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Evidence that insulin increases the proportion of polysomes that are bound to the cytoskeleton in 3T3 fibroblasts

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The association of polysome redistribution with changes in protein synthesis was investigated in insulin-stimulated fibroblasts. Free polysomes were released by Nonidet-P40 and 25 mM KCl, cytoskeletal-bound polysomes were retained at 25 mM KCl but released at 130 mM, while membrane-bound polysomes were released by deoxycholate. Insulin increased the proportion of polysomes which were retained at 25 mM KCl but had no effect when extraction was carried out at 130 mM KCl, suggesting that more polysomes were associated with the cytoskeleton. Insulin also reduced the amount of actin released from the detergent-insoluble cytoskeleton indicating that the hormone affects microfilament organization.

Ribosome; Actin; Insulin; Protein synthesis; Cytoskeleton

1. INTRODUCTION

It has been recognised for some years that polyribosomes (polysomes) are found both attached to the endoplasmic reticulum (membrane-bound) and in the cytosol (so-called 'free' polysomes). This distribution can undergo substantial alteration with changing extracellular milieu [1]. Recent evidence (reviewed in [2]) has suggested that a proportion or possibly all of the 'free' polysomes are in some way associated with the cytoskeleton [3,4]. The evidence comes both from electron microscopy and from the observed retention of polysomes in the insoluble cytoskeletal material obtained from cells treated with a non-ionic detergent. Such polysomes are retained in the cytoskeleton in the presence of 0.05% non-ionic detergent, but are released upon the addition of 1% deoxycholate [3,5]. This method is, however, very similar to that used to prepare membrane-bound polysomes [6,7], so that it would seem imprecise to define the cytoskeletal-bound polysomes on the basis of detergent extraction alone. Three observations from the literature led us to investigate whether increasing the salt concentration during non-ionic detergent treatment might allow separation of cytoskeletal and membrane-bound polysomes: (i) no polysomes are released from the non-ionic detergent insoluble material at low ionic strength [3]; (ii) in the presence of non-ionic detergent and high salt concentrations (100 mM NaCl) the cytoskeleton disintegrates [5]; and (iii) some polysomes are released at 130 mM KCl but the membrane-bound polysomes are retained in the non-ionic detergent insoluble material and are only released upon total solubilization of the endoplasmic reticulum [7,8].

The aims of the present work were two-fold; firstly to develop a method to distinguish between free, cytoskeletal-bound and membrane-bound polysomes in 3T3 fibroblasts; and secondly to investigate if stimulation of protein synthesis by insulin in quiescent cells was associated with any change in the distribution of polysomes between these three populations. One of the major biological effects of insulin is to stimulate muscle protein synthesis [9], and similarly addition of in-

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sulin to quiescent fibroblasts in cell culture causes a rapid increase (within 30 min) in protein synthesis which involves the activation of existing ribosomes [10,11].

2. MATERIALS AND METHODS

2.1. *Cell culture*

3T3 fibroblasts (Flow Laboratories, Irvine, Ayrshire, Scotland) were grown in Dulbecco's minimal Eagle's medium as described [11]. Cells were grown in 100 mm plastic Petri dishes containing 8 ml medium. At subculture, cells were seeded to achieve a cell density of $16–32 \times 10^5$/dish on the day of experiment; at this density the cultures were not confluent.

2.2. *Cell extraction and polysome profiles*

The medium was removed from the dishes and the cells were washed three times in 10 mM phosphate-buffered saline (PBS), pH 7.4. Approx. 10^7 cells were used for the isolation of polysomes and 2×10^6 for the measurement of actin content. Cells were scraped into ice-cold buffer (0.25 M sucrose, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.6, plus either 25 or 130 mM KCl) and then collected by centrifugation at $1000 \times g$ for 5 min. Cells were lysed by resuspension in appropriate buffer containing 0.05% Nonidet-P40 (NP-40), kept on ice for 1 min and then centrifuged at $1000 \times g$ for 5 min; the supernatant fluid contained released actin and polysomes while the pellet contained nuclei and remaining insolubilised membrane and cytoskeletal material. Further polysomes were released when the pellet was resuspended in buffer containing 1% NP-40 and 1% deoxycholate. After incubation at 4°C for 10 min the solubilised material was collected by centrifugation at $3000 \times g$ for 15 min. Samples were layered on 15–48% sucrose gradients and centrifuged at $130000 \times g$ for 145 min to separate polysomes from monosomes and ribosomal subunits. Profiles were monitored by measuring the optical density at 260 nm using a flow cell and a Pye Unicam SP8-400 recording spectrophotometer. The amount of polysomes was calculated from the area under the absorbance curve as indicated in fig.1.

2.3. *Analysis of actin*

SDS-polyacrylamide gel electrophoresis was performed using a discontinuous Tris/glycine buffer system [12]. Gels were either stained using Coomassie brilliant blue or the proteins were transferred electrophoretically to nitrocellulose paper [13] and probed for actin using a monoclonal antibody against actin (Amersham, England). The actin content of cell extracts was measured by an enzyme-linked immunosorbent assay (ELISA). Microtitre plates were coated (room temperature, overnight) with cell extracts at a protein concentration of 2 μg/ml. A linear assay curve was constructed using plates coated with muscle actin over the range 0.05–2 μg/ml. Plates were washed three times in PBS containing 0.05% Tween-20 (PBS-Tween) and then incubated with a monoclonal antibody against actin or normal mouse serum (non-immune control) at 1/10000 dilution in PBS-Tween for 2 h. After 4 washes in PBS-Tween the plate was then incubated with biotinylated anti-mouse antibody (2 h) and after three further washes with streptavidin peroxidase (45 min), both at 1/1000 dilution in PBS-Tween. After a final three washes the bound peroxidase activity was measured using *o*-phenylene diamine as substrate (15 min incubation) and the absorbance measured at 492 nm after the reaction had been stopped with 3 M H_2SO_4. All incubations were at room temperature.

3. RESULTS AND DISCUSSION

Proteins solubilised from cells by NP-40 treatment were analysed by SDS-polyacrylamide gel electrophoresis (fig.1a,b) and the results showed that with 130 mM KCl there was a particularly marked increase in the release of a protein of M_r ~45000. Immunoblotting (fig.1c) identified this protein as actin. Quantification of actin in the cell extracts using an ELISA technique showed that there was greater release of actin at the higher ionic strength (table 1). This suggests that treatment of cells with NP-40 in buffer containing 130 mM KCl causes an extensive disruption of the actin microfilament network. These observations are consistent with the earlier finding that the cytoskeleton is disrupted at salt concentrations above 100 mM [5]. In contrast at 25 mM KCl the cytoskeleton remains intact [5] and is insoluble in non-ionic detergent. Depolymerisation of actin filaments by pre-treatment of cells with cytochalasin B led to an increase in the actin content of low ionic strength extracts (table 2); this adds support to the interpretation that the release of actin seen at 130 mM KCl reflects microfilament disruption.

As shown in fig.2 the proportion of polysomes released during non-ionic detergent treatment of cells was influenced by the ionic strength of the extraction buffer. At low ionic strength (25 mM KCl) the majority of the polysomes were not released by NP-40 but were recovered in the fraction obtained after treatment with deoxycholate (fig.2a). In contrast, at higher ionic strength (130 mM KCl; fig.2b) most polysomes were released with NP-40 alone (tables 1 and 3). There are thus three populations of polysomes distinguishable by salt treatment: (i) polysomes released by non-ionic detergent at low salt (28% of total polysomes), (ii) polysomes which are retained at low salt but released at higher salt concentrations (46%, calculated as the difference between those released by 25 and 130 mM KCl), and (iii) polysomes which

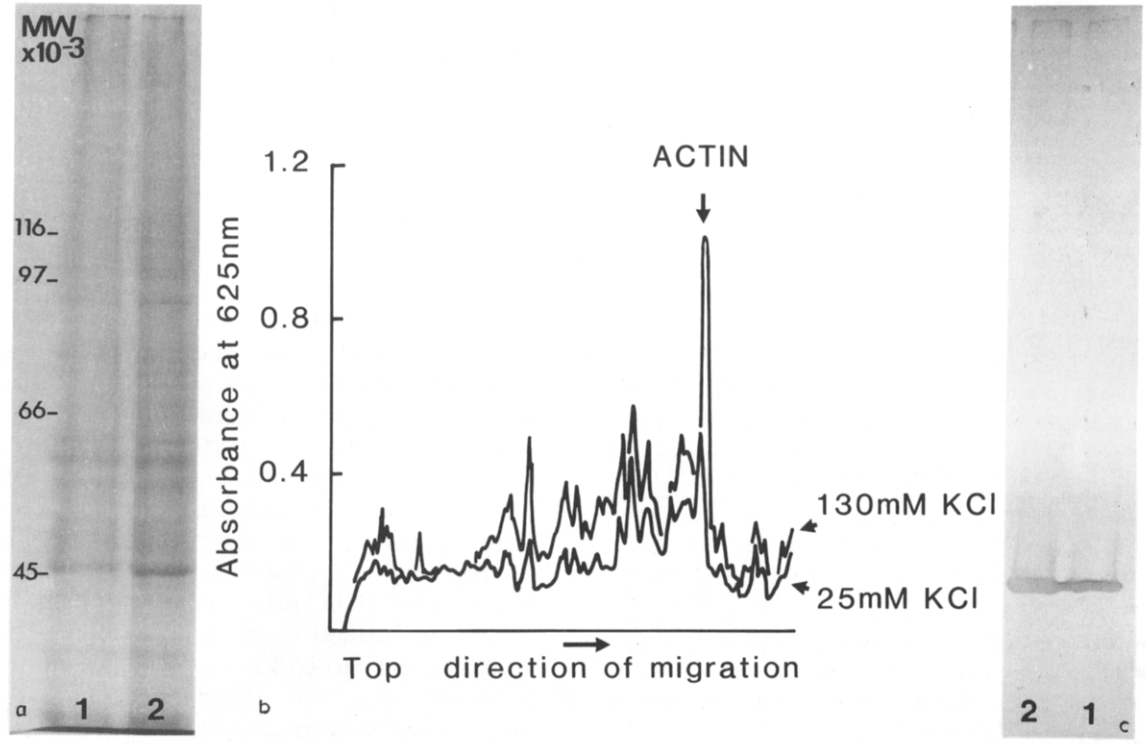

Fig.1. Identification of actin in non-ionic detergent extracts of 3T3 fibroblasts. (a) SDS-polyacrylamide (7.5%) gel of proteins extracted by NP-40. Lane 1: 25 mM KCl extraction; lane 2: 130 mM KCl extraction. Gel was stained with Coomassie brilliant blue. (b) Densitometric scan of similar gel. (c) Immunoblot of proteins separated in same gel system and subsequently transferred to nitrocellulose paper and incubated with antibodies against actin. Lane 1: 25 mM KCl extraction; lane 2: 130 mM KCl extraction.

are retained even at high salt but then can be released by treatment with deoxycholate (26%). These correspond to free, cytoskeletal-bound and membrane-bound polysomes, respectively.

The concomitant changes in actin release and polysome distribution suggest that the polysomes which are retained with the cytoskeleton at 25 mM salt but released together with actin at higher salt, are polysomes that are associated with the cytoskeleton; furthermore, polysomes that are only released by deoxycholate at 130 mM KCl have been shown to be membrane-bound [7,8]. Previous work has shown polysomes to be associated with the non-ionic detergent-insoluble cytoskeleton prepared from cells in culture [2,3,5]. As described here the use of two salt concentrations allows the cytoskeletal-bound and membrane-bound polysomes to be distinguished; the present results provide evidence that ~40% of the cell polysomes are associated with the cytoskeleton.

3T3 cells which have been stepped-down for 48 h in medium containing 4% serum and then incubated with insulin (1 mU/ml) show an increase in protein synthesis within 1 h [11]. Extraction of polysomes from such cells showed that insulin reduced the proportion of polysomes which were released by non-ionic detergent at low salt concentration (table 3); in contrast when extraction was performed at 130 mM KCl insulin had no effect on the distribution of polysomes. Insulin increased the proportion of polysomes which are retained at low salt concentration but are released at higher ionic strength, namely the cytoskeletal-bound polysomes, from 42 to 51% of total polysomes; the proportion of membrane-bound was unaffected. Investigation of the actin content of cell extracts (table 3) showed that insulin reduced by almost 50% the amount of actin found in low salt (25 mM KCl) extracts but had a much smaller and statistically non-significant effect on the actin

Table 1

Effect of different extraction conditions on polysome distribution and on the amount of actin released by 0.05% Nonidet-P40 (NP-40)

	KCl concentration in extraction buffer	
	25 mM	130 mM
% polysomes released by NP-40	25 ± 6 (6)	75 ± 3 (5)**
Actin (mg/mg extracted protein) released by NP-40	0.17 ± 0.02 (6)	0.30 ± 0.04* (6)

Results are means ± SE with number of experiments in parentheses. * $p < 0.02$, ** $p < 0.001$ compared to extraction at 25 mM KCl using a two-tailed t-test

recovered in 130 mM KCl extracts. The reduced release of actin, which suggests insulin increases actin polymerization, was accompanied by changes in the release of polysomes. This supports the view that the polysome population affected by insulin is associated with the cytoskeleton.

These results demonstrate an increased association of polysomes with the cytoskeleton under conditions of increased protein synthesis in response to insulin. It is possible that increased translational activity is associated with a shift of ribosomes from free polysomes to the cytoskeletal bound population. This is consistent with previous work which demonstrated fewer polysomes associated with the cytoskeleton after inhibition of protein synthesis or viral infection [3,5]. In addition, if, as suggested [14], different proteins are synthesized

Table 2

Effect of cytochalasin B (CB) on the amount of actin released by Nonidet-P40 at different salt concentrations

	KCl concentration in extraction buffer			
	25 mM		130 mM	
	Control	CB	Control	CB
Expt 1	0.14	0.30	0.36	0.35
Expt 2	0.10	0.33	0.27	0.38

Values given are mean actin contents (mg/mg extracted protein) from duplicate estimations. Cells were treated with either cytochalasin B (10 μg/ml) or methanol carrier for 20 min

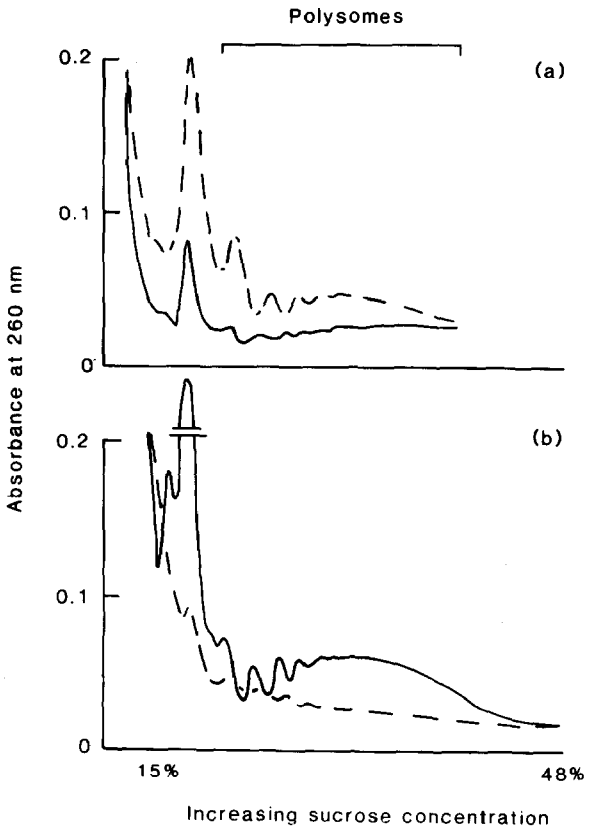

Fig.2. Polysome profiles from cells extracted with NP-40 at different salt concentrations: polysomes released by NP-40 alone (——) and subsequently by NP-40 and deoxycholate (– – –) using (a) 25 mM KCl or (b) 130 mM KCl in the extraction buffer.

on free and cytoskeletal-bound polysomes then the change in polysome distribution would presumably reflect an alteration in the pattern of protein synthesis.

The results also demonstrate that changes in microfilament organisation, due either to filament stabilisation or a stimulation of polymerisation, occur in response to insulin. The mechanisms involved are not yet known but might involve phosphorylation of actin-binding proteins; insulin affects the phosphorylation/dephosphorylation of several proteins [15], including the membrane-cytoskeletal protein spectrin [16]. One previous report [17] has shown insulin to stimulate membrane ruffling in KB cells and these membranes were associated with more actin. Changes in the cytoskeleton may be part of the overall response of

Table 3

The effect of insulin on the release of polysomes and actin by Nonidet-P40 (NP-40)

	KCl concentration in extraction buffer			
	25 mM		130 mM	
	Control	+ Insulin	Control	+ Insulin
% polysomes released by NP-40	31 ± 4 (4)	23 ± 6* (4)	73 ± 2 (3)	74 ± 3 (4)
Actin (mg/mg extracted protein) released by NP-40	0.19 ± 0.01 (4)	0.10 ± 0.02** (4)	0.30 ± 0.06 (4)	0.21 ± 0.05 (4)

Values are given as means ± SE with number of experiments in parentheses. * $p < 0.05$, ** $p < 0.02$ compared to control at same extraction conditions using a two-tailed paired t-test. Cells were treated with insulin (1 mU/ml) or PBS carrier for 1 h

cells to insulin, representing one facet of the stimulation of cell growth. Alternatively the changes in the cytoskeleton may be part of the mechanisms by which insulin exerts its biological effects; for example re-organization of the cytoskeleton may bring about changes in membrane transport [17] or modification of protein synthesis.

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