Demonstration of specific insulin binding to cytosolic proteins in H35 hepatoma cells, rat liver and skeletal muscle

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We previously demonstrated that internalized insulin enters the cytoplasm before accumulating in nuclei of H35 rat hepatoma cells. This finding raises the possibility that insulin may interact with cytosolic proteins in addition to insulin-degrading enzyme (IDE). In the present study, cytosol from H35 hepatoma cells, rat liver or muscle was incubated with A14- or B26-¹²⁵I-insulin at 4 °C for 5–120 min in the absence or presence of 25 µg/ml unlabelled insulin. ¹²⁵I-insulin was cross-linked to cytosolic proteins by disuccinimidyl suberate and analysed by reducing or non-reducing SDS/PAGE and autoradiography. Our results demonstrate the presence of both tissue-specific and common cytosolic proteins which specifically bind insulin. In muscle cytosol, only two proteins of 27 and 110 kDa were specifically labelled with B26-¹²⁵I-insulin. Seven major bands, of 27, 45, 55, 60, 76, 82 and 110 kDa, were labelled in rat liver cytosol. Detection of cytosolic insulin-binding proteins in H35-cell cytosol was dependent on cell-culture conditions. Labelling in cytosol from serum-deprived cells was decreased or absent compared with cytosol prepared from serum-fed or serum-deprived cells treated with 100 ng/ml insulin for 1 h before preparation of the

cytosol, in which six bands, of 32, 41, 45, 55, 82 and 110 kDa, were specifically labelled with B26-¹²⁵I-insulin. This result suggests that the concentration or binding activity of some cytosolic insulin-binding proteins is rapidly regulated. Labelling of both rat liver and H35 cytosolic insulin-binding proteins was time-dependent, and decreased or disappeared at 120 min in parallel with the degradation of labelled insulin. Fewer bands were specifically labelled with A14-¹²⁵I-insulin than with B26-¹²⁵I-insulin. The number of labelled bands observed under reducing and non-reducing conditions was not different in any of the cytosols. The 110 kDa band in all cytosols was identified as IDE by Western-blot analysis; the other proteins did not react with anti-IDE antibody and remain unidentified. 1,10-Phenanthroline (2 mM) increased IDE labelling, but decreased the labelling of 82 and 27 kDa bands. The marked difference in the number of cytosolic insulin-binding proteins in muscle and either H35 cells or liver suggests both that the labelling is specific and that these proteins serve a function and may be involved in some heretofore unknown mechanism of the signalling pathway by which insulin regulates cell growth or differentiation.

INTRODUCTION

It is well known that insulin affects nuclear events such as gene expression or cell growth. However, the mechanisms involved are relatively unknown. Many of insulin's actions require the binding of insulin to its plasma-membrane receptor, the auto-phosphorylation of the receptor β -subunit, and a resulting phosphorylation cascade of various substrates including IRS-1 (insulin-receptor substrate-1) [1]. It is also well known that insulin is internalized by many target cells. The functions ascribed to internalization have generally been limited to the regulation of the number of insulin receptors on the cell surface and/or the degradation of insulin. The existence of insulin receptors on intracellular organelles [2], particularly the nucleus [3–5], suggest that intracellular insulin could play an important role in insulin-regulated nuclear events if these receptors were exposed to insulin. This hypothesis is supported by studies from several laboratories. For example, microinjection of insulin into the cytoplasm of *Xenopus* oocytes increased RNA and protein synthesis [6]. When added to isolated nuclei, insulin affected various nuclear processes, such as nucleo-cytoplasmic transport of macromolecules [7], protein phosphorylation [8], enzymic activities [9] and mRNA release from nuclei [10].

In a series of biochemical and ultrastructural studies, we demonstrated that insulin, but not its plasma-membrane receptor, was translocated to and accumulated in the nucleus in several different cell types [11–13]. Insulin in the nucleus was intact and tightly attached to the nuclear matrix [14]. Since the nuclear matrix is the site of active gene transcription [15], these observations suggest that direct association of insulin with the nucleus may play an important role in gene regulation. Studies to demonstrate the mechanism or intracellular pathway resulting in the nuclear accumulation of insulin revealed the uptake was concentration-, time- and temperature-dependent, but energy-independent [16]. We also showed that, in addition to receptor-mediated internalization, fluid-phase endocytosis contributed to the accumulation of insulin at high concentrations of insulin [17]. However, the pathway that insulin follows from the endosome to the nucleus remained unknown. One possibility that we considered was that insulin might leave the endosome, enter the cytoplasm and, like steroid hormones, enter the nucleus complexed with a cytoplasmic protein or receptor. The feasibility of this pathway is supported by studies of insulin degradation from several laboratories.

The majority of cellular insulin degradation is the result of insulin's association with insulin-degrading enzyme (IDE) [18].

Abbreviations used: IDE, insulin-degrading enzyme; DSS, disuccinimidyl suberate; TCA, trichloroacetic acid; PMSF, phenylmethanesulphonyl fluoride; IR, insulin receptor; DTT, dithiothreitol.

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IDE is localized almost exclusively in the cytoplasm [19]. Hari et al. [20] showed the association of insulin with a cytosolic IDE in intact cells. In a recent study we reported that inhibition of IDE activity with the zinc chelator 1,10-phenanthroline resulted in a 5–10-fold increase in the nuclear accumulation of insulin [21]. These results provided evidence that internalized insulin does enter the cytoplasm before accumulating in the nucleus and suggested that IDE activity is an important factor regulating translocation of intact insulin to the nucleus.

In the present study, we tested the hypothesis that insulin might bind to cytosolic proteins in addition to IDE. The cytosol isolated from rat liver, muscle, or H35 rat hepatoma cells was incubated with A14- or B26-¹²⁵I-insulin, and cross-linked to detect the association of insulin with the cytosolic proteins. Our results demonstrate there are cytosolic insulin-binding proteins other than IDE in each of these tissues. In cytosol isolated from H35 hepatoma cells, some of these proteins were regulated by hormonal conditions. Although it is still not clear how insulin enters the cytoplasm, this paper suggests that the direct interaction of insulin with proteins in the cytoplasm, in addition to the previously reported IDE, may play a role in insulin's regulation of cellular processes.

EXPERIMENTAL

Preparation of cytosolic fractions

Cytosol fractions were prepared as previously described from 120 g male Sprague–Dawley rat (fed *ad lib.*) liver and skeletal muscle [22], or H35 rat hepatoma cells [21]. In brief, the rat liver or skeletal muscle was minced and homogenized in homogenizing buffer [0.25 M sucrose, 50 mM Mops, pH 7.4, 1 mM EDTA, 10 μ M leupeptin, 5 μ M pepstatin A and 1 mM phenylmethanesulphonyl fluoride (PMSF)] with a motor-driven homogenizer. The homogenate was strained through surgical gauze, and centrifuged at 30000 *g* for 20 min. The supernatant was centrifuged at 100000 *g* for 90 min at 4 °C. The supernatant was concentrated on an Amicon ultrafiltration membrane (5000-molecular-mass cut-off) with 50 mM sodium phosphate buffer (pH 7.6). H35 cells were cultured for 3 days after passage as described previously [17]. In some experiments, the cells were cultured overnight in fresh Dulbecco's modified Eagle's medium with 0.1% BSA (serum-deprived cells) or with 10% serum (serum-fed cells) before harvesting of cells. Insulin-treated cells were incubated with 100 ng/ml insulin for 60 min at 37 °C after overnight serum deprivation before harvesting of cells. Cytosol fractions were prepared by homogenizing cells in cytosolic incubation buffer (CIB; 5 mM phosphate buffer, pH 7.6, 5 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂) with 1 mM PMSF. The homogenate was centrifuged at 100000 *g* for 90 min at 4 °C over a 0.3 M sucrose cushion. The protein concentration of each cytosolic fraction was determined by the method of Lowry et al. [23].

Affinity labelling of cytosol with A14- or B26-¹²⁵I-insulin

A14- and B26-¹²⁵I-insulin were prepared by Iodo-Bead (Pierce Chemical Co., Rockford, IL, U.S.A.) catalysed iodination in the presence of 6.5 M urea as previously described [24]. A14- and B26-¹²⁵I-insulin were purified on a reverse-phase h.p.l.c. column (Vydac 218TP; 5 μ m; 4.6 mm $\times$ 250 mm RP-h.p.l.c.) and eluted with 26.5% CH₃CN in 0.2 M ammonium acetate, pH 5.5, at 1 ml/min. The cytosols were diluted to 1 mg/ml in CIB and incubated at 4 °C for 5–120 min with 10 ng/ml A14- or B26-¹²⁵I-insulin. Non-specific binding was assessed in the presence of 25 μ g/ml unlabelled insulin, as routinely used in studies of insulin binding to the plasma-membrane insulin receptor [21]. At

the end of the incubation, the cross-linking reagent disuccinimidyl suberate (DSS; Pierce Chemical Co.) was added to a final concentration of 0.5 mM [25]. After cross-linking for 15 min, the samples were dissolved in Laemmli buffer with or without 50 mM dithiothreitol (DTT) and boiled for 3 min. A 25 μ g portion of total protein from each sample was electrophoresed on SDS/10%-polyacrylamide gels [26] along with molecular-mass standards. Proteins were detected by Coomassie Blue R, and ¹²⁵I-insulin was detected by autoradiography.

ECL–autoradiogram overlay

Portions (10 μ g) of protein from the same samples as above were subjected to SDS/PAGE on 10% gels and electro-transferred on to nitrocellulose membranes. After the B26-¹²⁵I-insulin-binding proteins were detected by routine autoradiography on Kodak X-AR film, the nitrocellulose membranes were blocked with 5% BSA in 50 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.01% thimerosal and probed with 10 μ g/ml anti-IDE antibody 9B12 (kindly provided by Dr. Richard A. Roth, Stanford University). Bound antibodies were localized with horseradish-peroxidase-conjugated anti-mouse IgG (1:20000) and detected by ECL (Amersham Corp., Arlington Heights, IL, U.S.A.). The autoradiogram and the ECL film were overlaid and the labelled bands were compared.

Estimation of insulin degradation

Cytosol (1 mg/ml) was incubated with 10 ng/ml A14- or B26-¹²⁵I-insulin for various times at 4 °C, and the percentage of intact insulin was estimated by trichloroacetic acid (TCA) precipitation as described previously [21]. Some of the samples were also analysed by h.p.l.c.. After the incubation with A14- or B26-¹²⁵I-insulin, insulin-degradation activity was stopped by adding excess unlabelled insulin and 1,10-phenanthroline at final concentrations of 25 μ g/ml and 5 mM, respectively. The samples were applied on to the h.p.l.c. as described above and analysed by counting the radioactivity in 1 ml fractions.

RESULTS

We first measured ¹²⁵I-insulin degradation in isolated cytosol prepared from rat liver, skeletal muscle, and H35 rat hepatoma cells. Initial experiments revealed that insulin-degradation rates were dependent on cytosol protein concentration (0.1–1.0 mg/ml), temperature (4–37 °C) and time of incubation (0–120 min). More degradation was found as the protein, temperature and duration of incubation were increased (results not shown). Furthermore, insulin degradation was inhibited by a number of agents, including *N*-ethylmaleimide (1 mM), bacitracin (0.5 mM), 1,10-phenanthroline (2 mM) and unlabelled insulin (1–25 μ g/ml), as reported previously [21,27] (results not shown). *N*-Ethylmaleimide, bacitracin and 1,10-phenanthroline have been reported to affect insulin binding to plasma-membrane receptors [28,29] or IDE [30]. Therefore, none of these inhibitors were used in the present studies, except where specifically indicated (see Figure 5, below). Under the conditions used in the cross-linking studies, as shown in Figure 1, H35 hepatoma-cell cytosol degraded insulin very rapidly; less than 30% of the insulin was intact by 60 min at 4 °C. Insulin was degraded less rapidly in rat liver cytosol than in H35 cells, but 40–50% was degraded by 120 min. The percentage of the precipitable A14-¹²⁵I-insulin was lower than that of B26-¹²⁵I-insulin in both rat liver and H35-cell cytosol. To determine whether this difference is because A14-¹²⁵I-insulin (Figure 1a) was degraded faster than B26-¹²⁵I-insulin

Figure 1 Time course of ¹²⁵I-insulin degradation in the cytosol

Rat liver (■), muscle (●) or H35-cell cytosol (▲) (1 mg/ml) was incubated with A14- (a) or B26- (b) ¹²⁵I-insulin at 4 °C for the indicated times, and the percentage of TCA-precipitable ¹²⁵I-insulin was determined.

(Figure 1b), or the TCA-precipitated B-chain may contain more partial degradation products than the A-chain, we applied the samples of H35-cell cytosol incubated with A14- or B26-¹²⁵I-insulin for 20 min at 4 °C on to h.p.l.c. and analysed real percentages of degraded insulin: 12.0 % of A14- and 30.0 % of B26-¹²⁵I-insulin total radioactivity was eluted as an intact insulin peak. These results indicated that A14-¹²⁵I-insulin was degraded more rapidly than B26-¹²⁵I-insulin, although the absolute numbers that we found from the TCA-precipitation technique and h.p.l.c. analysis were different. No significant degradation of insulin was detected in muscle cytosol in incubations up to 120 min at 4 °C (Figures 1a and 1b).

We next examined affinity labelling of cytosolic proteins with ¹²⁵I-insulin. Rat liver cytosol was incubated with A14- or B26-¹²⁵I-insulin for 5–120 min at 4 °C in the presence or absence of 25 µg/ml unlabelled insulin and covalently cross-linked by DSS. As shown in Figures 2(a) and 2(b) respectively, both A14- and B26-¹²⁵I-insulin were cross-linked to several different proteins. The largest number of proteins was labelled at 30 min, and labelling intensity and the number of bands labelled decreased by 120 min. This decrease in labelling after 120 min corresponded to the decreased amount of intact insulin, as shown above. There were differences in the labelling of proteins by A14- and B26-¹²⁵I-insulin. With A14-¹²⁵I-insulin (Figure 2a), proteins at ~ 110, 66, 55, 45, 41, 34, 31 and 27 kDa were radiolabelled at 30 min. Unlabelled insulin blocked the labelling of the bands at 110, 34, 31 and 27 kDa, suggesting that only these four proteins are specific cytosolic insulin-binding proteins. Based on reports from other laboratories [27,30–32], we believed that the 110 kDa protein was probably IDE (see Figure 6, below). Non-specific labelling of a band at 66 kDa was of the BSA in the ¹²⁵I-insulin

Figure 2 Affinity labelling of rat liver cytosol with ¹²⁵I-insulin

Rat liver cytosol (1 mg/ml) was incubated with A14- (a) or B26- (b) ¹²⁵I-insulin in the absence (odd-numbered lanes) or presence (even-numbered lanes) of 25 µg/ml unlabelled insulin at 4 °C for 5 min (lanes 1 and 2), 30 min (lanes 3 and 4), or 120 min (lanes 5 and 6) as described in the Experimental section. The affinity labelling was analysed by SDS/PAGE (10% gel) and autoradiography after cross-linking with DSS. In this and subsequent Figures 'kDa' values refer to standards. On the right-hand side are shown CIBP p82 etc., numbers referring to apparent molecular masses in kDa.

solution. Interestingly, labelling of the 41 kDa band was actually increased in the presence of unlabelled insulin. By comparison, B26-¹²⁵I-insulin (Figure 2b) specifically labelled seven major bands at ~ 110, 82, 76, 60, 55, 45 and 27 kDa at 30 min (the apparent molecular masses of proteins specifically labelled with B26-¹²⁵I-insulin are summarized in Table 1). There were also two weakly labelled bands, at ~ 30 and 25 kDa, which showed specificity. Labelling of all proteins in the liver cytosol was maximal at 30 min, and disappeared or decreased by 120 min, which was consistent with the decrease in the concentration of intact insulin in the incubation mixture.

In H35 hepatoma cells, as shown in Figures 3(a) and 3(b) respectively, both A14- and B26-¹²⁵I-insulin were cross-linked to

Table 1 Comparison of specific cytoplasmic insulin-binding proteins affinity-labelled with B26-¹²⁵I-insulin in H35 hepatoma cells, rat liver and muscle

Molecular mass (kDa)		
H35 cells	Rat liver	Skeletal muscle
110 ^{1,2}	110 ^{1,2}	110 ¹
82 ³	82 ³	
	76	
	60	
55	55	
45 ⁴	45	
41 ⁴	41 ⁵	41 ⁵
32 ⁴		
	30 ⁶	
	27 ³	27
	25 ⁶	

¹ Protein identified as insulin degrading enzyme by Western-blot analysis.

² Binding of B26-¹²⁵I-insulin increased in the presence of 1,10-phenanthroline.

³ Binding of B26-¹²⁵I-insulin decreased in the presence of 1,10-phenanthroline.

⁴ Observed in insulin-treated or serum-fed cells but not in serum-deprived cells.

⁵ Binding of B26-¹²⁵I-insulin was not completely displaced by unlabelled insulin.

⁶ Weakly labelled proteins.

three cytosolic proteins at ~ 110, 82 and 55 kDa. The labelling of these bands was maximal at 10 min, was completely displaced by unlabelled insulin, and decreased in intensity by 60 min in response to the degradation of insulin. Using cytosol prepared from H35 cells cultured 3 days after passage, we could not consistently detect lower-molecular-mass bands, in contrast with the results seen in rat liver cytosol. However, as shown below (see Figure 7), low-molecular-mass bands in H35 cells were labelled under different culture conditions.

Rat muscle cytosol also was incubated for affinity labelling and, as shown in Figure 4(b), lanes 9–12, we observed only two highly specific cytosolic insulin-binding proteins. The labelling of a 110 kDa band was less intense than in liver or H35 cells, but was displaced by unlabelled insulin. Unlabelled insulin completely decreased labelling of the 27 kDa band, partially decreased that of the 41 kDa band, but that of the 47 kDa band was not insulin-displaceable.

We examined whether or not some of the lower-molecular-mass cytosolic insulin-binding proteins were reduced products of other binding proteins. Cytosol fractions from rat muscle, liver and H35 hepatoma cells were incubated with B26-¹²⁵I-insulin for 60, 30 or 10 min respectively, to achieve maximal labelling. The cross-linked insulin-binding proteins were analysed by both reducing and non-reducing SDS/PAGE. As shown in Figure 4, the resolution in the non-reducing condition (Figure 4a) was not as clear as that under reducing conditions (Figure 4b). However, the number of affinity-labelled proteins was identical in both conditions. These results suggest that the lower-molecular-mass cytoplasmic insulin-binding proteins labelled with B26-¹²⁵I-insulin are individual proteins and not smaller fragments of larger proteins, particularly IDE.

We next examined the effect of 1,10-phenanthroline, an IDE inhibitor [27], on the affinity labelling of the proteins. Other laboratories have reported that 1,10-phenanthroline increased the amount of ¹²⁵I-insulin cross-linked to IDE [30]. Rat muscle, liver, or H35-cell cytosol was incubated with B26-¹²⁵I-insulin at 4 °C for 60, 30 or 10 min respectively, with no addition, 2 mM 1,10-phenanthroline, or 25 µg/ml insulin. As shown in Figure 5,

Figure 3 Affinity labelling of H35-hepatoma-cell cytosol with ¹²⁵I-insulin

H35-cell cytosol (1 mg/ml) was incubated with A14- (a) or B26- (b) ¹²⁵I-insulin in the absence (odd-numbered lanes) or presence (even-numbered lanes) of 25 µg/ml unlabelled insulin at 4 °C for 5 min (lanes 1 and 2), 10 min (lanes 3 and 4), or 60 min (lanes 5 and 6) as described in the Experimental section. The affinity labelling was analysed by SDS/PAGE (10% gel) and autoradiography after cross-linking with DSS.

1,10-phenanthroline increased ¹²⁵I-insulin binding to the 110 kDa band in H35-cell cytosol. After 30 min, 1,10-phenanthroline had no significant effect on the labelling of the 110 kDa band in liver cytosol. However, 1,10-phenanthroline did increase labelling of the 110 kDa band when the cytosol was incubated for 120 min (results not shown). 1,10-Phenanthroline inhibited ¹²⁵I-insulin binding to some of the binding proteins. Labelling of the 82 and 27 kDa bands in liver cytosol and the 82 kDa band in H35-cell cytosol was blocked by 1,10-phenanthroline. In contrast, 1,10-phenanthroline did not have any significant effects on insulin binding in muscle cytosol, even after 120 min of incubation. The results, particularly with liver and H35 cells, were an additional indication that the 110 kDa band was IDE. These data also demonstrate that 1,10-phenanthroline, perhaps because it chelates zinc, selectively blocked specific binding of insulin to some cytosolic proteins. The different effect of 1,10-phenanthroline on insulin binding suggests that these proteins are not IDE or related protein fragments.

To confirm that the 110 kDa band was IDE and that the other proteins were not IDE fragments, affinity-labelled cytosolic proteins from H35 cells were transferred on to a nitrocellulose

Figure 4 Comparison of affinity labelling under reducing and non-reducing conditions

Liver cytosol (lanes 1–4), H35-cell cytosol (lanes 5–8) or muscle cytosol (lanes 9–12) was incubated at 4 °C for 30, 10 or 60 min, respectively, with A14- (lanes 1, 2, 5, 6, 9 and 10) or B26- (lanes 3, 4, 7, 8, 11 and 12) ^{125}I -insulin in the absence (odd-numbered lanes) or presence (even-numbered lanes) of 25 $\mu\text{g}/\text{ml}$ unlabelled insulin. The affinity-labelled samples were subjected to SDS/PAGE (10% gel) without (a) or with (b) DTT after cross-linking with DSS.

membrane after SDS/PAGE, and immunoblotting by the ECL technique (Figure 6a) and autoradiography (Figure 6b) were done on the same sample (ECL–autoradiogram overlay). As shown in Figure 6(a), anti-IDE antibody recognized 110 kDa bands which were affinity-labelled with ^{125}I -insulin in H35 cells. No other proteins reacted with the anti-IDE antibody. Similar results were obtained with rat liver and muscle cytosol, although the amount of IDE was less than that in H35 cells (results not shown). These results confirmed that the 110 kDa band was IDE and the other proteins affinity-labelled with ^{125}I -insulin were not immunoreactive fragments of IDE.

To rule out the possibility of insulin-receptor (IR) contamination in the cytosolic fraction, we incubated H35-cell cytosol with 600 ng/ml insulin in the presence of ATP (25 μM or 1 mM), 100 μM NaVO_4 , 10 mM MgCl_2 , 5 mM MnCl_2 and 25 mM Hepes, pH 7.4. The samples were electrophoresed along with wheat-germ-agglutinin (WGA)-purified IR and immunoblotted with anti-phosphotyrosine antibody (rabbit polyclonal, kindly provided by Dr. P. L. Rothenberg). No phosphotyrosine was

Figure 5 Effect of 2 mM 1,10-phenanthroline on affinity labelling

Cytosol (1 mg/ml) from rat liver (lanes 1–3), muscle (lanes 7–9) or H35 cells (lanes 4–6) was incubated at 4 °C for 30, 10 or 60 min, respectively, with B26- ^{125}I -insulin with no addition (lanes 1, 4 and 7), 2 mM 1,10-phenanthroline (lanes 2, 5 and 8), or 25 $\mu\text{g}/\text{ml}$ unlabelled insulin (lanes 3, 6 and 9). The affinity labelling was analysed by SDS/PAGE (10% gel) and autoradiography after cross-linking with DSS.

Figure 6 Immunoblotting of affinity-labelled proteins with anti-IDE antibody

H35-cell cytosol (1 mg/ml) was affinity-labelled with B26- ^{125}I -insulin for 10 min at 4 °C with no addition (lane 1), 2 mM 1,10-phenanthroline (lane 2), or 25 $\mu\text{g}/\text{ml}$ unlabelled insulin (lane 3). ^{125}I -insulin was cross-linked with DSS and the samples were subjected to SDS/PAGE (10% gel). The proteins were transferred to a nitrocellulose membrane, and immunoblotting (a) and autoradiography (b) were performed on the same membrane as described in the Experimental section.

Figure 7 Effect of serum feeding, serum deprivation or insulin treatment on the affinity labelling in H35-cell cytosol

H35 hepatoma cells were maintained in culture for 3 days after passage. The culture medium was replaced with Dulbecco's modified Eagle's medium containing 10% serum (serum-fed cells, lanes 1 and 2) or 0.1% BSA (serum-deprived cells, lanes 3 and 4) and the cells were cultured overnight. In addition, a portion of the serum-deprived cells was treated with 100 ng/ml insulin for 60 min before harvesting and preparing the cytosol (insulin-treated, lanes 5 and 6). The cytosols were affinity-labelled with B26-¹²⁵I-insulin in the absence (odd-numbered lanes) or presence (even-numbered lanes) of 25 µg/ml unlabelled insulin, and analysed by SDS/PAGE (10% gel) and autoradiography.

detected in the cytosol, whereas WGA-purified IR β subunit was tyrosine-phosphorylated and detected by the antibody (results not shown). These data indicate that there is no functional IR [or insulin-like growth factor-I (IGF-I) receptor] in cytosolic fractions. Furthermore, we immunoblotted cytosols affinity-labelled with B26-¹²⁵I-insulin (60 µg/lane) with anti-(IR α -subunit) antibody (UBI 06-112, rabbit polyclonal) and revealed bands by using anti-rabbit IgG-horseradish peroxidase and the ECL detection system. No bands were detected by the antibody at the region where ¹²⁵I-insulin was affinity-labelled (results not shown). These results suggest that the bands affinity-labelled with ¹²⁵I-insulin are not insulin or IGF-I receptors or their fragments.

Lastly, we examined whether the cytosolic insulin-binding proteins were regulated by physiological conditions. H35 hepatoma cells were maintained in culture for 3 days after passage. The culture medium was replaced with Dulbecco's modified Eagle's medium containing 0.1% BSA (serum-deprived) or 10% serum (serum-fed) and the cells were cultured overnight. In addition, a sample of the serum-deprived cells was treated with 100 ng/ml insulin for 60 min before harvesting and preparing the cytosol. Affinity labelling of the cytosols from these three conditions revealed that refeeding or insulin treatment increased insulin binding activity compared with serum-deprived cells, as shown in Figure 7. In contrast with the results shown in Figure

3 using cells maintained in culture for 3 days, three lower-molecular-mass bands of 45, 41 and 32 kDa were detected in serum-fed or insulin-treated cells in addition to the previously demonstrated 110, 82 and 55 kDa bands. Serum deprivation decreased the labelling of all proteins compared with cytosol of serum-fed cells. Insulin degradation, assessed by TCA precipitation, was not different in any of the three conditions (results not shown), indicating that the differences among the cytosols in Figure 7 were not due to differences in the amount of intact insulin as was seen in time-course experiments shown in Figures 2–4. These results suggest that the concentration or binding activity of the cytosolic insulin-binding proteins in H35 cells is rapidly regulated by hormonal (including growth factors) conditions.

DISCUSSION

In a series of biochemical and ultrastructural studies we have demonstrated that insulin, but not its plasma-membrane receptor, was translocated to and accumulated in the cell nucleus in 3T3-L1 adipocytes [11], cells of mouse embryo blastocysts [12], H35 hepatoma cells and HIR 3.5 fibroblasts [13]. Insulin in the nucleus was intact and tightly attached to the nuclear matrix [14], the active site of gene transcription [15], suggesting that direct association of insulin with the nucleus may play an important role in gene regulation. In a recent study [21], we reported that the inhibition of cytosolic IDE activity with the zinc chelator 1,10-phenanthroline resulted in a 5–10-fold increase in the nuclear accumulation of insulin. However, the non-zinc-chelating isomer 1,7-phenanthroline did not have any effect on insulin degradation or nuclear accumulation of insulin [33]. These results suggested that IDE is one of the important factors which regulate translocation of intact insulin to the nucleus. Previous reports using cell fractionation techniques have found most of the IDE activity in the cytoplasm of cells [19]. Immuno-electron-microscopy studies also have demonstrated IDE in the cytoplasm [34]. In addition, studies of IDE-related insulin degradation from other laboratories [19,31] have previously provided strong circumstantial evidence that insulin enters the cytoplasm to be degraded by IDE. In fact, a study using a cross-linker in intact cells showed association of ¹²⁵I-insulin with a cytosolic IDE *in vivo* [20]. Although it is not clear how insulin, or other extracellular ligands known to enter the cytoplasm of cells, crosses the endosomal membrane, our previous results were consistent with a pathway requiring insulin to enter the cytoplasm before entering the nucleus. We hypothesized that, in addition to IDE, insulin might associate with other cytosolic proteins. These putative insulin-binding cytosolic proteins might be translocated to the nucleus complexed with insulin in the same manner as steroids and their cytosolic receptors, or might serve a signal-transduction role and be responsible for some of insulin's intracellular effects.

In the present study, we utilized h.p.l.c.-purified A14- and B26-¹²⁵I-insulin and demonstrated specific insulin-binding activity to cytosolic proteins in rat liver, muscle and H35 rat hepatoma cells other than IDE. There were differences in the labelling observed with the two ligands. Affinity labelling with B26-¹²⁵I-insulin showed better specificity and dependence on intact insulin than did A14-¹²⁵I-insulin. These differences may be due, in part, to an increase in the efficiency of detection of the proteins labelled with B26-¹²⁵I-insulin when cross-linked with DSS through the B29-lysine. Particularly under reducing conditions, the insulin A chain, and therefore the A14-¹²⁵I, may be separated from some of the cytosolic binding proteins during SDS/PAGE if the disulphide bonds connecting the A and B chains are reduced.

Our results demonstrate insulin binding to the 110 kDa IDE, as reported by others [27,30–32], but also show other binding proteins not previously observed. In our studies, incubations were carried out at 4 °C for shorter periods than in other reports [27,30–32] to minimize insulin degradation. In fact, under conditions where the amount of intact insulin was significantly degraded, labelling of cytoplasmic insulin-binding proteins was not detected. Differences in the incubation conditions may be responsible, in part, for the differences between the results from other laboratories [27,30–32] and from ours.

The apparent molecular masses of specific cytosolic insulin-binding proteins labelled with B26-¹²⁵I-insulin are summarized in Table 1. The molecular masses estimated here include the cross-linked insulin molecule, so they are overestimated by 5700 Da, if insulin was not reduced, or by 3300 Da, if reduced and B-chain-labelled, compared with the unoccupied proteins. At least six specific bands other than IDE were affinity labelled with B26-insulin in rat liver cytosol, and these are not reduced products of other proteins, as shown in Figure 4. These bands were not breakdown products of IDE, but are apparently independent and unique proteins, based on the following observations. Firstly, the time course of insulin binding to those lower-molecular-mass proteins was different from that of IDE labelling, as shown in Figure 3. Secondly, 1,10-phenanthroline had different effects on ¹²⁵I-insulin binding to these bands from those on binding to the 110 kDa IDE, as shown in Figure 5. Lastly, only the 110 kDa band reacted with the anti-IDE antibody in Western blots, as shown in Figure 6. Based on the immunoblotting with anti-IR α -subunit, the labelled bands were not likely to be IR or breakdown products of IR. Furthermore, by anti-phosphotyrosine immunoblotting, functional IRs or IGF-I receptors were not detected in the cytosolic fraction.

In H35-cell cytosol prepared from cells maintained in culture 3 days after passage, only two bands were affinity-labelled other than IDE (Figure 3). However, when the cytosol was isolated from the cells fed with fresh serum overnight (serum-fed) or treated with insulin for 1 h (insulin-treated), affinity labelling revealed three additional low-molecular-mass bands (Figure 7). These results suggest that the concentration or insulin-binding activity of insulin-binding proteins in H35-cell cytosol is rapidly regulated by hormonal (including growth factors) conditions. In muscle cytosol, fewer bands were specifically labelled (Figure 4b, lanes 9–12) compared with liver or hepatoma cells. This difference suggests tissue specificity in the expression of cytosolic insulin-binding proteins. This tissue specificity was also apparent in the relative amount of binding to the IDE band, which reflects the differences in insulin-degradation activity in the cytosol of muscle compared with liver or H35-cell cytosol.

Interestingly, in liver cytosol, the labelling of the 41 kDa band with A14-¹²⁵I-insulin was not displaced, but actually increased, in the presence of unlabelled insulin (Figure 2). This observation can be explained if insulin binding to that protein is non-saturable. By inhibiting ¹²⁵I-insulin degradation with unlabelled insulin, the amount of intact ¹²⁵I-insulin in the cytosol would increase, as would the non-specific association of insulin with the protein. In fact, a similar increase in binding to this protein was seen in the presence of 1,10-phenanthroline, supporting this hypothesis.

The mechanisms underlying the effects of 1,10-phenanthroline, a zinc chelator, on insulin binding to some of the cytosolic proteins are not known. It has been previously reported that 1,10-phenanthroline increased insulin association with IDE, while at the same time inhibiting the degradation of insulin [30]. The increased binding to IDE may be due to an increase in the concentration of intact insulin secondary to the decreased

degradation, or a change in the conformation of IDE which simultaneously affects its degradative activity and binding affinity. 1,10-Phenanthroline inhibited ¹²⁵I-insulin binding to some of the binding proteins. Labelling of the 82 kDa band in both liver and H35-cell cytosol and of 27 kDa bands in liver cytosol was blocked by 1,10-phenanthroline. The decreased binding of insulin to these cytosolic proteins in the presence of 1,10-phenanthroline raises the possibility that those proteins may bind insulin fragments resulting from insulin degradation. If insulin-degradation products are binding to these proteins, decreased degradation in the presence of 1,10-phenanthroline could result in decreased association of ¹²⁵I-labelled fragments with the protein. However, we do not think that these proteins are binding insulin-degradation products. The time course of ¹²⁵I-insulin binding to these proteins does not parallel the appearance of insulin-degradation products in the cytosol. Furthermore, all attempts to inhibit A14- or B26-¹²⁵I-insulin binding to cytosolic proteins with 25 μ g/ml reduced A or B chains of insulin were not successful (results not shown). A more likely explanation is that zinc chelation may affect the conformation of those proteins and decrease their affinity for insulin.

Although we have demonstrated that, under appropriate conditions *in vitro*, insulin specifically binds to several cytosolic proteins, we have yet to demonstrate that such binding does occur in intact cells or tissues. In intact cells and under physiological conditions, insulin may bind to these proteins, but be undetectable with techniques currently being used. Previous attempts to show significant amounts of intact insulin in the cytoplasm of cells have been unsuccessful, although degraded insulin has been detected [35]. The lack of success may, at least in part, be due to the high rate of insulin degradation by cytoplasmic IDE in intact cells at physiological temperatures required to obtain either receptor-mediated or fluid-phase endocytosis of the insulin. Attempts to block insulin degradation, particularly with 1,10-phenanthroline and *N*-ethylmaleimide, change the binding affinity of binding proteins, as demonstrated here and elsewhere [28–30]. These effects preclude using these inhibitors in an attempt to block insulin degradation *in vivo* and demonstrate cytoplasmic insulin-binding proteins.

In summary, we have demonstrated tissue-specific and apparently common cytosolic insulin-binding proteins other than IDE which specifically bind insulin in rat liver, muscle and H35 hepatoma cells. In H35 hepatoma cells, the concentration or binding activity of at least three of these proteins appeared to be regulated by hormonal conditions (including growth factors). Although the identity and physiological significance of these proteins remain to be determined, their ability to be rapidly regulated suggests that they may be involved in some manner in the mechanism or signalling pathway by which insulin regulates cell growth. Further investigation of these proteins and their potential role in insulin action may provide a justification, other than degradation, for the significant internalization of insulin in most target tissues.

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