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# Effects of Chromium on Basal and Insulin-Induced Tyrosine Phosphorylation in H4 Hepatoma Cells: Comparison with Phorbol-12-myristate-13-acetate and Sodium Orthovanadate

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## SUMMARY

Chromium, in its various forms, is recognized both as a human carcinogen and as a nutrient essential in glucose homeostasis. Although the genotoxicity of this element is associated with its carcinogenic properties, the manner in which chromium mediates its epigenetic effects on cells, including its ability to potentiate insulin action, is not known. In the current studies, Western blotting with antiphosphotyrosine antibodies was used to study the effects of chromium on protein tyrosine phosphorylation in intact H4 rat hepatoma cells. Treatment of cells with hexavalent chromium [Cr(VI)] was found to induce the tyrosine phosphorylation of three prominent sets of proteins, having median molecular masses of 210, 125, and 87 kDa. Cr(VI) pretreatment also inhibited the insulin-induced tyrosine phosphorylation of the major substrate of the insulin receptor kinase, insulin receptor substrate-1, and its subsequent association with the 85-kDa regulatory subunit (p85) of phosphatidylinositol 3′-kinase. Furthermore, Cr(VI) was found to alter the pattern of other p85-binding (insulin-induced) phosphoproteins that were distributed throughout the soluble and particulate fractions of cells. Virtually all of the alterations in basal and insulin-induced phosphorylations associated with Cr(VI) treatment were also observed in cells treated with the protein kinase C (PKC) agonist phorbol-12-myristate-13-acetate. However, the effects of Cr(VI) were determined to be independent of PKC activity, because they were sustained in PKC-depleted cells. The pattern of phosphoproteins induced by Cr(VI) also had similarities to the pattern generated in response to the phosphatase inhibitor sodium orthovanadate. However, several specific differences, including the ability of vanadate to increase insulin receptor $\beta$ subunit autophosphorylation [i.e., an effect not observed with Cr(VI)], indicated that these agents modulate phosphorylation by distinct mechanisms. The ability of Cr(VI) to alter the phosphorylation state of key regulatory proteins in a manner similar to that of other biologically active agents suggests a mechanism by which this element can modulate the growth and metabolism of cells.

Chromium is known to have diverse biological effects on cells and organisms. Although the hexavalent form of this element is well established as a carcinogen (1), the paucity of epidemiological data involving human exposure has made it difficult to determine the manner in which this element acts to induce and/or influence the growth of tumors. The ability of this metal to produce various types of DNA lesions (2) has supported the notion that chromium functions as an initiating agent in carcinogenic mechanisms. However, there are numerous biological effects of chromium that are independent of its genotoxic properties (3, 4). It is possible that some of the epigenetic effects of this element contribute to the induction of human neoplasms.

Chromium, in its trivalent form, is also recognized as an essential trace element in human nutrition (4). Dietary chromium is apparently required for glucose homeostasis and has been found to normalize both hypoglycemic and hyperglycemic states (5, 6). The observed ability of this element to decrease the amount of insulin required to maintain glucose tolerance indicates that it functions as a potentiator of insulin action. It has been suggested that chromium may act by binding to the insulin hormone molecule, thereby stabilizing its secondary structure in a form that resists degradation by proteases (7). The extended serum half-life of these insulin-chromium complexes could presumably increase the effective concentration of this hormone and prolong the action of insulin. Other investigators have found that chromium facilitates the binding of insulin to its cell surface receptor and promotes receptor aggregation (8). Furthermore, specific organic forms of this element (i.e., chromium picolinate) have

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**ABBREVIATIONS:** IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol 3-kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; p85, 85-kDa regulatory subunit of PI3K.

been found to increase membrane fluidity (9). Such alterations in plasma membranes are thought to enhance the lateral mobility and internalization of the activated insulin receptor, which may increase the efficiency of signal generation. Although the results of these and other studies may explain the enhanced insulin action observed in cells and animals supplemented with various forms of chromium, it is possible that this element influences more distal events in the insulin-dependent signal transduction cascade.

It is known that insulin binds to its cell surface receptor and activates the receptor-associated tyrosine kinase activity (10). After autophosphorylation, the insulin receptor can phosphorylate other cellular proteins. Although many such proteins have been characterized (11), one extensively studied substrate for the insulin receptor kinase is known as IRS-1 (12). The insulin-mediated phosphorylation of IRS-1 within specific amino acid motifs (i.e., Tyr-Xxx-Xxx-Met) promotes the interaction of these particular regions of this protein with the *src* homology-2 domains of numerous signal-transducing molecules (for review, see Ref. 12). Functional alterations of these molecules during formation of these protein complexes are thought to be responsible for the transduction of the insulin signal throughout the cell. For example, such an interaction between phosphorylated IRS-1 and a *src* homology-2 domain of p85, the putative regulatory subunit of PI3K, induces the activation of the 110-kDa catalytic subunit of this holoenzyme, stimulating the generation of distal mediators of insulin action (12, 13). Characterization of the influence of chromium-containing compounds on these and other aspects of the insulin signal cascade should increase our understanding of the mechanism by which these agents potentiate the action of insulin. In the present studies, we have examined the effect of inorganic forms of chromium on the basal and insulin-induced tyrosine phosphorylation of cellular proteins in rat H4 hepatoma cells. The present results demonstrate that many of the biological effects of chromium may be due to the ability of this element to alter the phosphorylation state and function of specific mitogenic/metabolic regulatory proteins.

## Materials and Methods

**Cell culture.** H4-II-E rat hepatoma cells (American Type Culture Collection, Rockville, MD) were grown in Swim's S-77 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 2 mM L-glutamine, 20% (v/v) horse serum, 5% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Confluent plates (10 cm) of hepatoma cells were deprived of serum for 20 hr before treatment. The cells were then treated with various agents [100 μM $CrCl_3$ [Cr(III)], 50 μM $K_2Cr_2O_7$ [Cr(VI)], 160 nM PMA, or 100 μM $Na_3VO_4$] for 1 hr. Some plates received insulin (100 nM; Squibb Novo) for 2 min before harvesting. For harvesting, plates of cells were washed twice with 10 ml of ice-cold phosphate-buffered saline and then an extraction buffer (750 μl) containing 50 mM Tris·HCl, pH 7.4, 10 mM EDTA, 150 mM NaCl, 2 mM $Na_3VO_4$, 1% Nonidet P-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin (Sigma), and 1 mM phenylmethylsulfonyl fluoride (Sigma) was added to each plate. The cells were removed from the plate by using a rubber spatula and were transferred to a microfuge tube. The cell homogenates were then mixed vigorously for 1 min and the insoluble material was removed by centrifugation at 13,000 × *g* for 10 min at 4°. Aliquots of the supernatants, matched for protein content according to standard procedures (14), were added to an equal volume of 2× sample buffer (62.5 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 40 mM dithiothreitol) and placed in a boiling water bath for 5 min. The phosphotyrosine-containing proteins in the sample were separated using SDS-PAGE and immunoblotted (see below).

**Subcellular fractionation.** For subcellular fractionation studies, plates of treated hepatoma cells were washed with 10 ml of ice-cold phosphate-buffered saline, removed from the plate in 750 μl of a hypotonic fractionation buffer [5 mM Tris·HCl, pH 7.8, 2 mM $Na_2VO_4$, 1 mM EDTA, and protease inhibitors (see above)], transferred to a Dounce tissue grinder (Wheaton catalogue number 357542), and subjected to 60 disruptive strokes using a type B pestle. Immediately after disruption, the samples were adjusted to 50 mM Tris·HCl, pH 7.4, 10 mM EDTA, and 150 mM NaCl, transferred to a microfuge tube, and rotated (end over end) for 15 min at 4°. In some studies, the samples were adjusted to 500 mM NaCl to disrupt strong electrostatic interactions between soluble proteins and the constituents of the particulate cellular material. The samples were then centrifuged (100,000 × *g*, Beckman tabletop ultracentrifuge) for 10 min at 4° to separate the soluble and particulate fractions of the cells. An aliquot of each resulting supernatant, containing the soluble cytosolic proteins, was added to an equal volume of 2× sample buffer and prepared for SDS-PAGE as indicated above (soluble fractions were adjusted to 1% Nonidet P-40 before use in immunoprecipitation studies; see below). The remaining pellets were washed, without suspension, with 1 ml of fractionation buffer. Extraction buffer (750 μl) was then added to the isolated pellets and the cellular material was carefully resuspended using a microfuge tube pestle. The samples were then centrifuged for 10 min at 4° and the resulting supernatants, containing the solubilized plasma membranes, were processed for SDS-PAGE as described above. The remaining nuclear pellets were washed with 1 ml of extraction buffer, solubilized in 1× sample buffer, sonicated (three 5-sec pulses, at 4°), and processed for SDS-PAGE. Protein concentrations were determined colorimetrically, as described (14).

**Immunoprecipitation from H4 cell extracts.** For immunoprecipitation, 3 μg of the appropriate antibody were added to equal protein aliquots of the supernatants described above. After incubation for 16 hr at 4°, 60 μl of Protein A-Sepharose (40%, w/v; Sigma) were added. After 2 hr, immunocomplexed proteins obtained after centrifugation were washed three times with extraction buffer (see above), resuspended in 1× sample buffer (see above), and placed in a boiling water bath for 5 min. Solubilized proteins in the samples or in crude cell homogenates were analyzed by SDS-PAGE, according to the method of Laemmli (15) (7.5% polyacrylamide in the separating gel). Proteins separated by the gel system were transferred electrophoretically to nitrocellulose membranes. For visualization of the phosphoproteins, the membranes were first incubated in blocking buffer (50 mM Tris·HCl, pH 7.5, containing 3% nonfat dry milk, 0.1% Tween-20, and 150 mM NaCl) for 2 hr. The blots were then probed for 1 hr with a monoclonal antiphosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY), in blocking buffer without milk. The blots were washed four times and antibody reactions were detected using horseradish peroxidase-conjugated sheep anti-mouse IgG and a chemiluminescence detection system (Amersham, Arlington Heights, IL). Blots were also probed with other antibodies according to the manufacturer's directions. Polyclonal antiserum to PI3K and the anti-IRS-1 antibodies used for immunoprecipitation (catalogue number 06–165) and immunoblotting (catalogue number 06–248) were obtained from Upstate Biotechnology. The PKC-specific monoclonal antibody (clone MC5) used in these studies was purchased from Leinco Technologies (St. Louis, MO). PMA was obtained from LC Laboratories (Woburn, MA). Reagents associated with electrophoresis were from Bio-Rad (Richmond, CA).

## Results

**Chromium-induced protein tyrosine phosphorylation in H4 cells.** Antiphosphotyrosine immunoblots of H4 cell extracts revealed that Cr(VI) treatment induced the ty-

rosine phosphorylation of multiple cellular proteins, compared with the basal phosphoprotein profile of control cells (Fig. 1A). The three prominent sets of proteins phosphorylated in response to this form of chromium had median molecular masses of 210, 125, and 87 kDa (Fig. 1A, *lane 2*, *brackets a*, *b*, and *c*, respectively). Dose-dependent induction of the phosphoproteins was observed, with phosphorylations initially being detected at concentrations of Cr(VI) as low as 3 μM (1-hr dosing period) (Fig. 1B), whereas maximal phosphorylation was detected at concentrations of 100 μM. Kinetic analysis indicated that Cr(VI) (100 μM) caused a gradual induction of phosphoproteins, with detectable levels being observed after 5 min and maximal phosphorylation of all sets of proteins occurring within 1 hr (Fig. 1C). In these studies, the increase in phosphorylation of the 125-kDa set of phosphoproteins preceded the appearance of the 210- and 87-kDa bands. Furthermore, phosphoproteins remained detectable throughout the test period, with nearly maximal levels being observed after 4 hr of dosing. It is important to note that these levels of Cr(VI) had no significant effect on cell viability throughout the experimental period.

In these experiments, the ability of chromium to influence tyrosine phosphorylation was specific for the hexavalent form of the element, because trivalent chromium [i.e., Cr(III), $CrCl_3$] was found to have no observable effect on basal tyrosine phosphorylation (Fig. 2A, compare *lanes 1*, *3*, and *5*). Interestingly, a pattern of phosphoproteins similar to that generated by Cr(VI) was observed in H4 hepatocytes that were treated with the PKC agonist PMA (Fig. 2A, compare *lanes 5* and *7*). The 210-kDa band appearing in PMA-treated cells was indistinguishable from the corresponding band observed in cells after Cr(VI) exposure. Similarities were also observed in the sets of phosphoproteins appearing at approximately 125 and 87 kDa. A 210-kDa phosphotyrosine-containing band appeared in cells treated with the phosphatase inhibitor sodium orthovanadate and had the same molecular mass as the corresponding band in cells treated with Cr(VI) (Fig. 2A, *lanes 5* and *9*). However, the 125-kDa and 87-kDa sets of proteins that appeared in Cr(VI)-treated cells were much less apparent in cells treated with equimolar concentrations of vanadate. These data indicate that Cr(VI) can induce the tyrosine phosphorylation of numerous cellular proteins. Furthermore, the electrophoretic mobility and blotting characteristics of several of these phosphoproteins, especially the components of the Cr(VI)-induced 210-kDa band, are similar to those of corresponding phosphoproteins appearing in cells treated with either PMA or vanadate.

**Effects of chromium on insulin-induced protein phosphorylation.** The insulin-potentiating properties of numerous chromium-containing agents prompted us to investigate the effect of inorganic forms of chromium on insulin-mediated tyrosine phosphorylation. Furthermore, because both PMA and vanadate are known to mimic (16, 17) and potentiate (18) the action of insulin and insulin-like growth factors, a comparison of the influence of these agents on insulin-induced protein phosphorylation with that of Cr(VI) was also conducted. In these studies, the addition of insulin to the hepatocytes was found to induce the appearance of two phosphotyrosine-containing bands (Fig. 2A, *lanes 1* and *2*). One band appeared at 95 kDa and is known to represent the autophosphorylated β subunit of the insulin receptor (19). The other band had an apparent molecular

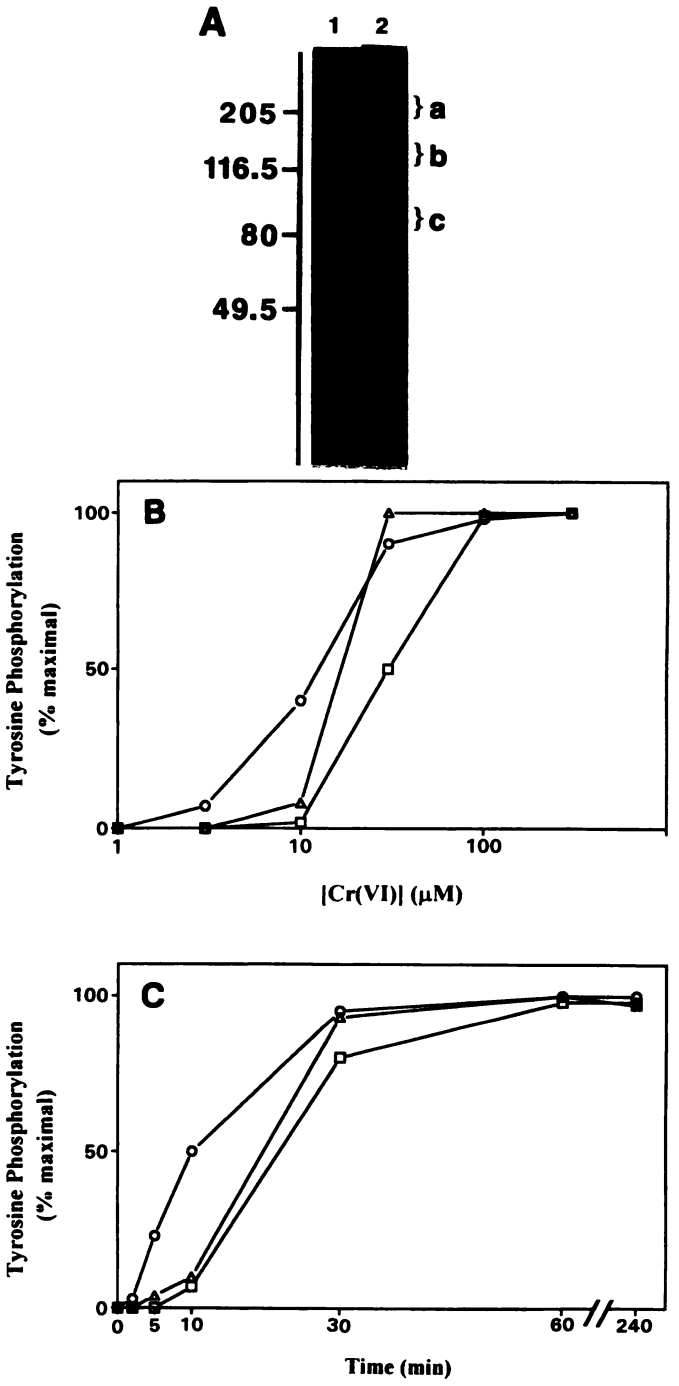


**Fig. 1.** Chromium-induced tyrosine phosphorylation of cellular proteins. A, H4 cells were treated without (*lane 1*) or with (*lane 2*) 50 μM $K_2Cr_2O_7$ [i.e., 100 μM Cr(VI)] for 1 hr. The phosphoproteins in the crude cell extracts were subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose membranes, and the blots were incubated with phosphotyrosine-specific monoclonal antibodies. Phosphoproteins reacting with the antibody were visualized using peroxidase-conjugated sheep anti-mouse secondary antibodies, as described in Materials and Methods. *Brackets a*, *b*, and *c*, positions of the prominent sets of Cr(VI)-induced phosphoproteins, having median molecular masses of 210, 125, and 87 kDa, respectively. B and C, Concentration-dependent (1-hr treatment) (B) and time-dependent [100 μM Cr(VI) treatment] (C) induction of bracketed phosphoproteins (□, bracket a; ○, bracket b; △, bracket c) were measured by densitometric scanning of developed film.

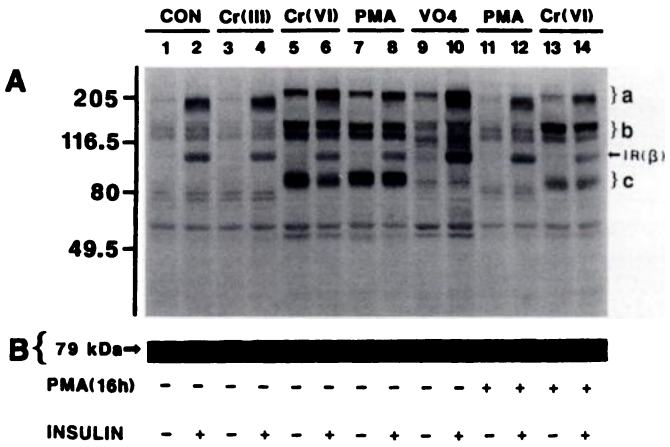


**Fig. 2.** Effect of various agents on basal and insulin-induced tyrosine phosphorylation in H4 cells. A, Confluent plates of serum-deprived (20 hr) H4 rat hepatoma cells were treated with various agents for 1 hr [*lanes 1* and *2*, control; *lanes 3* and *4*, 100 μM $CrCl_3$ [Cr(III)]; *lanes 5* and *6*, 50 μM $K_2Cr_2O_7$ [Cr(VI)]; *lanes 7* and *8*, 160 nM PMA; *lanes 9* and *10*, 100 μM $Na_3VO_4$]. Control and treated cells were then incubated without (*lanes 1*, *3*, *5*, *7*, and *9*) or with (*lanes 2*, *4*, *6*, *8*, and *10*) insulin (100 nM) for 2 min before harvesting. The cell lysates were subjected to Western blotting with antiphosphotyrosine antibodies as described for Fig. 1. In some treatment groups, cellular levels of PKC were down-regulated by treatment with PMA for 16 hr (*lanes 11-14*). Sets of plates were then treated with either additional PMA (*lanes 11* and *12*) or Cr(VI) (*lanes 13* and *14*) for 1 hr. One plate in each set was then treated with insulin (2 min) and the phosphotyrosine-containing proteins in crude cell extracts were analyzed as described above. *IR(β)*, insulin receptor β subunit. B, To confirm that cellular levels of PKC were depleted by this treatment, duplicate blots were developed using the PKC-specific monoclonal antibody MC5. The PKC-specific band appearing at approximately 79 kDa (*arrow*) in each treatment group is shown. Data are representative of five independent experiments.

mass of 185 kDa and has been characterized in another hepatoma cell line as consisting of several insulin-induced phosphoproteins, including the extensively characterized insulin receptor kinase substrate known as IRS-1 (20). Addition of insulin to cells pretreated with Cr(VI) was found to increase the phosphotyrosine content of the 210-kDa band that was observed in cells receiving Cr(VI) alone (Fig. 2A, *lanes 5* and *6*). This band was significantly higher in apparent molecular mass than the 185-kDa species that were tyrosine-phosphorylated in cells treated with insulin alone (Fig. 2A, compare *lanes 2* and *6*). The phosphotyrosine content of the 210-kDa band was the only aspect of the Cr(VI)-dependent phosphotyrosine pattern affected by insulin. Furthermore, Cr(VI) pretreatment had no observable effect on the insulin-induced phosphorylation of the insulin receptor β subunit, compared with cells treated with insulin alone. Similar observations were made for PMA-pretreated cells, in that further treatment with insulin increased the phosphotyrosine content of the 210-kDa band without altering any of the other phosphotyrosine-containing proteins induced by PMA. Addition of insulin to cells pretreated with vanadate was also associated with an elevation in the antiphosphotyrosine immunoreactivity of the 210-kDa band. However, the general appearance of this band suggested that it contained additional components having significant differences in electrophoretic mobility, compared with the corresponding band in cells treated with either Cr(VI)/insulin or PMA/insulin. Furthermore, vanadate treatment produced an increase (~43%, as determined by densitometric analysis of developed film) in the insulin-induced phosphotyrosine content of the insulin receptor β subunit, whereas Cr(VI) was found to have no significant effect on tyrosine phosphorylation of the β subunit. The results of this study suggest that Cr(VI) can alter aspects of insulin-induced phosphorylation of high molecular mass proteins in a manner similar to that of agents that have insulinomimetic properties.

**PKC independence of the effects of Cr(VI).** Because the pattern of phosphotyrosine-containing proteins appearing in Cr(VI)-treated cells was strikingly similar to the pattern generated by PKC-activating phorbol esters, the effect of PKC down-regulation on Cr(VI)-induced protein phosphorylation was investigated. For these studies, PKC was down-regulated in cultured H4 cells by prolonged incubation (16 hr) with PMA (160 nM). Extended exposure to PMA is known to specifically down-modulate PKC in many cell types, by characterized mechanisms (21, 22). After chronic PMA exposure, sets of culture plates were treated with either additional PMA or Cr(VI) for 1 hr. One plate from each set was then exposed to insulin and the phosphoproteins in the cell extracts were analyzed as described above. In these studies, prolonged PMA exposure was found to have no significant influence on either basal or insulin-induced tyrosine phosphorylation in H4 cells (data not shown). As expected, additional PMA treatment (1 hr) failed to produce alterations in the pattern of phosphotyrosine-containing proteins, relative to control (Fig. 2A, *lanes 11* and *12* versus *lanes 1* and *2*), further indicating that the cells were refractory to PMA stimulation. The attenuated responsiveness was apparently due to the down-regulation of PKC, because immunoblots probed with MC5 (22), an antibody that recognizes isoforms of this enzyme, failed to detect the 79-kDa PKC-specific band in cells chronically exposed to PMA (Fig. 2B, *lanes 11-14*). In these studies, PKC depletion had no apparent effect on the overall pattern of Cr(VI)-induced phosphoproteins in H4 cells (Fig. 2A, compare *lanes 13* and *5*). Furthermore, PKC down-regulation did not appear to influence the Cr(VI)-dependent alteration of protein tyrosine phosphorylation in response to insulin (Fig. 2A, compare *lanes 6* and *14*). Although the patterns of phosphoproteins generated in response to Cr(VI) and PMA appear to be similar, these results suggest that the appearance of the Cr(VI)-induced phosphoproteins, unlike those generated by PMA, is not dependent on isoforms of PKC that are down-regulated by phorbol esters.

**Subcellular distribution of phosphotyrosine-containing proteins.** Subcellular fractionation studies were conducted to further differentiate the various phosphoproteins observed in our analysis. Antiphosphotyrosine immunoblots of soluble and particulate (non-nuclear) fractions of H4 cells indicated that the 95-kDa autophosphorylated β subunit of the insulin receptor observed after insulin treatment was absent from the soluble fractions of cells (Fig. 3, *lane 2*). This finding is consistent with the fact that the insulin receptor is a membrane-associated tetrameric protein complex that is localized in the particulate fraction of cell homogenates (10). Furthermore, it was observed that essentially all detectable components of the phosphoprotein band appearing at 185 kDa in insulin-treated cells partitioned into the soluble cytoplasmic fractions. Because major components of this band are known to represent soluble, insulin-induced phosphoproteins, including the extensively characterized IRS-1 (20), the distribution of these proteins in the cytosol

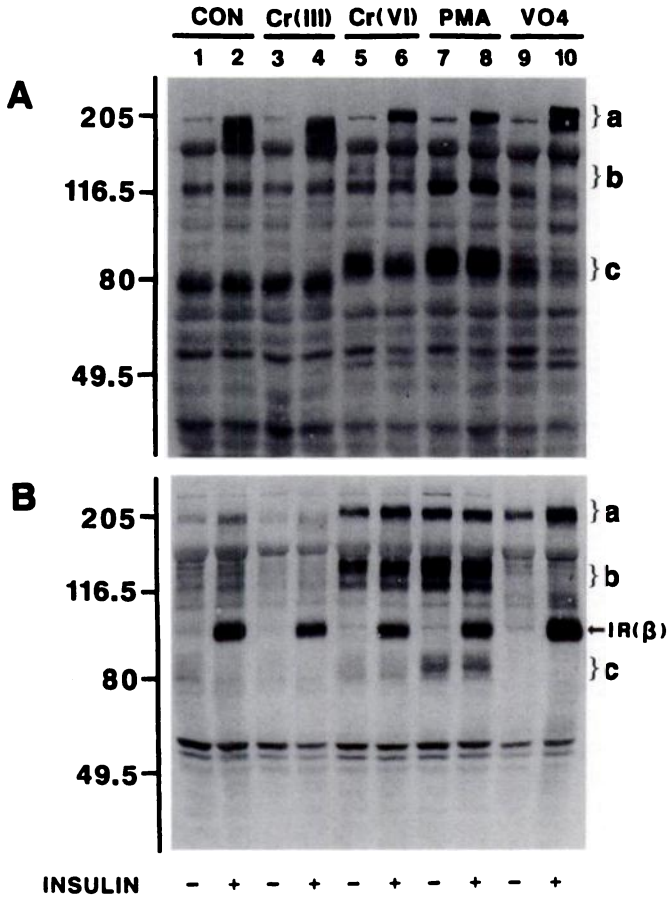


**Fig. 3.** Subcellular distribution of the phosphotyrosine-containing proteins appearing in treated H4 cells. H4 cells, treated as indicated for Fig. 1, were scraped from the plate (with a rubber spatula) into hypotonic fractionation buffer and subjected to Dounce homogenization. Immediately after disruption, the samples were adjusted to 50 mM Tris, pH 9.0, 10 mM EDTA, and 150 mM NaCl and rotated (end over end) for 15 min at 4°. After centrifugation, the proteins in the supernatant, representing the soluble fraction of cells, were processed for SDS-PAGE (see above), whereas the pellet was washed with fractionation buffer and subjected to extraction with a buffer containing the nonionic detergent Nonidet P-40. The insoluble material (i.e., nuclei) was removed by centrifugation and the supernatant, containing plasma membranes, was prepared for SDS-PAGE. After electrophoresis, the resolved phosphoproteins in the soluble (A) and particulate (non-nuclear) (B) fractions of cells were transferred to nitrocellulose membranes and visualized using the phosphotyrosine-specific immunodetection procedure described above. *IR(β)*, insulin receptor β subunit.

confirms effective fractionation. The results from these experiments also indicated that the 210-kDa phosphotyrosine-containing band appearing in cells treated with Cr(VI) was situated exclusively in the particulate fraction of the cells (Fig. 3, *lane 5*, *bracket a*). The interaction of this phosphoprotein with other particulate cellular material was not electrostatic in nature, because buffers containing 500 mM NaCl failed to disrupt the association (data not shown). Interestingly, the 210-kDa band appearing in cells treated with both Cr(VI) and insulin was found to represent at least two discrete components. One constituent of the band partitioned into the particulate fraction of cell homogenates and was identical in mass and phosphotyrosine content to the 210-kDa band observed in cells treated with Cr(VI) alone (Fig. 3B, *lanes 5* and *6*, *bracket a*). The other constituent of the band, which was responsible for its insulin-induced increase in phosphotyrosine content, was localized in the soluble fraction (Fig. 3A, *lanes 5* and *6*, *bracket a*). Identical results were observed for the subcellular fractionation experiments performed using PMA-treated cells, with only the insulin-induced component of the 210-kDa band partitioning into the soluble fraction (Fig. 3, *lanes 7* and *8*). Similarities were also noted for the subcellular distribution of the other sets of phosphoproteins induced by Cr(VI) and PMA. For example, the set of phosphoproteins at approximately 125 kDa in both Cr(VI)- and PMA-treated H4 hepatocytes was found exclusively in the particulate fraction of the cells (Fig. 3B, *lanes 5-8*, *bracket b*). Furthermore, major components of the 87-kDa set appearing in both treatment groups partitioned into the soluble fraction of cells (Fig. 3A, *lanes 5-8*, *bracket c*). The subcellular distribution of the high molecular mass phosphoproteins generated in response to vanadate treatment was similar to that of phosphoproteins induced by Cr(VI), with some exceptions. For example, as observed for Cr(VI), the 210-kDa phosphoproteins generated in vanadate-treated H4 cells were associated with the particulate fraction of the cells (Fig. 3, *lane 9*, *bracket a*). Furthermore, the insulin-induced, high molecular mass, phosphotyrosine-containing band partitioned into the soluble fraction of vanadate-treated cells (Fig. 3A, *lane 10*, *bracket a*). However, this band was composed of at least two phosphoproteins with significant differences in electrophoretic mobility, whereas the corresponding phosphoproteins in cells treated with Cr(VI) and insulin appeared as a single band. It is important to note that there were no major differences in the patterns of resolved phosphoproteins in the nuclear fractions from control and treated cells (data not shown). Furthermore, the subcellular distribution of the phosphoproteins generated in these experiments was not significantly altered by changes in the ionic strength of the fractionation buffer (i.e., 150–500 mM NaCl) (data not shown). Our results indicated that the fractionation behavior of the various corresponding sets of phosphoproteins generated in response to Cr(VI), PMA, and vanadate was virtually identical.

**Effect of Cr(VI) on insulin-dependent phosphorylation and PI3K binding of IRS-1.** Because insulin is known to induce the tyrosine phosphorylation of a soluble 180-kDa protein referred to as IRS-1, the possibility that this protein represents one of the high molecular mass phosphoproteins generated in cells treated with the various agents was investigated. In these studies, IRS-1-specific antibodies were found to immunoprecipitate a 180-kDa phosphotyrosine-containing protein from unfractionated lysates of cells receiving insulin (Fig. 4A). Analysis of the immunoisolates on blots probed with IRS-1-specific antibodies indicated that these bands represented phosphorylated forms of IRS-1 (Fig. 4B). Cr(VI) and PMA pretreatments were found to decrease (~30%) the insulin-induced tyrosine phosphorylation of IRS-1, relative to cells treated with insulin alone, whereas vanadate pretreatment was consistantly found to increase (~20%) IRS-1 phosphorylation. Results of these experiments are consistent with the observations of other investigators who found that PMA can inhibit (23), whereas vanadate can enhance (17), insulin-stimulated kinase activity. Our findings also extend the similarity between Cr(VI) and PMA pretreatment to include the ability of each agent to affect the various aspects of insulin-induced signaling events that involve IRS-1.

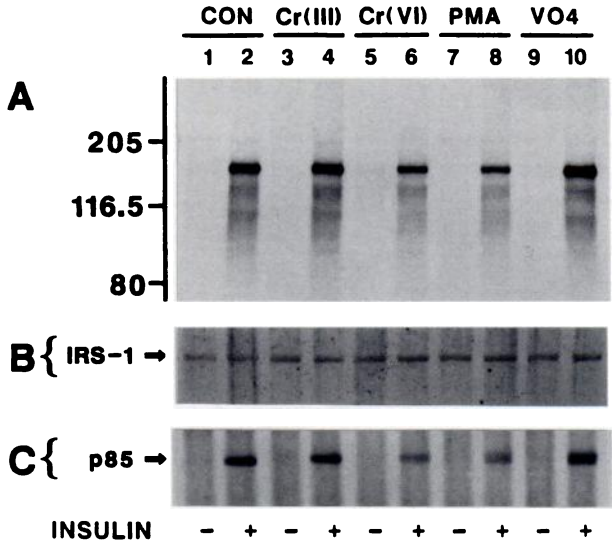


**Fig. 4.** Characterization of insulin-induced phosphorylation and PI3K binding of IRS-1 after treatment with various agents. Polyclonal antibodies specific for IRS-1 were used to immunoprecipitate this protein from unfractionated cell lysates of treated H4 cells. The effect of each pretreatment on the insulin-induced phosphorylation of IRS-1 was analyzed on antiphosphotyrosine immunoblots (A). Amounts of IRS-1 in the immunoisolates were determined by probing duplicate membranes with IRS-1-specific antibodies (B), whereas the physical association (i.e., co-precipitation) of PI3K with anti-IRS-1-precipitated material was determined on blots developed with polyclonal antibodies specific for the regulatory subunit of PI3K (i.e., p85) (C). The proteins reacting with the specific blotting antibodies were all visualized using an appropriate peroxidase-conjugated secondary antibody and a chemiluminescent detection system, as described in Materials and Methods. Data are representative of three independent experiments.

Interestingly, IRS-1 immunoisolates did not contain the insulin-induced, high molecular mass phosphoproteins (185–210 kDa) that were readily apparent in the soluble fractions of cells pretreated with either Cr(VI), PMA, or vanadate (Fig. 3A, *bracket a*). Indeed, a detailed comparison of the electrophoretic mobility and blotting characteristics of IRS-1 (Fig. 4, A and B) and the other insulin-induced phosphoproteins appearing in all treatment groups indicated that IRS-1 has an apparent molecular mass that is 5–25 kDa lower than those of the other, more prominent phosphoproteins. In addition, the results indicate that IRS-1 is a minor phosphoprotein component of the 185-kDa band in insulin-treated H4 cells that can be visualized on antiphosphotyrosine immunoblots only after immunoconcentration.

It is known that, after insulin-induced tyrosine phosphorylation, IRS-1 binds to and activates PI3K (13). To analyze the insulin-induced association of these proteins after treatment with various agents, Western blots containing the anti-IRS-1-recoverable material were probed with antibodies specific for the regulatory subunit of PI3K (i.e., p85). As expected, p85 was detected in all treatment groups after insulin stimulation (Fig. 4C). A decrease in the amount of p85 was detected in the IRS-1 immunoisolates of cells treated with Cr(VI)/insulin or PMA/insulin, compared with cells treated with insulin alone (Fig. 4C, compare *lanes 2*, *6*, and *8*). However, this decrease was correlated with the observed reduction in precipitable quantities of phosphorylated IRS-1 detected in these extracts (Fig. 4A).

**Characterization of additional p85-associated phosphoproteins.** Additional experiments were performed to characterize the p85 binding properties of other phosphoproteins generated in these studies. For this analysis, p85 was immunoprecipitated from either soluble or particulate fractions of cells and the proteins that were associated with p85-recoverable material were then analyzed by immunoblotting with antibodies to phosphotyrosine, IRS-1, and p85. In these studies, the 185–210-kDa insulin-induced phosphoproteins present in the soluble fractions of cells from the various treatment groups (Fig. 3A, *bracket a*) were found to co-precipitate with p85 (Fig. 5A, *I*). Surprisingly, anti-IRS-1 immunoblots, containing anti-p85-precipitated material, failed to detect the co-precipitation of IRS-1 in any treatment group (Fig. 5A, *II*). This finding was unexpected, because p85 was found to co-precipitate with IRS-1 in insulin-treated cells when IRS-1-specific antibodies were used (Fig. 4C). The reason for this discrepancy was evident in anti-p85 immunoblots of the supernatants of the anti-p85 immunoprecipitates, which indicated that a significant portion of p85 remained

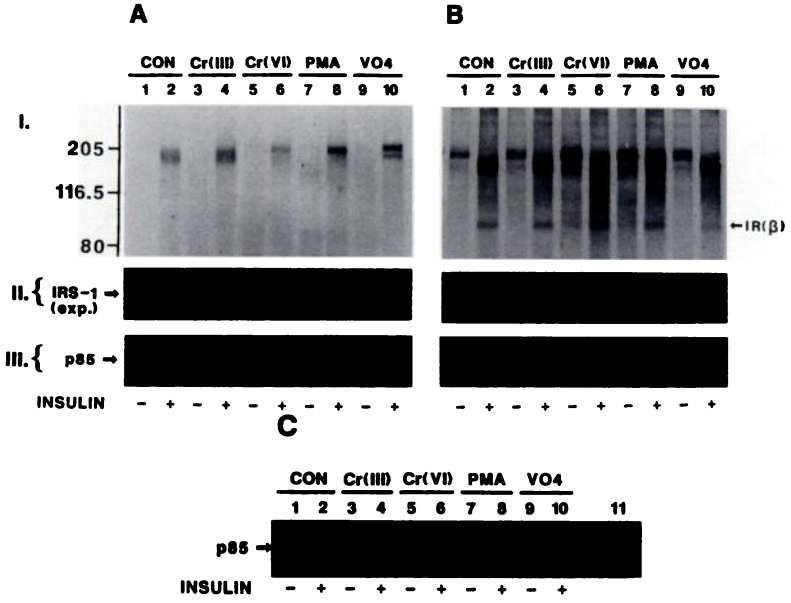


**Fig. 5.** Characterization of p85-binding phosphoproteins in soluble and particulate fractions of treated H4 cells. Antiserum specific for the regulatory subunit of PI3K (i.e., p85) was used in immunoprecipitation reactions using the soluble (A) and particulate (B) fractions of treated H4 cells. The proteins in the immunoisolates were resolved by SDS-PAGE and then analyzed on antiphosphotyrosine (*I*), anti-IRS-1 (*II*), and anti-p85 (*III*) immunoblots. *IR(β)*, insulin receptor β subunit. The unexpected absence of IRS-1 in the p85 immunoisolates of insulin-treated cells [A, *II*, *arrow*, expected (*exp.*) position of IRS-1 on immunoblot] prompted further investigation into the reason for this inconsistency. For this investigation, proteins in the supernatants resulting from anti-p85 immunoprecipitation were analyzed on anti-p85 immunoblots (C). *Lane 11*, immunopurified p85 as standard. The increased quantity of unprecipitated p85 observed in samples of insulin-treated cells substantiates other findings that demonstrate the inability of the anti-p85 antibodies to immunoprecipitate this protein when it is involved in a complex with IRS-1.

unprecipitated in samples receiving insulin treatment (Fig. 5C). This fraction of p85 could subsequently be precipitated from these samples using IRS-1-specific antibodies (data not shown). These results indicate that polyclonal anti-p85 antibodies do not quantitatively react with molecules of p85 that are involved in a complex with phosphorylated IRS-1. It is important to note that similar observations were made by other investigators using a different source of p85-specific polyclonal antibodies (13). The fortuitous selectivity of these antibodies has enabled us to confirm that the insulin-induced phosphoproteins associated with the anti-p85-recoverable material in soluble fractions are distinct from IRS-1.

Phosphotyrosine-containing proteins with a median molecular mass of 205 kDa were also found to co-precipitate with p85 from the particulate fraction of control untreated cells (Fig. 5B, *I*). Interestingly, the phosphotyrosine content of these proteins was increased in response to Cr(VI) and PMA, compared with control and vanadate-treated cells (Fig. 5B, compare *lanes 5* and *7* with *lanes 1*, *3*, and *9*). Furthermore, dramatic changes in the pattern of these p85-binding proteins were induced in response to insulin. Because most of these phosphoproteins could be visualized on antiphosphotyrosine immunoblots only after enrichment in the anti-p85 immunoisolates, it is likely that they are proteins of low abundance. Furthermore, analysis of the anti-p85-recoverable material on anti-IRS-1 immunoblots failed to detect IRS-1 in these samples (Fig. 5B, *II*). These findings indicate that there exist p85-binding phosphoproteins in the particulate fraction of H4 cells that are distinct from IRS-1.

Interestingly, a 95-kDa phosphoprotein corresponding to the $\beta$ subunit of the insulin receptor was visualized in the antiphosphotyrosine blots of the p85 immunoisolates from particulate fractions of insulin-treated cells (Fig. 5B, *I*, *lanes 2*, *4*, *6*, *8*, and *10*, *arrow*). These findings are consistent with the observations of other investigators who postulated signaling complexes involving the insulin receptor, PI3K, and PI3K-binding (phospho)proteins (24, 25). However, ternary signal complexes involving IRS-1 (24) were not detected in the current study, because this protein was absent from the anti-p85 immunoisolates (Fig. 5B, *II*). It should be noted that in immunodepletion studies, in which cell fractions were processed through two rounds of immunoprecipitation, anti-p85 antibodies failed to totally remove the phosphotyrosine signals appearing at 185–210 kDa from the fractionated cell extracts, leaving approximately 30% and 80% of the signal unprecipitated in the soluble and particulate fractions, respectively (data not shown). Interestingly, as observed for the soluble fractions (Fig. 5C), anti-p85 antibodies failed to immunoprecipitate all of the p85-immunoreactive material from the (nondenatured) particulate fraction of insulin-treated cells. Therefore, it is possible that other phosphoproteins, including the prominent 210-kDa species appearing in Cr(VI)-, PMA-, and vanadate-treated cells, are physically associated with p85 but fail to be precipitated due to their ability to conceal p85 from the precipitating antibody. The results from these studies suggest that there are several distinguishable types of p85-binding phosphoproteins that are distributed throughout the soluble and particulate fractions of H4 cells. The ability of Cr(VI) to induce alterations in the pattern of these phosphoproteins, in a manner similar to that of other biologically active agents, suggests that this may be a mechanism by which this element can alter the growth and metabolism of cells.

## Discussion

The present studies were conducted to explore the effect of chromium on tyrosine phosphorylation of cellular proteins. In particular, because chromium-containing agents are known to possess insulin-potentiating qualities, the effect of chromium on various aspects of the insulin-dependent signaling pathway (i.e., insulin-induced tyrosine phosphorylation) was evaluated. The PKC agonist PMA and the phosphatase inhibitor sodium orthovanadate were also included in our analysis. These agents are known to induce phosphoproteins in hepatocytes by distinct mechanisms (26, 27), as well as exhibiting insulinomimetic activities (16, 17). Therefore, comparison of the phosphoprotein profiles generated using these agents and that seen with chromium may suggest a mechanism by which this element affects protein phosphorylation and the insulin signaling cascade.

Hexavalent chromium is of importance because of its implication in carcinogenesis in humans (1), whereas trivalent chromium is recognized as being an essential trace element that is involved in the maintenance of normal glucose tolerance (4). Although the genotoxic and mutagenic properties of various forms of chromium suggest that it functions as an initiating agent in the overall carcinogenic process, little is understood about the epigenetic effects of this element on the growth and metabolism of cells. Our finding that Cr(VI) induces the tyrosine phosphorylation of several sets of cellular proteins suggests a mechanism by which this element can affect cellular processes. Tyrosine phosphorylation of key regulatory proteins is considered to be a major event in growth factor-mediated signal transduction (28). Therefore, Cr(VI)-induced alterations in protein phosphorylation may modulate various regulatory pathways of the cell, as well as altering their responsiveness to growth factors. Our observation that Cr(VI) induces a nearly identical pattern of phosphotyrosine-containing proteins in H4 cells, compared with the phorbol ester PMA, suggests that the two agents function by a common mechanism. PMA is known to induce its biological effects by binding to and activating a serine/threonine kinase, PKC (29). The substrates of this enzyme appear to be components of signal-transducing pathways [i.e., other kinases (30), phosphatases (31), etc.] involved in the regulation of cellular growth and metabolism. It is possible that PKC-mediated phosphorylation of these types of proteins results in the activation of specific tyrosine kinases or, alternatively, the inhibition of protein tyrosine phosphatases that mediate the generation of the phosphotyrosine-containing proteins observed in PMA-treated cells (26). In a manner analogous to that of PMA, Cr(VI) may also affect tyrosine phosphorylation by initially activating a serine/threonine kinase; however, because many of the effects of chromium observed in our study occur in PKC-depleted cells, it is likely that this proposed chromium-activated kinase is distinct from the isoforms of PKC that are down-regulated by chronic PMA exposure (21).

It is important to note that we have not conducted the detailed analysis necessary to definitely prove that the phosphoproteins appearing in Cr(VI)-treated cells are, in fact, the same as those generated after PMA treatment. However,

because several of the phosphoproteins generated in response to these two agents are indistinguishable in many respects (i.e., electrophoretic mobility, antiphosphotyrosine immunoreactivity, subcellular location, and insulin-induced phosphorylation), it is likely that many of the phosphoproteins induced in the two treatment groups are identical.

Because hexavalent chromium freely enters cells through a relatively nonspecific anion channel (32), is rapidly converted to pentavalent chromium, and then is further reduced to the trivalent state (33), it is not certain which form of this element is responsible for inducing protein tyrosine phosphorylation in H4 cells. Our observation that inorganic trivalent chromium fails to alter either basal or insulin-induced tyrosine phosphorylation of proteins was not unexpected, because cellular uptake of this species is poor (34). However, Cr(III) is known to accumulate at cation binding sites on the external surface of the cell (35). In earlier work, it was suggested that ternary complexes can form between chromium, membrane-bound receptor kinases, and their respective ligands, thus initiating biological activity (5). Because $CrCl_3$ treatment failed to alter tyrosine phosphorylation in H4 cells, it appears unlikely that the surface effects of trivalent chromium induce relevant alterations in the activity of membrane-associated kinases.

Chromium is recognized as being an essential trace element in human nutrition (4). Furthermore, supplemental chromium has been found to normalize both hypoglycemic and hyperglycemic states (5, 6). These normalizing effects have been associated with an increased efficiency of insulin action, characterizing chromium as a cofactor in glucose metabolism. It has been determined that chromium exists in the trivalent state in many dietary sources of this element (36, 37). It is thought that the organic components of dietary chromium function to chelate the trivalent chromium ion, facilitating its passage through the cell membrane. Addition of these factors to cells (37, 38) or supplementation of the diets of diabetic animals with these factors (36) is known to have dramatic effects on glucose uptake and metabolism. Conversely, studies involving inorganic unchelated Cr(III) (i.e., $CrCl_3$) fail to show consistent influences on glucose homeostasis (39). It is our hypothesis that inorganic hexavalent chromium shares many biological effects with the various organic (chelated) trivalent forms of this element, when analyzed in cultured cells, because it can readily enter cells, where it is rapidly converted to the trivalent form. This may not occur *in vivo*, because Cr(VI) is rapidly converted to membrane-impermeant forms [i.e., Cr(III)] in biological solutions (e.g., plasma) (40). Inclusion of stable organic forms of trivalent chromium in future studies may reveal which oxidation states are important as modulators of tyrosine phosphorylation and as cofactors in glucose homeostasis.

Several groups of investigators have associated the insulin-potentiating properties of chromium with its ability to stabilize the ligand (7), enhance ligand binding (8), and promote receptor internalization (9). In the current studies, Cr(VI) was found to alter more distal components of the insulin signal transduction cascade. For example, Cr(VI) inhibited the insulin-induced tyrosine phosphorylation of IRS-1 and its subsequent association with p85. Although the significance of these observations for the insulin signaling pathway was not determined in the current studies, our results demonstrate the ability of chromium to influence specific, insulin-mediated signaling events. Cr(VI) was also found to have an effect on insulin-mediated phosphorylation through its ability to alter the electrophoretic mobility of the high molecular mass phosphoproteins that were generated in response to insulin (Fig. 3A, *bracket a*). The insulin-induced phosphoproteins appearing in the soluble fraction of Cr(VI)-pretreated cells were approximately 15–25 kDa larger in apparent molecular mass than the corresponding phosphoproteins observed in cells treated with insulin alone. The reason for the observed alteration in gel migration may be associated with the possibility that Cr(VI) induces the post-translational modification of these proteins, via mechanisms described above (i.e., serine/threonine phosphorylation). In this case, the altered phosphoamino acid content may be responsible for the apparent change in gel mobility. It is also possible that the insulin-induced 210-kDa species generated in Cr(VI)-pretreated cells represent alternative substrates of the insulin receptor kinase that are distinct from the 185-kDa phosphoproteins appearing in cells treated with insulin alone. Although the identity and function of these proteins are unknown, phosphoproteins in this band are similar to the high molecular weight, $M_r$ 185,000 phosphoprotein described by other investigators using the Fao hepatoma cell line, in that these phosphoproteins are phosphorylated in response to insulin treatment and they are distinct from IRS-1 (20). However, unlike the high molecular weight, $M_r$ 185,000 phosphoprotein, a significant proportion (~65%) of the high molecular mass phosphoproteins observed in our analysis was found to co-precipitate with p85 (PI3K) (Fig. 5A, *I*).

The physical interaction between these phosphoproteins and PI3K may have biological significance. In a manner similar to that of IRS-1, insulin-mediated phosphorylation of these proteins may promote the binding and activation of PI3K (13). For example, it is possible that these proteins contain Tyr-Xxx-Xxx-Met motifs, which mediate p85 binding and PI3K activation by characterized mechanisms (12) (see the introduction). Cr(VI)-induced alterations of these PI3K-binding proteins, as reflected in their altered apparent molecular masses, may translate into an increased PI3K-activating potential of these proteins after insulin-mediated tyrosine phosphorylation. Modulation of insulin-stimulated PI3K activity in such a manner may explain the ability of some chromium-containing compounds to potentiate insulin action.

An interesting aspect of these studies involves the p85-binding phosphoproteins that were observed in the particulate fractions of control and treated cells (Fig. 5B, *I*). Although the 95-kDa phosphoprotein appearing in insulin-treated cells has been determined to be the $\beta$ subunit of the insulin receptor (24, 25), the identities of the phosphoproteins having higher relative masses (i.e., 180–205 kDa) are unknown. The failure to detect significant amounts of IRS-1 in these fractions suggests that this particular protein is not a major component of these observed phosphoproteins. Interestingly, the phosphotyrosine content of these proteins was altered by Cr(VI) and PMA. Furthermore, dramatic alterations in the patterns of these proteins were observed after insulin treatment, suggesting that these p85-binding proteins may be involved in modulating the responsiveness of the cells to various effectors of the insulin signal cascade. Recently it has been determined that, in addition to acting as the regulatory subunit of PI3K, p85 can serve as an adaptor

molecule, linking various types of proteins that are involved in signal transduction (41). If our results reflect the formation of such complexes, it is apparent that Cr(VI) alters the phosphorylation state of a constituent protein. Detailed characterization of these p85-containing complexes and the manner in which chromium affects their formation and/or function are the subjects of future studies.

Chromium has many chemical, physical, and biological properties that are similar to those of vanadate. For example, both are transition metals, occupying adjacent positions in the periodic table. In addition, both elements have multiple oxidation states and are considered to be essential nutrients (4). Furthermore, various forms of these elements have been characterized as being either potentiators of insulin action (18, 42) or insulinomimetics (17). In the current studies, both agents were found to induce the tyrosine phosphorylation of a 210-kDa protein that partitioned into the particulate fraction of H4 cells. Despite these similarities, our detailed comparison of the abilities of these agents to influence tyrosine phosphorylation revealed major differences (see Results). The effect of vanadate on protein phosphorylation has been attributed to the ability of this agent to inhibit specific tyrosine phosphatases (17, 27). In contrast, chromium does not function as a phosphotyrosine phosphatase inhibitor in *in vitro* phosphatase assays (17). Therefore, these agents appear to modulate protein phosphorylation by distinct mechanisms. Further characterization of Cr(VI)-induced phosphoproteins in H4 cells and alterations in their properties subsequent to phosphorylation should help to determine the mechanism by which chromium-containing agents affect cell growth and metabolism.

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