Evidence that a 90-kDa phosphoprotein, an associated kinase, and a specific phosphatase are involved in the regulation of Cloudman melanoma cell proliferation by insulin

(protein phosphorylation–dephosphorylation/hormones/somatic cell hybridization/phosphotyrosine)

ROBERT D. FLEISCHMANN AND JOHN M. PAWELEK

Department of Dermatology, Yale University School of Medicine, New Haven, CT 06510

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ABSTRACT Proliferation of wild-type Cloudman S91 melanoma cells is inhibited when insulin is included in the culture medium. Using growth inhibition as a selective marker, we isolated variant cell lines that are either resistant to insulin or dependent upon insulin for growth. We have studied the effects of insulin on proliferation by using combined genetic and biochemical approaches. Through a series of genetic hybridization analyses, we have identified three complementation groups and determined that, in general, insulin-sensitivity is dominant to insulin-resistance. Through analyses of *in vitro* protein phosphorylation reactions, we have identified a protein of ≈ 90 kDa (pp90) whose phosphorylation is a function of at least one of the complementation groups. Although pp90 is not phosphorylated in extracts of insulin-resistant variants, it is phosphorylated in extracts of insulin-sensitive hybrids formed between complementing resistant variants. Insulin itself exhibits little or no regulation over the phosphorylation of pp90; rather, the ability to phosphorylate pp90 correlates with the ability of cells to respond to insulin. Migration in NaDod-SO₄/polyacrylamide gels, solubility characteristics, and divalent cation requirements indicate that pp90 is distinct from the 95-kDa β -subunit of the insulin receptor. Both pp90 and its associated phosphoprotein kinase are found in 30,000 $\times g$ pellets of sonicated cell lysates, whereas a specific pp90 phosphoprotein phosphatase activity is found in 30,000 $\times g$ supernatant fractions. Phosphorylation of pp90 occurs at tyrosine and serine residues. Our evidence indicates that the state of phosphorylation of pp90 is an important determinant in the regulation of cellular proliferation by insulin.

Many cellular processes are affected through interactions between insulin and high-affinity receptors for insulin (1–4). The mechanisms by which cellular functions are altered remain unclear, although there is considerable evidence that insulin may act through the regulation of protein phosphorylation–dephosphorylation reactions. For example: (i) insulin stimulates the phosphorylation of ribosomal protein S6 in HeLa cells and 3T3-L1 adipocytes (5, 6); (ii) rat adipocytes contain an insulin-sensitive 140-kDa phosphoprotein (7); (iii) preparations of membranes and mitochondria from rat adipocytes contain an insulin-sensitive 120- and 42-kDa phosphoprotein, respectively (8, 9); and (iv) it has been shown that insulin stimulates the autophosphorylation of the 95-kDa β -subunit of the insulin receptor after formation of the insulin–receptor complex. Phosphorylation of the receptor was found to occur at least in part at tyrosine residues and has been observed in human lymphocytes, rat hepatoma cells, 3T3-L1 adipocytes, human placenta, and Cloudman murine melanoma cells (refs. 10–12 and unpublished re-

sults). The kinase activity copurifies with the insulin receptor, suggesting an insulin receptor–kinase complex analogous to the epidermal growth factor receptor–kinase complex (13, 14).

Although the above observations demonstrate that protein phosphorylation–dephosphorylation reactions are influenced by insulin, the physiological relevance of these reactions is difficult to establish. This is due in part to the lack of cell systems responsive to insulin in which biochemical and genetic approaches can be applied simultaneously. With this problem in mind, techniques were established for isolating Cloudman melanoma cells with differing responses to insulin (15). We now present results on comparisons of phosphoproteins in insulin-sensitive and insulin-resistant cell lines.

The proliferation of wild-type Cloudman melanoma cell line is inhibited by insulin. The effect appears to be mediated through the interaction of insulin and specific high-affinity receptors. Mutant cell lines have been selected which differ from the wild-type in their ability to grow in the presence of insulin (15). In addition, a system of somatic cell hybridization has been developed which allows the study of dominant/recessive relationships of the insulin phenotype and the identification of genetic complementation groups (16). By using this system of wild-type, mutant, and hybrid cell lines, three genetic complementation groups involved in the regulation of proliferation have thus far been identified. In attempting to correlate our genetic observations with specific biochemical events, we have identified a phosphoprotein of approximately 90 kDa (pp90) whose phosphorylation correlated strongly with the sensitivity of the cells to insulin. The phosphoprotein was rapidly phosphorylated in the 30,000 $\times g$ pellet fraction of insulin-sensitive cells (both wild type and hybrids) when incubated *in vitro* with [γ -³²P]ATP but was rarely labeled in insulin-resistant cell lines. Partial acid hydrolysis of pp90 revealed both phosphotyrosine and phosphoserine residues. A phosphoprotein phosphatase specific for pp90 also was detected. Our evidence suggests that phosphorylation and dephosphorylation of pp90 are important steps in the insulin-mediated control of proliferation of Cloudman melanoma cells and that regulation of these processes is a function of at least one of the above mentioned complementation groups.

MATERIALS AND METHODS

Cell Culture. Four cell lines and two hybrid cell lines were used in these studies: 1A (17); Ou4 (16); 111 (16); OuIns-1 (16); 1A $\times$ OuIns-1; and 111 $\times$ OuIns-1 (16). The phenotypes and selective genetic markers are listed in Table 1. The cells were cultured in monolayer in Ham's F-10 medium contain-

Table 1. Cell lines, genetic markers, and insulin phenotype

Cell Line	Ouabain	HAT*	Insulin
1A	Sensitive	Resistant	Inhibited
111	Sensitive	Resistant	Resistant
Ou4	Resistant	Sensitive	Inhibited
OuIns-1	Resistant	Sensitive	Resistant
1A×OuIns-1	Resistant	Resistant	Inhibited
111×OuIns-1	Resistant	Resistant	Inhibited

*HAT, 0.1 mM hypoxanthine/0.4 μ M aminopterin/16 μ M thymidine/3 μ M glycine.

ing 2–10% horse serum or in clonal culture as described previously (16). Selection for mutants and isolation of hybrid cell lines also have been described (15, 16).

Determination of Generation Times. To determine rates of proliferation under various culture conditions, cells (2×10^4) were inoculated into 25-cm² Corning tissue culture flasks in 4 ml of medium, which was changed three times per week. Cultures were harvested in triplicate, and cells were counted in a Coulter counter every 2 or 3 days for up to 2 weeks. The generation time was determined from the rate of division in log phase growth according to the following equation: Generation time = $0.69(t)/\ln(C_2/C_1)$, where t equals the time in hours between the initial cell number (C_1) and the final cell number (C_2) over the linear portion of the growth curve.

Labeling and Analyses of Phosphoproteins in Lysed-Cell Preparations. Cells ($2.5\text{--}6 \times 10^6$) were inoculated into 150-cm² Corning tissue culture flasks in Ham's F-10 culture medium supplemented with horse serum (2–10%). After an appropriate period of growth with or without insulin (0.065–0.65 μ M), the cells were harvested in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Joklik's nutrient solution containing EDTA (1 mM), washed in buffered saline (35 mM Tris Cl, pH 7.5/146 mM NaCl/12 mM D-glucose), and resuspended in sonication buffer (30 mM Tris Cl, pH 7.6/154 mM NaCl/10 mM MgCl_2) at a concentration of 2.5×10^7 cells per ml. Cells were lysed by sonication (Ultrasonics Sonifier Cell Disruptor, 75 mV for 30 sec, using a microprobe). Remaining intact cells were removed by low-speed centrifugation ($300 \times g$ for 5 min). Supernatant fraction designated "whole cell lysate" was retained and, where indicated, was further separated into supernatant and pellet fractions by centrifugation at $30,000 \times g$ for 10 min. The $30,000 \times g$ pellets were resuspended in sonication buffer as described above (i.e., pellets from 2.5×10^7 cells were resuspended in 1 ml of sonication buffer).

Phosphorylation reactions were initiated by the addition of [$\gamma\text{-}^{32}\text{P}$]ATP (Amersham, 3000 Ci/mmol, 1 Ci = 37 GBq; 4–40 μ M final concentration). The [^{32}P]ATP (10 μ l) was added to either whole cell lysates (240 μ l) or the resuspended $30,000 \times g$ pellets (240 μ l). The mixtures were incubated at room temperature. At various times the samples were placed on ice and then centrifuged at $30,000 \times g$ for 10 min, and the $30,000 \times g$ pellets were boiled for 2 min in Laemmli sample buffer (62.5 mM Tris Cl, pH 6.8/2% NaDodSO₄/10% glycerol/5% 2-mercaptoethanol/0.001% bromophenol blue). The samples then were subjected to NaDodSO₄/PAGE (18). After electrophoresis, the gels were stained for proteins with Coomassie blue (19), dried, and exposed to Kodak X-Omat AR film for autoradiography.

Identification of Phosphotyrosine Residues by Treatment of Gels with Alkali. Phospho-tyrosine bonds are generally more resistant to alkaline hydrolysis than are phospho-serine or -threonine bonds. Thus, a rapid technique for determining whether proteins might be phosphorylated at tyrosine residues is to incubate gels in alkali to differentially remove phosphates attached to serine or threonine residues. The procedure was as follows. Cell lysates were incubated with [^{32}P]ATP as described above. The samples were divided into two equal portions and analyzed on NaDodSO₄/polyacryla-

mid gels. After electrophoresis and staining, one of the two gels was immersed in 1 M NaOH for 15 min at room temperature and then for 1 hr at 40°C. The gels then were rinsed and incubated for 2 hr in 10% acetic acid, dried, and autoradiographed. Comparisons of autoradiograms from treated and untreated gels gave an indication of which proteins were likely to be phosphorylated at tyrosine residues. However, it should be noted that the alkaline treatment provides an indication but not a proof of phosphotyrosine residues. Proof requires direct analysis of the phospho amino acids obtained from acid hydrolyzates of protein bands (see below).

Identification of Phospho Amino Acids by Electrophoresis on Thin-Layer Cellulose. Phosphoproteins labeled with ^{32}P and separated by NaDodSO₄/PAGE were extracted from the polyacrylamide gels (20), frozen, and lyophilized to dryness. The proteins then were redissolved in 6 M HCl, sealed under vacuum, and incubated for 2 hr at 110°C. Phospho amino acid standards (phosphoserine, phosphothreonine, and phosphotyrosine) were added and samples were separated by high-voltage electrophoresis on thin-layer cellulose in a pH 3.5 buffer (acetic acid/pyridine/water, 50:5:945, vol/vol) for 2.5 hr at 400 V (21). The standards were visualized by spraying the plates with ninhydrin (0.3% in 1-butanol). The plate then was used to prepare an autoradiogram to localize the ^{32}P radioactivity.

RESULTS

Proliferation of Cloudman Cells in Response to Insulin. Proliferation of the cell lines 1A and Ou4 was inhibited when insulin (0.65 μ M) was added to the culture medium (Fig. 1 *a* and *b*). The proliferation of two insulin-resistant variants, 111 and OuIns-1, was unaffected by the presence of insulin (Fig. 1 *c* and *d*). The doubling times of these two variants in the presence or absence of insulin were similar to those for cell lines 1A and Ou4 in the absence of insulin.

FIG. 1. Growth curves of insulin-inhibited parental cell lines 1A and Ou4 and of insulin-resistant variants 111 and OuIns-1. (*a*) Cell line 1A was insulin-inhibited, HAT-resistant, and killed by ouabain. (*b*) Cell line Ou4 was insulin-inhibited, killed by HAT, and ouabain-resistant. (*c*) Cell line 111 was identical to 1A except that it was insulin-resistant. (*d*) Cell line OuIns-1 was identical to Ou4 except that it was insulin-resistant.

The insulin-resistant variant OuIns-1 also was resistant to ouabain (up to 3 mM) and killed by medium containing HAT. Therefore, this cell line could be used in somatic cell fusions with the insulin-inhibited wild-type cell line 1A and the insulin-resistant variant 111. These two cell lines were ouabain-sensitive and grew in the presence of medium containing HAT. Hybrids formed between 1A and Ou Ins-1 (1A×OuIns-1) were insulin-inhibited, indicating a dominant effect of the 1A insulin-inhibited genotype in this hybrid cell line. Hybrids formed between the insulin-resistant variants 111 and OuIns-1 (111×OuIns-1) also were insulin-inhibited, resulting from genetic complementation between these insulin-resistant variants (Table 2).

In Vitro Protein Phosphorylation. By comparing insulin-inhibited and insulin-resistant cell lines, we hoped to identify phosphoprotein(s) whose phosphorylation might be involved in the regulation of cell proliferation by insulin. The 30,000 × g pellet proteins prepared from insulin-inhibited cell line 1A and its insulin-resistant variant 111 formed identical gel patterns when separated by NaDodSO₄/PAGE and stained with Coomassie blue (Fig. 2, left 4 lanes). However, when the phosphoproteins labeled with [³²P]ATP were visualized by autoradiography, one consistent difference was revealed. A protein of ≈90 kDa (pp90) was labeled in insulin-inhibited cell lines but not in insulin-resistant variants (Fig. 2, middle 4 lanes). Note in this figure that data are presented for one insulin-inhibited line (1A) and one insulin-resistant variant (111). Not shown here are our findings that pp90 also was observed in another insulin-inhibited line (Ou4) but not in another insulin-resistant line (OuIns-1).

The determination of the molecular mass of this protein was based on its relative migration as compared to protein standards in NaDodSO₄/polyacrylamide gels of ≈8% acrylamide. The apparent molecular mass varied (80–95 kDa), depending on the duration of the electrophoresis. The pp90 protein migrated to a region of the gels that stained only faintly for proteins. When insulin-inhibited and insulin-resistant cell lines were labeled metabolically with [³⁵S]methionine and the 30,000 × g pellet proteins were analyzed by NaDodSO₄/PAGE and autoradiography, no obvious differences could be detected in the proteins synthesized by the two cell lines (results not shown).

The phosphorylation of pp90 took place when [³²P]ATP was added to 30,000 × g pellet protein. The incorporation of ³²P was complete after 2 min. The incorporation of ³²P from ATP into pp90 was dependent on either Mg²⁺ or Mn²⁺—that is, the presence of either divalent cation was sufficient for the reaction to occur.

The insulin-inhibited hybrid cell lines 1A×OuIns-1 and 111×OuIns-1 were examined for the ability of whole cell lysates to phosphorylate pp90. The hybrid cell lines phosphorylated pp90 *in vitro* (Fig. 3). Therefore, the restoration of the insulin-inhibited phenotype by genetic means correlated with the ability of these cell lines to phosphorylate a 90-kDa phosphoprotein.

Phospho Amino Acid Residues of pp90. The nature of the phosphorylation of pp90 was examined by two methods. Initially, NaDodSO₄/polyacrylamide gels were incubated in NaOH to dephosphorylate phosphoserines and phosphothreonines. This is a rapid method to screen for proteins phosphorylated at tyrosine residues. There was little or no reduc-

FIG. 2. NaDodSO₄/PAGE analysis of 30,000 × g pellet proteins, showing the presence of ³²P-labeled pp90 in cell line 1A (insulin-inhibited) and the absence of labeled pp90 in cell line 111 (insulin-resistant). Both cell lines were grown in the presence (+, 0.65 μM) or absence (–) of insulin. Pellets were labeled *in vitro* with [³²P]ATP. Left four lanes: Coomassie blue-stained NaDodSO₄/polyacrylamide gel. Middle four lanes: an autoradiogram prepared from the left four lanes. Right four lanes: an autoradiogram prepared from a gel identical to left four lanes but having undergone alkaline treatment (see *Materials and Methods*).

tion in pp90 ³²P radioactivity after treatment with NaOH, suggesting that pp90 contained phosphotyrosine (Fig. 2, right 4 lanes). The phosphoprotein was further examined by slicing a number of pp90 bands from identical gel lanes of the 30,000 × g pellet fraction of cell line 1A. The protein was eluted and analyzed for phospho amino acid content as described in *Materials and Methods*. This analysis revealed the presence of both phosphotyrosine and phosphoserine in the extracted protein (Fig. 4).

Phosphoprotein Phosphatase Activity with Specificity Toward pp90. Labeling of pp90 with [³²P]ATP was highly reproducible in 30,000 × g pellet fractions of insulin-inhibited cells but usually was not detectable in unfractionated whole

FIG. 3. An autoradiogram prepared from an NaDodSO₄/polyacrylamide gel identifying labeled pp90 in insulin-inhibited wild-type and hybrid cell lines. Cell lines 1A (insulin-inhibited), 111 (insulin-resistant) and OuIns-1 (insulin-resistant) and hybrids 1A×OuIns-1 (insulin-inhibited) and 111×OuIns-1 (insulin-inhibited) were grown in the absence of insulin. Extracts were labeled *in vitro* with [³²P]ATP and the 30,000 × g pellet proteins were analyzed by NaDodSO₄/PAGE.

Table 2. Clonal analyses of hybrids between cell lines expressing insulin-inhibited and insulin-resistant phenotypes

Hybrid	Flasks with clones		Total clones	
	–Insulin	+ Insulin	–Insulin	+ Insulin
1A×OuIns-1	20/20	1/20	134	1
111×OuIns-1	18/20	2/19	49	6

FIG. 4. An autoradiogram showing high-voltage electrophoretic analysis on thin-layer cellulose of a partial acid hydrolysate of pp90. The protein was labeled with [32 P]ATP, sliced from gels, rehydrated, and extracted. The extracted material was partially acid hydrolyzed and subjected to electrophoresis at pH 3.5 on thin-layer cellulose. The dotted lines indicate the positions of the phospho amino acid standards P-Tyr (phosphotyrosine), P-Thr (phosphothreonine), and P-Ser (phosphoserine).

cell lysates. We found that the $30,000 \times g$ supernatant fraction contained an activity that caused the loss of 32 P from pp90 after it had been labeled in the pellet fraction (Fig. 5a). In this experiment, pp90 was labeled in a $30,000 \times g$ pellet fraction of 1A cells as described above. After the labeling, the pellet was washed by centrifugation to remove 32 P. Then either the $30,000 \times g$ supernatant fraction or the equivalent amount of lysis buffer as a control were mixed with the labeled pellet and incubated at room temperature. Note that by 30 min of incubation with the supernatant fraction but not the lysis buffer, labeled pp90 had disappeared. This finding suggested that the $30,000 \times g$ supernatant fraction contained either a protease activity specific for pp90 or a phosphoprotein phosphatase activity specific for pp90. To distinguish between these possibilities, cells were labeled *in vitro* with [32 P]ATP at different times and in the presence of various combinations of pellet and supernatant fractions (Fig. 5b). The experiments were carried out with cell lines 1A and 111. Lanes 1 (cell line 1A) and 2 (cell line 111) are from experiments in which whole cell lysates were incubated with [32 P]ATP and the $30,000 \times g$ pellet fractions were analyzed by NaDodSO₄/PAGE and autoradiography. Note that pp90 is not labeled. Lanes 3 (cell line 1A) and 4 (cell line 111) are from an experiment in which the $30,000 \times g$ pellets were incubated with $30,000 \times g$ supernatant fractions for 1 hr, reisolated by centrifugation at $30,000 \times g$, and then labeled with [32 P]ATP. Note that pp90 is labeled in the $30,000 \times g$ pellet fraction from cell line 1A but not in the $30,000 \times g$ pellet fraction from cell line 111. Lanes 5 and 6 are from an experiment in which the $30,000 \times g$ fraction from cell line 1A was isolated by centrifugation, labeled with [32 P]ATP, washed three times to remove unreacted [32 P]ATP and [32 P]P_i, and then incubated an additional 30 min with either buffer (lane 5) or $30,000 \times g$ supernatant fraction (lane 6). Note that as in Fig. 5a, incubation with the supernatant fraction but not with the buffer resulted in a specific loss of the pp90 band. Lane 7 is from an experiment in which the $30,000 \times g$ pellet fraction from cell line 1A was labeled with [32 P]ATP, washed, incubated for 30 min with the $30,000 \times g$ supernatant fraction as in lane 6, recentrifuged at $30,000 \times g$, and then reincubated with [32 P]ATP. Note that the pp90 band can be rephosphorylated after removal of the supernatant fraction. The results indicate that the supernatant frac-

FIG. 5. Evidence for a phosphatase activity specific for pp90. (a) pp90 was labeled with [32 P]ATP in a $30,000 \times g$ pellet fraction of cell line 1A. The pellet was washed three times by centrifugation to remove unreacted [32 P]ATP and [32 P]P_i and then either placed on ice (lane 1), incubated for 30 min at room temperature with lysis buffer (lane 2), or incubated for 30 min at room temperature with an equivalent amount of $30,000 \times g$ supernatant fraction (lane 3). Aliquots then were withdrawn and subjected to NaDodSO₄/PAGE and autoradiography. Note that pp90 labeling is reduced after incubation with the supernatant fraction (lane 3). (b) Lanes 1 and 2: $30,000 \times g$ pellet proteins obtained from whole cell lysates of cell lines 1A (lane 1) and 111 (lane 2). The whole cell lysates were labeled with [32 P]ATP. Note that pp90 was not labeled. Lanes 3 and 4: $30,000 \times g$ pellet proteins from cell lines 1A (lane 3) and 111 (lane 4). The $30,000 \times g$ pellets were labeled with [32 P]ATP after preincubation with the $30,000 \times g$ supernatant fraction. Note that pp90 was labeled in cell line 1A. Lanes 5 and 6: $30,000 \times g$ pellet fractions from cell line 1A were labeled with [32 P]ATP. The pellets were washed to remove unreacted [32 P]ATP and [32 P]P_i and then incubated an additional 30 min with either buffer (lane 5) or an equivalent amount of $30,000 \times g$ supernatant fraction (lane 6). Note the decrease in label specifically associated with pp90 after incubation with the supernatant fraction. Lane 7: $30,000 \times g$ pellet fraction from cell line 1A was labeled with [32 P]ATP, washed, incubated an additional 30 min with $30,000 \times g$ supernatant fraction as in lane 6, recentrifuged, and relabeled with [32 P]ATP. Note the reappearance of labeled pp90 (compare lanes 6 and 7).

tion contains a phosphoprotein phosphatase activity with specificity toward pp90.

DISCUSSION

A combined biochemical and genetic approach using wild-type, variant, and hybrid Cloudman melanoma cells has been applied to the study of insulin action and the regulation of proliferation. We have identified three factors that are apparently involved in regulating an insulin-sensitive proliferative response: (i) an ≈ 90 -kDa phosphoprotein (pp90) located in $30,000 \times g$ pellets; (ii) a kinase activity that modifies pp90, also located in $30,000 \times g$ pellets; and (iii) a soluble phosphatase activity, located in $30,000 \times g$ supernatants, showing specificity for pp90. Insulin itself exhibited little or no regulation over the phosphorylation of pp90. However, by using insulin-resistant variants, we correlated the phosphorylation of pp90 with the insulin-inhibited phenotype. This protein was phosphorylated *in vitro* in cell lines exhibiting the insulin-inhibited phenotype (1A and Ou4) but not phosphorylated in two insulin-resistant variant cell lines (111 and OuIns-

1). In addition, two hybrids were examined in which the insulin-inhibited phenotype was restored genetically, one through dominance (1A×OuIns-1) and the other through complementation (111×OuIns-1). In both cases the phosphorylation of pp90 also was restored.

The 30,000 × g supernatant fraction prevented the phosphorylation of pp90 in cell line 1A and caused the specific loss of the pp90 band in pre-labeled 30,000 × g pellets. Experiments in which 30,000 × g supernatant and pellet fractions were mixed at various times before and after incubation with [³²P]ATP indicate that the 30,000 × g supernatant fraction contains a phosphoprotein phosphatase activity with specificity toward pp90. Thus, the phosphorylation of pp90 *in vitro* is under the regulatory control of both kinase and phosphatase activities.

Cellular transformation by a number of classes of RNA tumor viruses is mediated by protein kinases specific for tyrosine residues. An increase in phosphotyrosine content of cellular proteins is also observed when cells are treated with epidermal growth factor, platelet-derived growth factor, or insulin, three factors that are capable of regulating cell growth. Our experiments identified phosphotyrosine and phosphoserine amino acid residues in pp90. The presence of phosphotyrosine and phosphoserine may indicate that more than one phosphoprotein was present in and extracted from the pp90 region of the gel. Alternatively, pp90 may contain both phospho amino acids and may exist in a number of modified forms. The latter possibility suggests that pp90 may be a substrate for more than one protein kinase or that a single protein kinase may be capable of phosphorylating tyrosine and serine residues. These same considerations apply to the phosphatase activity in the 30,000 × g supernatant fraction.

The insulin-resistant cell line 111 has recently been shown to contain an insulin receptor with an altered insulin-binding capacity and a decreased autophosphorylation of the β -subunit of the insulin receptor at one specific tryptic peptide (22). The autophosphorylation of the β -subunit and the phosphorylation of pp90 both appear to be correlated with the regulation of cell proliferation by insulin. However, as yet, a relationship between these two phosphorylation events has not been found. Although pp90 is similar in molecular weight to the β -subunit of the insulin receptor, the two are unlikely to be identical, for the following reasons (a) ³²P-labeled pp90 is readily detectable in 30,000 × g pellet fractions prepared from 10⁶ cells, whereas ³²P-labeled β -subunit of the insulin receptor is not. (b) The presence of Mg²⁺ as a cofactor is sufficient for the phosphorylation of pp90, while Mn²⁺ is necessary for the autophosphorylation of the insulin receptor (10, 11). (c) The insulin receptor is readily solubilized from membrane preparations of Cloudman melanoma cells treated with Triton X-100, but Triton treatment of 30,000 × g pellets prepared from Cloudman cells is not effective in solubilizing pp90 (unpublished results). (d) When ³²P-labeled pp90 was electrophoresed on gels side-by-side with ³²P-labeled insulin receptor prepared from Cloudman melanoma

cells, their rates of migration were clearly different, pp90 being the faster-moving protein (unpublished results).

We have identified a relationship in Cloudman melanoma cells between the regulation of cell proliferation by insulin and the phosphorylation *in vitro* of a 90-kDa phosphoprotein. The role of pp90 in mediating insulin sensitivity and the possible relationship between autophosphorylation of the β -subunit of the insulin receptor and pp90 phosphorylation remain to be determined.

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