Protein Kinases in Brown Adipose Tissue of Developing Rats

ELECTROPHORETIC SEPARATION AND ASSAY OF SOLUBLE PROTEIN KINASES ON POLYACRYLAMIDE GELS AND A STUDY OF THEIR PROPERTIES AND CHANGES DURING DEVELOPMENT*

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BRIAN L. KNIGHT and JOSEPH P. SKALAS
From the Medical Research Council Lipid Metabolism Unit, Hammersmith Hospital, London W12 OHS, United Kingdom, and the Centre for Developmental Medicine, Department of Paediatrics, University of British Columbia, Vancouver, British Columbia, Canada, V5Z 1L7

Protein kinase activity was detected and assayed directly on polyacrylamide gels after disc electrophoresis of the 100,000 × g supernatant fraction of brown adipose tissue of infant rats. Nine major bands of activity were detected, eight of which could be stimulated by cAMP or inhibited by the cAMP-dependent protein kinase inhibitor protein. This electrophoretic technique revealed heterogeneity in the cAMP-dependent protein kinase activity eluted from DEAE-cellulose by high concentrations of salt, but not in the peak of activity eluted by low concentrations of salt. The catalytic properties and substrate specificities of the kinases in the various bands were studied while the enzymes were still in the gels. The activity in each band differed from each of the others in at least one of these properties.

The activities of the protein kinases in brown fat changed as the animals grew, and each band exhibited a distinct and unique developmental pattern. The major changes in kinase activities occurred in the immediate post-parturition period, then at 15 days after birth and at weaning. These developmental stages coincide with the periods during which the tissue undergoes changes in the rate of its proliferation, differentiation, and functional activity.

The main, and probably the only, function of brown adipose tissue is to produce heat (1). In rats, the tissue rapidly proliferates and differentiates after birth until a maximal capacity for heat production is achieved at about 10 days. This high functional capacity is then maintained for approximately 1 week, after which the tissue begins a gradual process of involution which coincides with the decreasing requirements for nonshivering heat production (2). It is thought that the regulation of heat production (3-5) and of tissue proliferation and differentiation (6-9) are associated with the phosphorylation of specific proteins or enzymes by cyclic nucleotide-dependent protein kinases.

A wide variety of proteins have been shown to act as substrates for cyclic nucleotide-dependent protein kinases, but it has not proved possible to demonstrate a corresponding variety of protein kinases with different specificities for protein substrates (8, 10). However, a small number of protein kinases with similar catalytic properties can often be separated from a single tissue by chromatography. Brown fat, for instance, contains at least three cAMP-dependent protein kinases and one cyclic nucleotide-independent protein kinase (4, 11). It is not known whether these enzymes have different functions in the tissue despite their apparent similarity. If the enzymes in brown fat are associated with different processes, then their individual activities might change during development in accord with the changes occurring in the relative activities of the various processes. We have, at least partially, investigated this possibility using the microsome-free supernatant fraction from brown fat. In order to avoid differential losses on chromatography and because of the small quantity of tissue that was available, we separated protein kinases by electrophoresis on polyacrylamide and assayed their activities directly on the gels. A number of protein kinases could be separated from brown fat by this method and this paper reports their properties and the changes in their relative activities during development.

Experimental Procedures

Tissue Extracts—Wistar rats were used throughout. They were kept at 23° in a 10-h light, 14-h dark lighting cycle. Newborn animals (8/litter) were left with their mothers until weaned at 30 days.

Brown adipose tissue was obtained from the interscapular and cervical regions and tissue from a number of rats of the same age was pooled (5). The tissue was homogenized in 2 volumes of ice cold 0.25 M sucrose containing 1 mM EDTA and 10 mM Tris/HCl, pH 7.6, using a glass Potter-Elvehjem homogenizer fitted with a motor-driven Teflon pestle. The homogenate was centrifuged at 10,000 × g for 20 min at 4°. The supernatant between the precipitate and the floating fat was then centrifuged at 100,000 × g for 60 min in a Beckman L2-65B ultracentrifuge using an SW-50.1 rotor at 4°. The clear supernatant between the precipitate and the floating fat was then centrifuged at 100,000 × g for 60 min in a Beckman L2-65B ultracentrifuge using an SW-50.1 rotor at 4°. The clear supernatant so obtained was assayed for protein by the method of Lowry et al. (12) and for protein kinase activity as described previously (13).

The assay medium contained 30 mM 2-(N-morpholino)ethane-sulfonic acid buffer (pH 6.5), 0.3 mM ethylene glycol bis(β-aminoethyl ether)N,N′-tetraacetic acid (EGTA), 0.2 mM EDTA, 2 mM theophylline, 10 mM magnesium acetate, 0.2 mM γ-[32P]ATP, 15 mg/ml of histone (type II), and up to 20 μl of supernatant in a total volume of 100 μl. The reaction was started by the addition of enzyme and continued for 10 min at 30°. The 100,000 × g supernatant fraction...
was dispersed into small tubes and frozen. Once thawed, any unused sample was discarded.

**Separation Methods** - Ion exchange chromatography was performed on a column (10 x 0.9 cm) of DEAE-cellulose equilibrated at 4° with 10 mM (N-morpholino)ethanesulfonic acid, pH 6.5, containing 1 mM EDTA. After application of 2.5 ml of the 100,000 x g supernatant, the column was washed with 2 bed volumes of equilibration buffer and eluted with a linear gradient (0 to 400 mM) of NaCl in the same buffer.

Electrophoresis was performed in vertical slabs of polyacrylamide gel using the discontinuous system described by Davis (14). The slabs were prepared in glass cassettes with internal dimensions 8.2 x 8.2 x 0.28 cm. They consisted of 6 cm of a 7% polyacrylamide (pH 8.9) separating gel under 2 cm of a 2.5% polyacrylamide (pH 6.7) spacer gel. Samples were applied into wells (1 cm deep by approximately 0.3 cm wide) in the spacer gel. There were normally eight wells per slab, each containing 20 μl of sample. Up to four slabs could be run at the same time. Heat produced during electrophoresis was dissipated into the electrophoresis buffer which surrounded the slabs. The buffer was continuously circulated through a cooling coil in ice cold water and the whole apparatus was placed at 0°, so that the temperature of the slabs never rose above 4°. A potential of 300 V was applied which produced 20 mA/gel under these conditions. Electrophoresis was continued until marker haemoglobin (R, 0.4081) had run to 1 cm from the bottom of the separating gel (approximately 4 h).

**Detection of Protein Kinase Activity on Polyacrylamide Gels** - The assay for protein kinase activity on the gels was developed from the method described by Hirsch and Rosen (15) for locating the kinases after electrophoresis. The gels were removed from their cassettes and soaked in 50 ml of ice cold 250 mM Tris/HCl, pH 7.0, for 30 min with two changes of buffer. They were washed for 10 min in ice cold 50 mM Tris/HCl, pH 7.0, and cut longitudinally into strips if required. The gels were then incubated with gentle shaking for 30 min in 10 ml of ice cold 50 mM Tris/HCl, pH 7.0, containing 15 mg/ml of histone type II A. The gel and histone solution were warmed to 37° in a water bath and 4.3 ml of concentrated assay mixture was added to give a final concentration of 50 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.5), 0.3 mM ethylene glycol bis(β-aminoethyl ether)N,N',N'-tetraacetic acid, 0.2 mM EDTA, 5 mM theophylline, 10 mM magnesium acetate, 10 μM CAMP, and 50 μM [γ-32P]ATP (specific activity, 100 to 200 dpm/μmol). After 15 to 30 min of gentle shaking at 37°, the reaction mixture was removed and the gels were rinsed twice in ice cold 5% trichloroacetic acid and left soaking with gentle agitation in 1 liter of 5% trichloroacetic acid overnight. The trichloroacetic acid solution was replaced and the washing continued for 7 to 8 h. The gels were then soaked in 10% acetic acid overnight, dried on filter paper under vacuum, and autoradiographed. Radioactive bands were cut out and the radioactivity was measured in a liquid scintillation spectrometer with 0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene in toluene as scintillation fluid. Densitometer traces of autoradiograms were obtained with a Joyce-Loebl densitometer.

**Materials** - [γ-32P]ATP (10 to 15 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, United Kingdom. Nonradioactive nucleotides, haemoglobin, protamine, and histones were from Sigma Chemical Co., St. Louis, Mo. Vitamin-free casein and phosphotin were obtained from Nutritional Biochemicals Co., Cleveland, Ohio. Cyclic AMP-dependent protein kinase inhibitor protein was purified from beef muscle by the method of Gilman (16). Kodirex x-ray film was from Kodak Ltd., London, United Kingdom, and DEAE-cellulose (DE11) was from Whatman Biochemical Labs, Maidstone, United Kingdom.

**RESULTS**

**Separation of Protein Kinase Activities** - The microsoma-free supernatant fraction of brown fat was subjected to electrophoresis, and protein kinase activity was assayed directly on the gels as described under "Experimental Procedures." Radioactive phosphorylated substrate was present in the positions occupied by the protein kinases and was detected by autoradiography. Fig. 1 shows a densitometer trace of an autoradiogram produced by this procedure. Ten regions of radioactivity could normally be distinguished. Band 1 only just penetrated the gel. Band 2 was very faint and was not included in these studies. Bands 3, 4, and 5 were sharp and well separated, whereas Band 6 was more diffuse. Bands 7 and 8 were well defined and represented the major protein kinase activities in brown fat cytosol. Assay conditions for Fig. 1 were deliberately chosen so that the activities of Bands 7 and 8 were selectively reduced to allow the minor bands to be demonstrated more clearly. There were also two diffuse regions of kinase activity which we have called Bands 9 and 10.

The protein kinase activities separated by electrophoresis could be located in the various peaks of activity separated by the routine method of DEAE-cellulose chromatography. Protein kinase activity in the supernatant fraction of brown fat was eluted from DEAE-cellulose as five partially separated peaks, as shown in Fig. 2. Samples from each peak were subjected to electrophoresis and Fig. 3 is a representation of the results. Kinase activity in Peak I ran as a single band in a position corresponding to Band 3. This was clearly separated from Band 4 which was eluted in DEAE-cellulose Peaks IIA.
and IIB along with the major bands, 7 and 8. Chromatography did not completely separate Bands 7 and 8. Bands 1 and 6 were located almost exclusively in DEAE-cellulose Peaks IIC, whereas Bands 5, 9, and 10 were present in Peaks IIIC and III. A summary of RF values and behavior on DEAE-cellulose is given in Table I.

Assay of Protein Kinase Activities - The method for detecting protein kinase activity on polyacrylamide gels had been developed merely as a qualitative procedure (15). It had not been established whether the incorporation of radioactivity into the substrate protein was a reliable method for estimating the relative activities of the kinases in each band.

The bands were located by autoradiography and then cut out and the radioactivity assayed. Incorporation of 32P into the substrate protein was directly proportional to the length of the incubation at 37° for at least 90 min and to the amount of sample applied to the gel up to at least 500 μg of protein. There was some (<20% of that with histone) incorporation of radioactivity into Bands 1, 3, and 4 in the absence of added substrate protein. Incorporation into the other bands was dependent upon the addition of a protein substrate such as histone. For each band, the double-reciprocal plot of incorporation against concentration of histone was a straight line (Fig. 4). There was no significant difference between the bands in values of apparent $K_a$ for histone calculated from these lines (Table II). Incorporation of 32P was also affected by the concentration of ATP, and in this case there were quite large differences between bands in values of apparent $K_a$ (Fig. 5; Table II). The apparent affinity for ATP of the kinases represented by Bands 3 and 4 was almost 10-fold higher than that of Band 8 or 9. There were also differences between bands in the concentration of magnesium required to give the maximal incorporation (Table II).

These results indicated that protein kinases could be assayed directly upon polyacrylamide gels. Incorporation of radioactivity never varied by more than 10% between duplicate samples assayed at the same time. There was a similar consistency between duplicate samples assayed at different times if the incorporation was expressed as a percentage of the total.

Properties of Protein Kinases - Electrophoresis was performed at pH 8.3. The activities of most of the kinases were increased if the pH was lowered by soaking the gels in 250 mM Tris/HCl, pH 7.0 (Table III). Only the kinases in Bands 1 and 4 showed a significant increase in activity.

![Diagram of DEAE Cellulose Peaks](Fig. 3)

**Fig. 3.** Electrophoretic behavior of protein kinase activities in the peak fractions obtained from DEAE-cellulose chromatography. Samples from each DEAE-cellulose fraction (see Fig. 2) were dialyzed and subjected to electrophoresis and protein kinase assay as described under "Experimental Procedures." The figure is a diagrammatic representation of the positions and relative activities of the kinases detected.

**Table I**

| Band | $R_f$ on electrophoresis | Approximate concentration of NaCl required for elution from DEAE-cellulose | mM |
|------|-------------------------|---------------------------------|-----|
| 1    | 0                       |                                 | 260 |
| 3    | 0.067 ± 0.002           |                                 | 50  |
| 4    | 0.089 ± 0.002           |                                 | 160 |
| 5    | 0.159 ± 0.001           |                                 | 290 |
| 6    | 0.187 ± 0.002           |                                 | 260 |
| 7    | 0.240 ± 0.001           |                                 | 160 |
| 8    | 0.278 ± 0.002           |                                 | 200 |
| 9    | 0.341 ± 0.003           |                                 | 300 |
| 10   | 0.432 ± 0.004           |                                 | 300 |

![Table II](Table II)

**Table II**

| Band | Apparent $K_a$ for ATP | Histone | Optimal concentration of magnesium acetate |
|------|------------------------|---------|------------------------------------------|
|      | μM                     | mg/ml   | mM                                      |
| 1    | 47                     | 6.3     | 5                                       |
| 3    | 8                      | 2.8     | 20                                      |
| 4    | 8                      | 3.3     | 25                                      |
| 5    | 17                     | 4.7     | 40                                      |
| 6    | 15                     | 4.3     | 10                                      |
| 7    | 35                     | 2.4     | 5                                       |
| 8    | 72                     | 2.8     | 8                                       |
| 9    | 73                     | 4.3     | 10                                      |
| 10   | 68                     | 4.0     | 10                                      |

**Fig. 4.** Effect of histone concentration on the incorporation of radioactivity. Samples (20 μl) of 100,000 x g supernatant fractions from brown fat of 24-day-old rats were subjected to electrophoresis as described under "Experimental Procedures." The gel slabs were cut longitudinally into three-lane pieces which were assayed for protein kinase activity (see "Experimental Procedures" with different concentrations of histone type II A. The gels were dried and autoradiographed and the radioactivity in the bands was determined. Results from the three lanes were averaged and the reciprocal was plotted against the reciprocal of the concentration of histone. The figure shows the results for Band 5 as representative for all bands.
Effect of pH, salt concentration, and temperature on activity of protein kinases in electrophoretic bands.

The addition of the protein kinase inhibitor protein (17) inhibited the kinase activity in every band except Band 1 (Table IV). This inhibitor is specific for cAMP-dependent protein kinases. However, kinase Bands 4, 5, and 6 did not exhibit the expected stimulation by cAMP under normal assay conditions with histone as substrate (Table IV). The assay usually included a 30-min preincubation of the gel with the protein substrate. This allowed the substrate to diffuse into the gel and improved the incorporation of radioactivity. Some protein kinases were examples of this type of protein kinase. They could only be stimulated during a preincubation with histone (see "Experimental Procedures") with a different protein substrate. Concentrations of substrates employed were 10 μg/ml of histone type IIA, 5 μg/ml of arginine-rich histone, 10 μg/ml of lysine-rich histone, 1 μg/ml of protamine (pH 7.0), 5 μg/ml of phosvitin, and 5 μg/ml of hydrolyzed casein. Gels were dried and autoradiographed and the radioactivity in the bands was determined. Results from the two lanes were averaged, corrected for any basal activity, and expressed as a percentage of the incorporation with histone type IIA.

### Table IV

| Band | Activity ratio |
|------|---------------|
| 1    | 0.91          |
| 2    | 1.57          |
| 3    | 0.97          |
| 4    | 1.60          |
| 5    | 1.83          |
| 6    | 6.55          |
| 7    | 7.73          |
| 8    | 2.90          |
| 9    | 3.31          |

Values are per cent of activity with histone type IIA.

### Table V

| Band | Activity ratio |
|------|---------------|
| 1    | 0.82          |
| 2    | 1.17          |
| 3    | 0.96          |
| 4    | 1.73          |
| 5    | 1.59          |
| 6    | 2.25          |
| 7    | 1.75          |
| 8    | 2.04          |
| 9    | 1.93          |

Results shown are the average for three determinations.
stimulated by cAMP either with or without the preincubation with histone (Table IV). There was no difference between bands in the range of concentrations over which cAMP exerted its effect. Almost complete activation was achieved by raising the concentration of cAMP from 0.3 to 3.0 μM. If the gel was not preincubated with histone (Table IV) or was assayed without added substrate, the activity in Band 1 was inhibited by cAMP. This was a consistent result for which we have no immediate explanation.

Many of the kinases separated by electrophoresis had different preferences for protein substrates. Each substrate was used at a concentration that was 5-fold higher than the apparent $K_{m}$ determined with the original supernatant fraction, and the incorporation was calculated for each band as a percentage of that with histone type IIA (Table V). Band 1 readily phosphorylated histones and casein but not protamine or phosvitin. Band 3 favored arginine-rich histone whereas Band 4 was active towards the more basic lysine-rich histone or protamine. Band 5 was active with phosvitin or casein. The other bands used arginine-rich or mixed histones and did not significantly phosphorylate casein, phosvitin, protamine, or lysine-rich histone.

**Development of Protein Kinases** — Fig. 6 shows densitometer traces representing kinase activities in supernatant fractions from rats at three different ages. In these experiments, and in the subsequent developmental studies, protein kinase activity was assayed in the presence of cAMP with histone type IIA as protein substrate (see "Experimental Procedures"). There were clear differences in the proportions of the various bands between different ages. In particular Bands 5, 6, and 8 were greater in newborn animals and Bands 9, 10, and 3 were greater in adult animals. In order to discover when these changes occurred we prepared more detailed developmental curves. Each sample was subjected to electrophoresis and assayed on at least two separate occasions. Incorporation into each band was expressed as a percentage of the total for that sample and the average values for samples within each age group were combined and shown as mean ± standard error in Fig. 7. This demonstrates the relative activities of the enzymes at different ages. Band 7 was the most active kinase in brown fat from newborn animals and its proportion gradually increased as the animals grew. Bands 9 and 10 also increased during infancy, whereas most of the others declined. The

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**Fig. 7.** Relative activities of the protein kinases at different stages of development. Samples (20 μl) of 100,000 x g supernatant fractions of brown fat were subjected to electrophoresis and protein kinase activity was detected on the gels as described under "Experimental Procedures." Each sample was assayed on at least two separate occasions. Incorporation was expressed as a percentage of the total for that lane. Average values for samples within each age group were expressed as mean ± standard error. There were four separate pools of tissue from newborn and 21-day-old rats and three separate pools of tissue from rats at the other ages. Results are given in the upper part of the figure for Bands 1 (○), 9 (△), and 10 (▽), and in the lower part for Bands 3 (○), 4 (●), 5 (▲), 6 (■), 7 (▲), and 8 (▲).
activity in Band 3 increased dramatically after weaning at about 30 days. A better way to demonstrate the changes in the activities of the various kinases is to express each as a specific activity recalculated as a percentage of that found in brown fat from newborn animals. This does not give an indication of their relative activities, but takes into account changes in the total kinase activity in the tissue and treats each activity independently (Fig. 8). The activity in each band had a different pattern of development. Band 1 fell after 5 days, stayed low, then recovered in the adult. Band 3 also fell after 5 days, but its activity increased greatly in the adult. The activity in Band 4 fell from birth and stayed low, whereas that in Band 5 was unchanged for 2 weeks, fell, and recovered in the adult. Kinase activity in Band 7 rose during the second week and remained high. Activity in Band 8 rose from birth to reach a peak at 15 days and then rapidly fell in the third week. Bands 9 and 10 increased during the first 2 weeks and stayed high. The most dramatic changes in activity occurred at 5 or 15 days or on weaning at 30 days.

**DISCUSSION**

Nine major bands of protein kinase activity could be detected on polyacrylamide gels after disc electrophoresis of the 100,000 x g supernatant fraction of brown adipose tissue. Most of the kinases separated by the conventional technique of ion exchange chromatography gave more than one band on electrophoresis. This heterogeneity explains the apparently poor separation of protein kinase activities during chromatography on DEAE-cellulose. The protein kinase activity in each band differed from each of the others in at least one catalytic property. Band 1, which hardly entered the separating gel, contained a cAMP-independent protein kinase. This enzyme had been isolated previously by chromatography on phosphocellulose (11) and was particularly active with casein as substrate. Band 5 also contained a casein kinase, but this enzyme had a relatively greater activity than Band 1 with phosvitin and could be stimulated by cAMP. Bands 6 to 10 contained cAMP-dependent histone kinases which could be classified as type II kinases under the convention of Corbin et al. (19). Brown fat contained only one type I kinase (19), which migrated on electrophoresis as Band 3. The activities in Bands 3 and 4 were not greatly stimulated by cAMP, although they were inhibited by the inhibitor protein that is specific for cAMP-dependent enzymes. This suggests that they were true cAMP-dependent protein kinases but that they could not be stimulated by cAMP when on the gels. These bands exhibited some activity without the addition of protein substrate, so it is possible that substrate proteins which co-migrated on the gel had already activated the enzymes before the addition of cAMP. Certainly Bands 5 and 6 could be activated by substrate since they appeared to be insensitive to cAMP after a prolonged incubation with histone (Table IV). The endogenous substrates in Bands 1, 3, and 4 could not be completely removed by chromatography on DEAE-cellulose. However, the maximum activity shown by those bands was not determined by the concentration of the endogenous substrates, so their presence did not significantly affect any of the results reported here.

The properties of the protein kinases in the various bands, as measured on the gels, indicate that each is a different enzyme. Confirmation of this must await their further purification and characterization since it is possible that some still formed complexes with substrates or modulators which could affect their properties. The relative proportions of the bands were not affected during storage of the original tissue or by freezing and thawing or storage of the supernatant fraction. Moreover, the activity in each band showed a different pattern of development. Thus the different bands are not produced by proteolysis or as artifacts during homogenization or storage, but we cannot exclude the possibility that some of the bands represent different forms of the same enzyme that can be interconverted in vitro.

The results presented here show that the method of Hirsch and Rosen (15) for locating protein kinases on polyacrylamide gels can be readily adapted to provide an assay of the activities of the kinases. Since the properties of the enzymes were different, the choice of assay conditions was inevitably a compromise and the method could not be used to give absolute values of kinase activity. Nevertheless, it provided reliable information on changes in the activity of individual bands and the relative activities between bands. This technique could prove to be extremely useful in demonstrating changes in the activities of protein kinases, especially when only small amounts of tissue are available and chromatographic separation is impractical or undesirable.

The protein kinase activity in each band changed as the animal grew and brown adipose tissue proliferated and then involuted. From Fig. 8 it can be seen that the most dramatic changes in kinase activity occurred during the early neonatal stage, then at 15 days, and subsequently at weaning. These are the periods when there are pronounced changes in the rate of proliferation of the tissue, its capacity to produce heat, and type of substrate supplied to the tissue (2, 6, 7, 20). There is no

**Fig. 8.** Changes in specific activity of protein kinases during development. Incorporation for each band was expressed per mg of protein applied and this specific activity as a percentage of that of the same band in a sample from newborn rats assayed at the same time. Results are given for Bands 1 (x) through 10 (symbols used are the same as those in Fig. 7). For details of assay, see legend to Fig. 7.
direct evidence to link any change in kinase activity with a change in any particular process. However, each of the protein kinase activities had a distinct and unique pattern of development which presumably must in some way reflect alterations in the various processes in the tissue. Because of the specificity of the function of brown fat and its response to physiological stimuli during development or during acute and chronic cold exposure, it may eventually prove possible to ascribe different functions to the different kinases found in the tissue.

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B L Knight and J P Skala

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