi/ml. After an incubation with gentle shaking at 37°C for 1 h, the reaction was terminated by removal of the incubation mixture followed by two rapid rinses with 100 ml of cold 5% trichloroacetic acid, 1% phosphoric acid. The gel was soaked overnight in the cold with the acid solution, washed three more times with the same solution, fixed overnight in 10% acetic acid, 30% methanol, and dried in an oven at 60°C. Some gels were stained with Coomassie brilliant blue (R250, Biorad) added at 0.1% to the fixative. Dried gels were exposed to Kodak X-omat R film for radioautography. The gel/film was exposed in a cassette containing one detail screen (Cronex XTRA Life Detail, DuPont), for about 6-24 h, depending on the radioactivity. The radioautograph was screened on an LKB Laser Densitometer (Model 202 Ultroscan).

RESULTS AND DISCUSSION

When cytoplasmic extracts from ConA-stimulated cells were separated and assayed *in situ* using histone as a substrate, six bands of kinase activity were seen (Figs. 1,2). Bands 1 and 6 were barely detectable in short exposures of radioautographs. Bands 3, 4 and 5 were the most intense. Band 3 appeared to be a doublet. These five bands were present in stimulated and nonstimulated cells, but were of greater intensity in the stimulated cells.



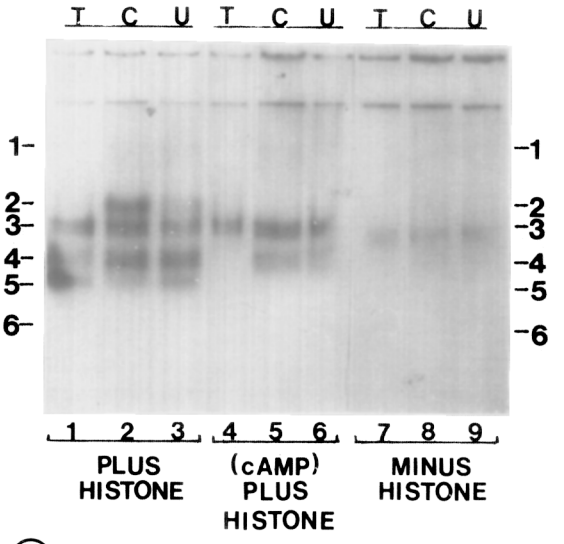


Figure 1. Cytoplasmic protein kinases of lymphocytes on nondenaturing gels in the presence of histone. Lymphocytes were left unstimulated (U), or stimulated either with ConA (7.4 µg/ml) (C), or TPA (10^{-7} M) (T), for 24 h, before extracts were separated on nondenaturing gels and phosphorylated in situ with histone as substrate. Shown is a radioautogram after 6 h of exposure. Also shown, for ConA-stimulated cells, is a densitometer trace of a scan of a radioautogram which was exposed 24 h in order to see bands 1 and 6.

Figure 2. Protein kinase activity in the presence and absence of substrate and after treatment with cyclic AMP. Cytoplasmic extracts from lymphocytes treated as described in the legend to Fig. 1 were separated on nondenaturing gels and phosphorylated plus or minus histone. One set of samples was incubated with cyclic AMP at 10^{-4} M for 5 min before application to the gel.

However, an additional kinase, band 2, was present in the ConA-treated cells, but faint or absent in the nonstimulated. The appearance of band 2, as well as an increase in intensity of the other bands, was most characteristic of the stimulated cells, using histone as the exogenous substrate.

When histone was omitted from the phosphorylation mixture, two faint bands were present (Fig. 2) in both stimulated and nonstimulated cells. These autophosphorylating proteins migrated in the same positions as bands 3 and 4, which were prominent in the presence of histone.

In order to identify cyclic AMP-dependent kinases, the homogenates were incubated with 10^{-4} M cyclic AMP before being electrophoresed. This treatment separates the catalytic and regulatory subunits of cyclic AMP-dependent protein kinase and prevents the catalytic subunit from migrating into the gel (6,7). Thus, cyclic AMP dependence is evidenced by the loss of a band(s) of

activity. After treatment of lymphocyte homogenates with cyclic AMP, three bands of activity (bands 2,5,6) disappeared from the gels (Fig. 2). Bands 3 and 4 did not disappear or change in intensity after treatment with cyclic AMP (Fig. 2) or cyclic GMP (data not shown). These kinases appear to belong to the general class of cyclic AMP-independent kinases.

We also examined the kinases of lymphocytes treated with phorbol ester in such a way as to block DNA synthesis (8). When homogenates of lymphocytes pretreated for 24 h with TPA were assayed for protein kinase activity, the pattern of phosphorylation in the presence of histone was generally the same as that of the unstimulated cells (Fig. 2). One exception was a decrease in intensity of band 4. Relative to the other bands, band 4 was faint or absent. Kwong and Mueller (11) have also reported the loss of phosphorylation of a membrane protein in TPA-treated bovine lymphocytes, but at this time it is unclear how the decrease in kinase activity seen in band 4 is related.

In order to determine the actual number of proteins in an area of kinase activity, each band was cut from the native gel after phosphorylation in the presence of histone and rerun on an SDS gel. The results for all six bands were similar. Shown are the results of band 3 (Fig. 3). As expected, the major bands visible by silver staining and by radioautography were the histones, the added endogenous substrate. In addition there were seven or eight bands faintly labeled. One of these, about 30 kilodaltons (kDa), is a contaminant of the histone preparation. The rest may be endogenous autophosphorylating components or substrates in the cell lysate.

In order to look at the autophosphorylating components more closely, band 3, the most prominent of these, was cut from a gel which had been phosphorylated without histone and was rerun on an SDS gel. A week's exposure to X-ray film revealed two labeled bands estimated to be approximately 78 and 52 kDa (Fig. 4). Interestingly, the autophosphorylating protein kinase C is reported to be about 75-80 kDa (13). The possibility that band 3 is a protein kinase C needs to be determined.

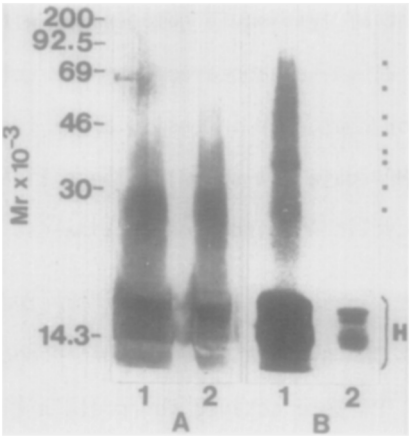


Figure 3. Radioautograph of an SDS gel electropherogram of band 3 kinase from ConA-stimulated lymphocytes after phosphorylation on a nondenaturing gel. Band 3 was cut from several lanes of a dried, nondenaturing gel after phosphorylation in the presence of histone, and rehydrated in distilled water. The slices were transferred to 100 µl of 0.1 M Tris-HCl, pH 6.8, homogenized in a small ground-glass homogenizer, and left overnight in the cold. To the extract was added SDS to 0.2%, and dithiothreitol (DTT) to 0.1 mM before it was heated for 1 min at 100°C. A drop of bromophenol blue and glycerol was added and two volumes of the samples were run on SDS gels according to Laemmli (10). The running gel was 10% acrylamide (13 cm) with a 4% stacking gel (1 cm). Molecular markers were a ^{14}C-methylated protein mixture (Amersham) containing myosin, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase and lysozyme. The gel was stained with silver stain by a modification of Merril's procedure (12) before radioautography. Lane 1 had three times as much extract as lane 2. A = silver stain; B = radioautograph; H = histone.

In order to examine the endogenous substrates for the cytoplasmic kinases, the cytoplasmic extracts were phosphorylated in a test tube with no added substrates or kinases and separated on an SDS gel. Numerous endogenous

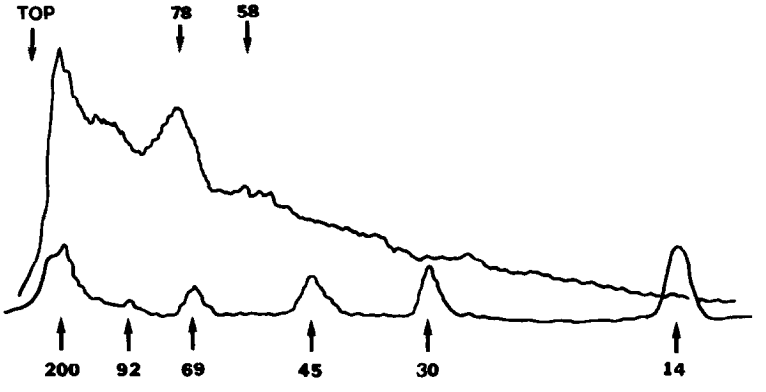


Figure 4. Autophosphorylating components of band 3 kinase. Band 3 was cut from several lanes of a nondenaturing gel after autophosphorylation and treated as described in the legend to Fig. 3. X-ray film was exposed to the gel for 7 days. Shown is a trace of the radioautogram and of the ^{14}C markers.

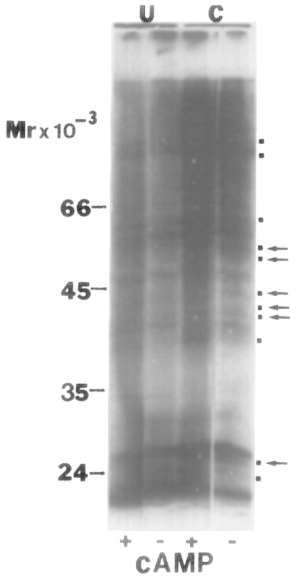


Figure 5. Radioautograph of proteins phosphorylated by endogenous protein kinases in cytoplasmic extracts of unstimulated and ConA-stimulated lymphocytes. The cytoplasmic fraction from cells stimulated 24 hr with ConA or from unstimulated cells was phosphorylated in test tubes before separation on SDS PAGE gels. The clarified 100,000 × g supernatant was dialyzed against phosphorylation buffer (14) and 100 µg protein was phosphorylated in 80 µl total volume with 10 µCi [γ^{32}P]ATP (2-3 Ci/mMole) for 10 min at 37°C. When indicated, cyclic AMP was present at 10^{-6} M. The reaction was stopped by the addition of SDS to 2% and DTT to 0.1 mM and treated as described in the legend to Fig. 3. Molecular standards were bovine serum albumin, egg ovalbumin, pepsin and trypsinogen.

substrates were seen by radioautography (Fig. 5). The ConA-stimulated cells showed at least 25 bands of labeled proteins. Cyclic AMP increased the intensity of several bands, e.g., those of approximately 83, 80, 61, 57, 50, 49 to 39, 29 and 24 kDa. One band of approximately 80 kDa was one of the most intense in the stimulated cells and may correspond to the 80 kDa protein which becomes highly phosphorylated in growth-factor stimulated 3T3 cells (15). The unstimulated lymphocytes showed a pattern similar to that of the stimulated cells but the intensity of the bands was much lower. However, several bands which were enhanced by cyclic AMP in the ConA-stimulated cells appeared to be absent or barely detectable in the unstimulated lymphocytes (approximately 57, 44 to 50, 39 and 24 kDa). It is not known at this time if the protein kinase of band 2 in the nondenaturing gel is responsible for the phosphorylation of these substrates in the stimulated cells. Addition of TPA directly to the phosphorylation mixture had no effect on the phosphorylation.

In summary, we have used a sensitive system to assay endogenous kinases in lymphocytes. Although there is plentiful evidence that lymphocytes have all of the machinery for regulation with protein kinases and phosphoprotein substrates, there has been no clear resolution of the numbers or role of either (see ref. 1 for review). Standard DEAE-chromatography techniques are not only difficult to use with small samples, but also have not given good resolution of classes of kinases in extracts of various cultured cells (6,7) or with lymphocytes (2). Although several techniques for assaying protein kinases on gels have been published (5,6,16,17), we have modified the technique to increase its sensitivity and resolution. For the first time it is possible to detect autophosphorylating components. Applying this technique to lymphocytes we have identified two autophosphorylating and six histone-dependent kinases. At least three bands of kinase activity were sensitive to cyclic AMP. One of these, band 2, is intensified in ConA-stimulated but not in unstimulated cells. Two major bands were not cyclic AMP-dependent. One cyclic AMP-independent band which showed significant activity may be protein kinase C, a cyclic AMP-independent kinase and the most abundant kinase in lymphocytes (18,19). Another cyclic AMP band was depressed when cells were treated with TPA and under the conditions where lymphocyte proliferation was also depressed. Further correlation between the kinases and the stimulatory and inhibitory reactions remains to be defined.

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