Reduction of Cell Attachment and Phosphorylation of Focal Adhesion Kinase Associated with Expression of a Mutant Insulin Receptor*

Nicky Konstantopoulos‡ and Stella Clark§ ¶

From the University of Melbourne, Department of Medicine, P. O. Royal Melbourne Hospital, Parkville 3050 and §Deakin University, Institute of Human Nutrition, Malvern, 3144 Victoria, Australia

Insulin signaling results in rapid changes to the cell cytoskeleton, and it has recently been shown that insulin stimulates the dephosphorylation of the cytoskeletal-associated tyrosine kinase, focal adhesion kinase (pp125FAK). We report here that mutation of two tryptic cleavage sites (Lys164 and Lys582) in the insulin receptor α-subunit results in a cell-line (CHO.2N-10) with altered morphology associated with an increase in cell size, a decrease in cell adhesiveness, and a decrease in pp125FAK tyrosine phosphorylation in the absence of insulin (45.2 ± 9.7% compared to nontransfected Chinese hamster ovary (CHO) cells). In contrast to pp125FAK, paxillin phosphorylation was similar in all cell lines despite lower levels (61.0 ± 10.4% compared to CHO cells) of paxillin protein in CHO.2N-10 cells. We observed comparable protein levels of pp125FAK and the structural focal adhesion protein, vinculin, in all cell lines. Despite under phosphorylation of pp125FAK in the basal state, insulin stimulation of CHO.2N-10 cells still resulted in dephosphorylation of pp125FAK. CHO.2N-10 and CHO.T (overexpress wild-type insulin receptor) cells have similar insulin binding characteristics, insulin-stimulated autokinase and peptide phosphorylation, and insulin-stimulated pp185/IRS-1 phosphorylation. Our results suggest that the insulin receptor may play an important role in cell-matrix interactions, such as modulating cell adhesion and inducing cell architecture changes.

Interactions between growth factors and integrin signaling pathways are probably involved in the regulation of cell proliferation, shape, adhesion and migration (1, 2). Integrins, the receptors for extracellular matrix proteins, provide both a physical link to the cytoskeleton (often at sites of focal adhesion) and transduce signals from the extracellular matrix. A connection is observed between integrins and stress fibers of polymerized actin, which is necessary for maintenance of cell integrity and appears to terminate at focal adhesion sites. The assemblies of structural proteins (such as α-actin, talin, vinculin, tensin, and paxillin) that co-localize with some integrins in focal adhesions are thought to play important roles in stabilizing cell adhesion and regulating cell shape, morphology, and mobility (1). These structural proteins may also serve as a framework for the association of signaling molecules that regulate signal transduction pathways leading to integrin-induced changes in cells (1). In addition, there are now many examples of growth factor-induced regulation of components involved in integrin signaling pathways. Growth factors may rapidly stimulate actin polymerization at the plasma membrane of many cell types to produce lamellipodia and edge-ruffles (e.g. PDGF and insulin) (3–5) and at later times promote actin stress fiber formation (e.g. PDGF) (6). Conversely, components of growth factor signaling pathways (such as the mitogen-activated protein kinase cascade) are probably utilized by integrins as part of their signal transduction pathways (7, 8).

A key enzyme in cytoskeletal rearrangements/interactions/architecture appears to be the phosphorylated, cytoplasmic tyrosine kinase, focal adhesion kinase (pp125FAK) which plays a central role in integrin-mediated signal transduction. Apart from activation by integrins, specific tyrosine phosphorylation of pp125FAK is also induced by several growth factors (9), lysophosphatidic acid (10) and neuropeptides (11, 12), suggesting that several diverse signaling pathways may converge at this point. The N-terminus of pp125FAK can bind synthetic peptides derived from several β-integrin cytoplasmic domains, although an in vivo association has not yet been demonstrated (12); indeed, an indirect linkage via talin (13) has recently been proposed. Within the C terminus of pp125FAK, a focal adhesion targeting sequence is both necessary and sufficient to localize pp125FAK to focal adhesions (14). The cytoskeletal protein, paxillin, also associates with pp125FAK through a C-terminal sequence of pp125FAK distinct from the focal adhesion targeting domain (15, 16). Upon integrin clustering, pp125FAK is autophosphorylated on tyrosine 397 (17), and its tyrosine kinase activity is enhanced, which may then lead to association of pp125FAK with SH2 domain-containing signaling proteins such as Src, GRB2, phosphatidylinositol 3-kinase (PI 3-kinase), C-terminal Src kinase, and phospholipase Cγ to potentially form large signaling complexes which can, for instance, activate mitogen-activated protein kinase. Growth factors such as PDGF stimulate tyrosine phosphorylation of paxillin and pp125FAK (9) and induce the association of PI 3-kinase with pp125FAK in adherent Swiss 3T3 cells (18). Lysophosphatidic acid and neuropeptides such as bombesins also stimulate tyrosine phosphorylation of pp125FAK and induce formation of focal adhesions and actin stress fibers in Swiss 3T3 cells (6, 10, 12). However, while associated with cytoskeletal rearrangements, a

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To whom correspondence should be addressed: Deakin University, Institute of Human Nutrition, P. O. Box 224, Malvern, 3144 Victoria, Australia. Tel.: 613-9244-5337; Fax: 613-9244-5338; E-mail: stella@deakin.edu.au.

The abbreviations used are: PDGF, platelet-derived growth factor; FAK, focal adhesion kinase; CHO, Chinese hamster ovary; 2N, insulin receptor with a double point mutation (K164N/K582N); IRS-1, insulin receptor substrate-1; PI 3-kinase, phosphatidylinositol 3-kinase; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; α-MEM, α-modified Eagle’s medium; FCS, fetal calf serum; BSA, bovine serum albumin.

1 The abbreviations used are: PDGF, platelet-derived growth factor; FAK, focal adhesion kinase; CHO, Chinese hamster ovary; 2N, insulin receptor with a double point mutation (K164N/K582N); IRS-1, insulin receptor substrate-1; PI 3-kinase, phosphatidylinositol 3-kinase; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; α-MEM, α-modified Eagle’s medium; FCS, fetal calf serum; BSA, bovine serum albumin.
definitive role for pp125FAK in signal transduction mechanisms that alter cytoskeletal properties remains to be determined.

As discussed above, most stimuli increase pp125FAK tyrosine phosphorylation. Surprisingly (and perhaps in contrast to its “PDGF-like” effect on cytoskeletal changes in some cells) (5), insulin stimulates the dephosphorylation of pp125FAK in rat 1 fibroblasts (19) and in CHO cells (20). Unlike the bell-shaped curve of pp125FAK phosphorylation in response to PDGF, pp125FAK dephosphorylation occurs at all insulin concentrations (21). Insulin stimulation was also reported to correlate with a marked decrease in the length and number of actin stress fibers in CHO cells overexpressing insulin receptors (22). We now report that expression, in CHO cells, of an insulin receptor mutated to change lysines 164 and 582 to asparagine leads, in the absence of insulin, to decreased tyrosine phosphorylation of pp125FAK; altered cell shape, and abrogated cell adhesion. These sites, which are putative tryptic cleavage sites (23, 24), were mutated as part of an ongoing study to examine the mechanism of tryptic activation of the insulin receptor (24). The unusual phenotype of the cell line expressing the mutant insulin receptor provides further evidence for the insulin receptor as a potential regulator of cell architecture and adhesion.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human insulin receptor containing Lys814→Asn, Lys562→Asn, or K164N/K582N (2N) substitutions were generated using polymerase chain reaction-based mutagenesis by Dr. L. Macaulay (CSIRO, Australia). Rat tail collagen (type I) was kindly provided by Dr. Bob Whitehead (Ludwig Institute, Melbourne, Australia). The mouse myeloid cell line (FDC-P1) was generously provided by Dr. A. Harris (Walter and Eliza Hall Institute, Melbourne, Australia). Monoclonal antibodies against the human insulin receptor, CT-1, and 18-44 were purchased from AbD Serotec, Oxford, UK. Antibodies 2A7 (monoclonal) and BCS (polyclonal) against pp125FAK were generously provided by Dr. J. T. Parsons, University of Virginia, Charlottesville. The mouse anti-hamster integrin α5 (clone 3B11) and anti-hamster integrin β3 (clone 7E2) monoclonal antibodies were generous gifts from Dr. R. Juliano, University of North Carolina, Chapel Hill. Lipofectin® and G418 (Genetec) were purchased from Life Technologies, Inc. Plasma fibronectin was purchased from Promega, Madison, WI. Insulin kinase substrate “FYF peptide” (RRDIFETDYFRK) was purchased from ICN Biomedicals, Cleveland, OH (clone PY20) or Chemicon International Inc., Temecula, CA. Enhanced chemiluminescence reagents were from Dupont NEN. All other reagents were of the highest grade available.

**Site-directed Mutagenesis—**A Lys814→Asn mutant receptor cDNA was constructed by inserting a mutagenic oligonucleotide (generated by a polymerase chain reaction, encoding the Lys814→Asn mutation between the Kpn1 and EcoRI restriction sites of the human insulin receptor cDNA) into a Kpn1-EcoRI digest of the insulin receptor expression vector pET (25). For mutagenesis of Lys562→Asn, a mutant oligonucleotide was generated by a polymerase chain reaction between the AccI and BamHI sites of the insulin receptor cDNA, and then inserted into the AccI-BamHI digest of a modified pET insulin receptor expression vector. The single mutations were combined to produce the 2N mutant by inserting the KpnI-XbaI fragment from Asn814→Asn562 into the Asn164 construct. All constructs were sequenced around the insertion, subjected to extensive restriction analysis, and shown to be correct.

**Cell Culture—**CHO cell lines were maintained in α-modified Eagle’s medium containing 10% v/v fetal calf serum (FCS). For immunofluorescence, cells were cultured on glass coverslips to 80% confluency. When required cells were serum-starved for 18 h with α-MEM containing either 0.5% v/v FCS or 0.1% w/v bovine serum albumin (BSA).

**Stable Expression of cDNA in CHO Cells—**CHO cell lines expressing the 2N (K164N/K582N) insulin receptor (and single point mutants) were obtained by co-transfection of 10 μg of plasmid DNA and 2 μg of pSVneo DNA using Lipofectin®. Transfected CHO cell lines were maintained in medium containing 800 μg/ml G418 sulfonate. Positive insulin receptor expressing clones were identified by enzyme-linked immunosorbent assay as described by Clark et al. (24). The only variation to the described method (24) was the addition of 10 mg/ml BSA after coating the microtiter plates with CT-1, to block nonspecific binding. Cell lines expressing high levels of mutant receptors were further selected by fluorescence-activated cell sorting (FACS) as described previously (20). FACS selected positive colonies were grown in 24-well plates and plated and incubated in Earle’s balanced salt solution, 25 mM Hepes, pH 7.4, 1% v/v 10% v/v BSA containing approximately 20% CHO cell insulin, in the absence or presence of increasing concentrations of unlabeled insulin (1–1000 ng/ml) for 4 h at 37 °C. Cells were washed and solubilized, and bound radioactivity was measured in an autogamma spectrometer as described previously (27). Nonspecific binding was measured in the presence of 10 μg/ml unlabeled insulin and was always less than 2% of total binding. Data were analyzed by the method of Scatchard (28).

**Receptor Autophosphorylation and Kinase Activity—**CHO cells were treated with or without insulin (172 nm) and lysed, and insulin receptor immunoprecipitates were analyzed for immunoprecipitated with monoclonal antibody CT-1 as described previously (21) in vitro autophosphorylation assays were carried out after addition of [γ−32P]ATP as described previously (27). To measure peptide phosphorylation, receptor immunoprecipitates were incubated in 10 μl of phosphorylation buffer (50 mM Hepes, pH 7.4, 12 mM MgCl2, 100 μM Na2VO4, 2 mM MnCl2, 150 mM NaCl, 0.2% w/v Triton X-100, 50 μM ATP, and 1 mM EGTA), and the reaction was started by the addition of 200 μM γ32P ATP peptide (RRDIFETDYFRK) and 0.4 μM of [γ−32P]ATP per sample for 5 min at 30 °C. At this time, duplicate 10-μl aliquots of each reaction were washed three times in 30% v/v glacial acetic acid, 0.05% w/v phosphoric acid, rinsed in 70% v/v ethanol, and dried, and radioactivity was measured in a liquid scintillation spectrometer. Blank values, in the absence of FYP peptide, were subtracted from each result.

**For IRS-1 immunoprecipitation, cells were cultured as described by Konstantopoulos and Clark (21). For insulin receptor β-subunit immunoprecipitation, cells were treated as described above and immunoprecipitated with monoclonal antibody 18-44 overnight at 4 °C. To detect tyrosine phosphorylation of either IRS-1 or insulin receptor β-subunit, membranes were probed with HRP-conjugated 4G10 (1:188).

**Measurements of Cell Growth Rate and Cell Attachment—**CHO cell lines were seeded at 10 × 103 cells in 24-well plates and incubated in α-MEM containing 10% v/v FCS. Cells from individual wells were washed twice with Versene (phosphate-buffered saline containing 0.2% EDTA), and attached cells were removed by incubation with 0.25% v/v trypsin/Versene for 3 min at 37 °C. Cells were counted (in triplicate) in a Coulter counter every 24 h over 5 days, and simultaneously, the Coulter counter measured the mean cell volume. For attachment assays plates were left uncoated or coated with 10 μg/ml fibronectin or 15 μg/ml rat tail collagen. Treated or untreated plates were blocked with 2 mg/ml BSA for 1 h at 37 °C and washed with phosphate-buffered saline prior to the addition of cells. Cells (10–30 × 103) were allowed to attach for either 2 or 24 h after plating in the presence of α-MEM containing 0.1% BSA.

**Actin Staining of CHO Cell Lines—**For filamentous actin localization, cells grown on coverslips were fixed in 4% formaldehyde in Tris-buffered saline for 8 min at 22 °C, rinsed in Tris-buffered saline, and permeabilized for 5 min in Tris-buffered saline containing 0.5% (v/v) Triton X-100. After washing in Tris-buffered saline, cells were incubated with rhodamine-phalloidin (0.1 μg/ml) for 20 min at 22 °C in a dark humidity chamber. After the final washes, the coverslips were
Cell Adhesion, FAK, Paxillin, and Mutant Insulin Receptors

**TABLE I**

| Cho | CHO-2N-10 (P5 to P10) | CHO-2N-10 |
|-----|------------------------|-----------|
| $R_K$ | $7.72 \times 10^3$ | $2.87 \times 10^5$ |
| $K_d$ | $6.95 \times 10^{-13} M$ | $9.13 \times 10^{-10} M$ |
| $K_a$ | $2.07 \times 10^{-6} M$ | $1.18 \times 10^{-6} M$ |

Expression of 2N cDNA in CHO Cells—Expression of a double point mutant of the insulin receptor (2N = K164N/K582N) was initially difficult to obtain, despite production of stable cell lines expressing either of the individual point mutations alone (Lys464 → Asn or Lys582 → Asn). To avoid overgrowth of 2N-expressing cells by non-2N-expressing cells, CHO-2N cells were simultaneously FACs sorted and single-cell cloned 10 days after transfection. This method provided eight independently derived clones expressing the 2N insulin receptor (up to 15 passages) which have been studied to varying degrees. One of the highest receptor-expressing clones (2N-10) was used in most of the studies, as its binding and phosphorylation characteristics (see below) were closest to those of CHO-T cells (overexpress wild-type human insulin receptors). The mutations in the external domain of the insulin receptor were of potential trypsin cleavage sites (23, 24) and mutated as part of another study (24). However, the unusual phenotype of the cells expressing the double point mutation (see below) led to the analysis presented here. In preliminary experiments, treatment of the cell lines (expressing mutant insulin receptors) with trypsin did not result in significant differences from CHO-T cells.

Insulin-binding Characteristics—The ability of two CHO.2N cell lines (clones 10 and 11) to bind insulin is shown in Fig. 1. The insulin binding of CHO.2N-10 and CHO.2N-11 cells was lower than that of CHO-T cells (clone 10 at passage 6, 9.8%, and clone 11 at passage 6, 13.2%, compared to wild-type, 42.0%, of total $125^I$-insulin added). In addition, $125^I$-insulin binding to CHO.2N-10 and 2N-11 cells decreased with prolonged passage, whereas the ability of CHO-T cells to bind $125^I$-insulin did not alter with passage. $125^I$-insulin binding always correlated with the level of insulin receptor expression measured by enzyme-linked immunoassay (data not shown). CHO.2N-10 cells express fewer insulin receptors per cell compared to CHO-T cells as determined by Scatchard analysis (Table I). The nontransfected parent CHO cells express approximately $3 \times 10^8$ hamster insulin receptors per cell and bind very low levels

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*2 M. Ginsberg and M. Hemler, personal communication.
*3 M. Ginsberg, personal communication.

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![Fig. 1. Specific $125^I$-Insulin binding to CHO.2N-10 and CHO.2N-11 cells with increasing passage number. $125^I$-Insulin binding to CHO.T (●), CHO (□), CHO.2N-10 (○), and CHO.2N-11 (▲) cells was measured over a number of cell passages as detailed under "Experimental Procedures." Results are expressed as a percentage of the total $125^I$-Insulin added and are the mean of triplicate determinations.](image-url)
of insulin (0.47% of total $^{125}$I-insulin added, see Fig. 1). The dissociation constants for insulin binding were comparable between the cell lines (Table I).

**Kinase Activity of CHO.2N Insulin Receptors**—The autophosphorylation of insulin-stimulated CHO.2N-10 insulin receptors (passages 6–10) was similar to wild-type insulin receptors (Fig. 2, lower panel) as measured by immunoblotting with anti-phosphotyrosine antibodies. Autophosphorylation of CHO.2N-10 receptors was stimulated 4.4-fold by insulin compared to 5.6-fold for wild-type receptors (Table I) in an in vitro phosphorylation assay. When the insulin-stimulated kinase activity of CHO.2N-10 and wild-type receptors toward a synthetic peptide (FYF, derived from the major autophosphorylation domain of the insulin receptor tyrosine kinase) was measured, phosphorylation was stimulated 20.1- and 19.9-fold, respectively (Table I). Insulin-stimulated phosphorylation of pp185/IRS-1 was also comparable between CHO.T and CHO.2N-10 (passage 6–8) cells (Fig. 2, upper panel).

**Cellular Morphology and Growth Characteristics**—CHO.2N-10 cells (as well as four other 2N clones) showed morphological differences which disappeared upon prolonged passage. These morphological changes were not dependent on cell density. Fig. 3A is a photomicrograph of CHO.2N-10 cells at passage 7; these cells formed a heterogenous monolayer with many large, flat cells containing large, granular nuclei. In contrast, the nontransfected, parental CHO cells (Fig. 3B) or CHO.T cells (not shown) grew as a homogenous monolayer of smaller cells. At late passages (>15 passages), CHO.2N-10 cells were indistinguishable from CHO cells (not shown). This reversion in morphology strongly correlated with the loss of insulin binding (Fig. 1) and insulin receptor expression of CHO.2N-10 cells at these later passages. Rhodamine-phalloidin staining of CHO.2N-10 cells at passage 9 shows the dense formation of actin stress fibers running the length of the mutant cells (Fig. 3C). In contrast, the nontransfected CHO cells (Fig. 3D) or CHO.T cells (not shown) stain intensely close to the plasma membrane region of the cells, which appears to be absent in the CHO.2N-10 cells (Fig. 3C).

The growth of CHO.2N cell lines, especially clone 10, appeared extremely slow compared to nontransfected CHO and wild-type CHO.T cells. However, the doubling time of CHO.2N-10 cells at all passages was similar to nontransfected CHO cells when corrected for the number of cells attached to dishes after 24 h (Fig. 4). In addition, there was a significant difference in the mean cell volume, with early passage CHO.2N-10 cells having a larger volume compared to CHO cells (1973 ± 42 fl compared to 1231 ± 42 fl, $p < 0.01, n = 5$) correlating with the observed differences in cell size (Fig. 3, A compared to B).

**Attachment of CHO.2N Cells to Tissue Culture Matrices**—The
apparent slower cell growth of CHO.2N-10 cells was explained by their inability to attach efficiently to tissue culture dishes at early passage. After 24 h (reflects both cell attachment and cell growth) CHO.2N-10 (passage 6) cell number was 31% of added cells compared to CHO.2N-10 cells at late passage (150% of added cells) or controls (140% of added cells) (Table II). This reduced ability to adhere to the tissue culture dishes was observed with six of the eight independently derived clones (data not shown). This was not due to differences in extracellular matrix production by early passage CHO.2N-10 cells as these cells still showed reduced attachment to matrix elaborated by CHO.T cells, and conversely CHO.T cells attached equally well to their own or a CHO.2N-10-elaborated matrix after 2 h (data not shown). In addition, early passage CHO.2N-10 cells attached less well to both fibronectin and type I collagen-coated plates after 2 h when compared to controls (Table II). Viability studies showed that the cells which did not attach to tissue culture plates were still viable (83 ± 4%), as determined by trypan blue exclusion.

Focal Adhesion Kinase (pp125FAK) and Other Focal Adhesion Proteins—Focal adhesions are cellular regions that, in vitro, provide contact points for cells with tissue culture dishes. They contain, among other proteins, a cytoplasmic tyrosine kinase, pp125FAK, which is phosphorylated upon cell attachment (32).

CHO.2N-10 cells at early passage have a significant reduction in tyrosine phosphorylation of pp125FAK in the absence of insulin (42.5 ± 9.7% of CHO cells, Figs. 5, panel A, and 6, upper panel). This difference held whether sample loading was corrected for either cell number (Fig. 5, panel A) or cell protein content (data not shown). It is interesting to note that expression of the wild-type insulin receptor in CHO cells (CHO.T) resulted in a consistent but not significant increase of pp125FAK tyrosine phosphorylation (168.9 ± 30.0%) compared to nontransfected CHO cells (Fig. 5, panel A). Focal adhesion kinase protein levels were comparable between CHO, CHO.T, and CHO.2N-10 cells (passages 5–8) (100 ± 12%, 77.7 ± 3.4%, and 77.1 ± 4.9%). We also measured pp125FAK tyrosine phosphorylation as a function of adhesion to fibronectin in all cell lines as cell attachment to fibronectin often leads to an increase in pp125FAK phosphorylation (32). However, none of the cell lines showed a further increase in pp125FAK phosphorylation following 2 h of plating onto fibronectin when compared to plating on noncoated dishes (data not shown). Alteration to the basal level of pp125FAK phosphorylation could result from the action of a phosphatase, we therefore preincubated CHO.2N cells with the tyrosine phosphatase inhibitor pervanadate (1 or 10 μM) for 1 h at 37 °C. The basal level of pp125FAK phosphorylation in CHO.2N cells increased significantly toward that of CHO.T cells (Fig. 5, panel B).

Recently, two laboratories have shown that insulin, unlike other growth factors, stimulates dephosphorylation of pp125FAK in cells overexpressing wild-type insulin receptors (19, 20). We have also examined the effect of insulin on pp125FAK in CHO, CHO.T, and CHO.2N-10 cells. Insulin decreased pp125FAK tyrosine phosphorylation in both CHO.T and CHO.2N-10 (passages 7 and 8) cells (Fig. 5, panels C and D) when compared to basal pp125FAK tyrosine phosphorylation (27.9 ± 7.9% and 52.2 ± 10.0% of nonstimulated cells, respectively). CHO cells which express only endogenous hamster insulin receptors showed negligible dephosphorylation of pp125FAK in response to insulin (91.0 ± 16.5%, Fig. 5, panels C and D). The protein levels of pp125FAK were not altered in response to insulin stimulation (data not shown). Results were similar whether cells were tested in complete medium or after 18 h of serum starvation.

Paxillin, a structural protein, is also phosphorylated on tyrosine residues, usually (15) but not always (33, 34) in parallel to pp125FAK. In our studies, in contrast to the decrease in pp125FAK tyrosine phosphorylation, there was no consistent change in paxillin phosphorylation (Fig. 6, middle panel) in CHO.2N-10 cells (passages 7–10). Over a number of experiments, less paxillin protein was immunoprecipitated from CHO.2N-10 cells (61.0 ± 10.4%, p < 0.01, n = 7) when compared to CHO and CHO.T cells. However, the ratio of paxillin phosphorylation to paxillin protein levels was similar for all

![Graph showing the growth curves of CHO.T, CHO, and CHO.2N-10 cells.](image)

**FIG. 4.** Growth curves of CHO.T, CHO, and CHO.2N-10 cells. CHO cell lines, initially plated at 10 × 10^4/dish were removed and counted every 24 h as described under “Experimental Procedures.” The number of cells present at each time point is expressed as a percentage of the 24-h count and is the mean ± S.E. of triplicate determinations. The S.E. at some time points is contained by the symbol. □, CHO.T; ▣, CHO; ●, CHO.2N-10 (passage 5); and ○, CHO.2N-10 (passage 19).

| Cell type (and passage number) | After 24 h | After 2 h |
|-------------------------------|-----------|----------|
|                               | Control   | Collagen |
|                               | ×10^3     |          |
| CHO                           | 14.2 ± 1.1* | 3.7 ± 1.4 |
| CHO.T                         | 14.0 ± 1.4  | 3.0 ± 0.8 |
| CHO.2N-10 (P6)                | 3.1 ± 0.3*  | 9.4 ± 0.5* |
| CHO.2N-10 (P22)               | 14.6 ± 1.3  | 30.2 ± 3.7 |

* Cells were seeded at 10 × 10^4/ml in 24-well plates, and after 24 h cells were counted as described.

† Cells were seeded at 30 × 10^3/ml in 24-well plates coated as described under “Experimental Procedures,” and after 2 h attached cells were counted.

All results are expressed as the mean of triplicate determinations ± standard error of the mean, and are representative of five separate experiments. *p < 0.05 when compared with CHO, CHO.T, and late passage CHO.2N-10 cells.
CHO.2N cells in the absence of pervanadate.

d) levelsin unstimulated CHO.T and CHO.2N-10 (passages 7–11) cells. Cellswereserum-starvedfor 18 h with
a serum starvation (0.5% FCS or 0.1% BSA). Phosphotyrosine and protein levels are expressed as a percentage of the CHO cells and are the mean ± S.E. of at least four separate experiments. **p < 0.01 when compared to CHO.2N cells and other CHO cell lines (Fig. 3, due to the gross differences in cell size between early passage cell lines. Paxillin binds to the rod domain of vinculin, another structural focal adhesion protein concentrated at the sites of cell-extracellular matrix adhesions involving integrins (35). Vinculin levels were also comparable between CHO, CHO.T, and CHO.2N-10 (passages 7 and 8) cells (Fig. 6, lower panel).

In order to estimate the relative number of focal contacts for each cell line we examined the cellular distribution of vinculin by indirect immunofluorescence. Quantitation was not possible due to the gross differences in cell size between early passage CHO.2N cells and other CHO cell lines (Fig. 3, A compared to B); however, no overall difference in cellular localization of vinculin was observed (data not shown). We also attempted to estimate the number of focal adhesions present in the adherent cells by immunolocalizing the focal adhesion and membrane ruffle marker, talin. Talin has not only been shown to be indirectly linked with pp125FAK (13) but also linked directly to the $\beta_1$ cytoplasmic portion of integrin (36, 37). CHO cell lines did not exhibit well developed focal contacts (also reported by Bauer et al., (38)) but rather showed an intense staining for talin around the periphery of the cell. This pattern of staining was not significantly different between CHO.2N, CHO.T, and CHO cells (data not shown).

Altemations to the expression of integrins (receptors for extracellular matrix proteins) could provide an explanation for the morphological changes in CHO.2N cells. We investigated any gross changes to the level or type of integrin expression in CHO.2N cells by FACS analysis. CHO cells endogenously express significant levels of $\alpha_5$ and $\beta_1$ integrins, and lower levels of $\alpha_5$, $\alpha_r$, and $\beta_4$ integrins. In general, no change to this pattern of expression was observed in CHO.2N cells compared with the other cell lines; however, there was a trend for an increase in the mean fluorescence of $\alpha_5$ integrins (319.8 ± 91.5%, n = 5) when compared to CHO.2N cells (181.3 ± 4.5% and 175.4 ± 16.2%, respectively, n = 5) (Fig. 7), but this did not reach statistical significance due to the larger standard error of the CHO.2N cells as a result of their heterogeneity. It was interesting to note the 2–3-fold increase ($p < 0.05$, n = 3) in expression of $\beta_4$ integrins in cell lines which overexpress insulin receptor constructs (CHO.T and CHO.2N) compared to CHO cells (Fig. 7).

DISCUSSION

We have shown that mutation of two potential tryptic cleavage sites (23) (lysines 164 and 582 to asparagine, CHO.2N) in
the insulin receptor α-subunit results in a cell line with altered morphology associated with an increase in cell size, a decrease in cell adhesiveness, a decrease in protein levels of paxillin, and a decrease in tyrosine phosphorylation of focal adhesion kinase. These features correlate with expression of the mutant 2N insulin receptor. The cells revert to normal CHO phenotype at later passages, at which time insulin binding and receptor expression returns to that of nontransfected CHO cells, presumably because the cells lose the capacity to express the mutant insulin receptor. However, decreased CHO.2N cell adhesion was not associated with altered insulin receptor functions, including insulin binding, receptor kinase activity, or IRS-1 phosphorylation, nor was it associated with changes to the structural focal adhesion protein, vinculin, or gross alterations in integrin expression. While a complete survey of focal adhesion and cytoskeletal proteins has not been conducted, the reduction of pp125FAK phosphorylation and of paxillin protein in CHO.2N cells and the known correlation of these proteins with focal contact formation (14, 39, 40) suggest changes to focal contacts formed by CHO.2N cells may impair their ability to interact initially with a substratum. Overall, these results support a role for the insulin receptor in modulating cytoskeletal interactions and cell adhesion.

The signals generated as a result of pp125FAK activation are currently unknown but it is apparent that autophosphorylation of pp125FAK results in co-association of signaling molecules such as Src, PI 3-kinase, and GRB2, in addition to proteins such as paxillin which, in turn, binds C-terminal Src kinase activity, or IRS-1 phosphorylation, nor was it associated with changes to the structural focal adhesion protein, vinculin, or gross alterations in integrin expression. While a complete survey of focal adhesion and cytoskeletal proteins has not been conducted, the reduction of pp125FAK phosphorylation and of paxillin protein in CHO.2N cells and the known correlation of these proteins with focal contact formation (14, 39, 40) suggest changes to focal contacts formed by CHO.2N cells may impair their ability to interact initially with a substratum. Overall, these results support a role for the insulin receptor in modulating cytoskeletal interactions and cell adhesion.

PP3-kinase is a major signaling molecule activated following growth factor stimulation and has been implicated in pathways leading to cytoskeletal rearrangements, possibly upstream of Rac (5, 43). The interaction of PI 3-kinase with pp125FAK can be stimulated in vivo by either PDGF (18) or cell adhesion (44) concomitant with pp125FAK activation; however, no increase in pp125FAK-associated PI 3-kinase was seen in insulin-stimulated cells (18). Recently, it has been shown that blocking PI 3-kinase activity with wortmannin inhibits PDGF-stimulated pp125FAK phosphorylation (45). While wortmannin and mutated p85 subunits of PI 3-kinase activity with wortmannin inhibits PDGF-stimulated pp125FAK phosphorylation (45). While wortmannin and mutated p85 subunits of PI 3-kinase block insulin-stimulated membrane ruffling (5), our preliminary data show that wortmannin (100 nM) had no effect on the ability of insulin to stimulate dephosphorylation of pp125FAK. Surprisingly in CHO.2N-10 cells, in the absence of insulin, a consistent increase in pp125FAK phosphorylation was observed after 10 min of incubation with wortmannin, implying some interaction between PI3-kinase and pp125FAK in these cells. Further investigations are under way to examine this linkage.

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commonly linked with insulin signaling and modulation of their activity, by various means, may either enhance (LAR (46) and PTP1B (47)) or block (Syk (20)) insulin action. Phosphatases have also been linked with cytoskeletal activities and, for instance, changes in cell density (which affect the cytoskeleton) also lead to changes in the level of LAR, PTP1B, and Syk (48). Syk, with two phosphotyrosine SH2 binding domains, has been shown to bind directly to both phosphorylated insulin receptor and IRS-1 (49) and, when overexpressed in CHO cells, a dominant negative Syk mutant induced a significant increase in basal pp125FAK phosphorylation (20). Furthermore, LAR has been localized to focal adhesions with a possible involvement in focal adhesion disassembly (50). Our initial data which shows that the tyrosine phosphatase inhibitor pervanadate can increase the basal phosphorylation of pp125FAK in CHO.2N cells is suggestive of phosphatase involvement. A study to isolate putative "pp125FAK" phosphatases is in progress.

The structural focal adhesion protein, paxillin, is closely linked with pp125FAK via an association site in the C terminus of pp125FAK. It is thought that paxillin is an in vivo substrate of pp125FAK (2), and the phosphorylation of these two proteins usually increases and decreases in concert. However, in CHO.2N-10 cells, despite decreased basal phosphorylation of pp125FAK, there was no parallel decrease in paxillin phosphorylation demonstrating that the two events are not always linked. A similar result was observed in phorbol 12-myristate 13-acetate down-regulated cells (33) and pp125FAK-deficient mice (34). Little is known about the overall mechanism for focal contact formation and the contribution of various structural proteins such as paxillin. In this context, we observed that the level of paxillin protein is decreased by 40% in CHO.2N-10 cells could explain (at least in part) the decreased ability of these cells to adhere, despite no alteration to the level of vinculin.

The mechanism whereby a double point mutation (K164N/K582N) in the external domain of the insulin receptor leads to the observed cell phenotype is unknown. One possibility is that the mutation results in a conformational change in the receptor's cytoplasmic domain such that a signal transduction pathway involved in cell architecture is modulated in the absence of insulin. Truncation of the insulin receptor C terminus and more specifically, mutation of tyrosines 1328 and 1334 in this region abolished the insulin responsive dephosphorylation of pp125FAK, suggesting that the C terminus and in particular phosphorylation of these two tyrosine residues is critical for insulin effects on pp125FAK phosphorylation (19). In addition, this region of the receptor and in particular Tyr1328 and Tyr1334 has been linked to Syk activation by insulin (51), and also to Shc (52), p85 subunit of PI-3-kinase (53), and Grb10 (54) association. Previous studies have demonstrated that insulin binding to its receptor induces a conformational change in the receptor's cytoplasmic domain such that a signal transduction pathway involved in cell architecture is modulated in the absence of insulin. Truncation of the insulin receptor C terminus and more specifically, mutation of tyrosines 1328 and 1334 in this region abolished the insulin responsive dephosphorylation of pp125FAK, suggesting that the C terminus and in particular phosphorylation of these two tyrosine residues is critical for insulin effects on pp125FAK phosphorylation (19). In addition, this region of the receptor and in particular Tyr1328 and Tyr1334 has been linked to Syk activation by insulin (51), and also to Shc (52), p85 subunit of PI-3-kinase (53), and Grb10 (54) association. Previous studies have demonstrated that insulin binding to its receptor induces a conformational change in the receptor's cytoplasmic domain such that a signal transduction pathway involved in cell architecture is modulated in the absence of insulin. Truncation of the insulin receptor C terminus and more specifically, mutation of tyrosines 1328 and 1334 in this region abolished the insulin responsive dephosphorylation of pp125FAK, suggesting that the C terminus and in particular phosphorylation of these two tyrosine residues is critical for insulin effects on pp125FAK phosphorylation (19). In addition, this region of the receptor and in particular Tyr1328 and Tyr1334 has been linked to Syk activation by insulin (51), and also to Shc (52), p85 subunit of PI-3-kinase (53), and Grb10 (54) association. Previous studies have demonstrated that insulin binding to its receptor induces a conformational change in the receptor's cytoplasmic domain such that a signal transduction pathway involved in cell architecture is modulated in the absence of insulin. Truncation of the insulin receptor C terminus and more specifically, mutation of tyrosines 1328 and 1334 in this region abolished the insulin responsive dephosphorylation of pp125FAK, suggesting that the C terminus and in particular phosphorylation of these two tyrosine residues is critical for insulin effects on pp125FAK phosphorylation (19). In addition, this region of the receptor and in particular Tyr1328 and Tyr1334 has been linked to Syk activation by insulin (51), and also to Shc (52), p85 subunit of PI-3-kinase (53), and Grb10 (54) association. Previous studies have demonstrated that insulin binding to its receptor induces a conformational change in the receptor's cytoplasmic domain such that a signal transduction pathway involved in cell architecture is modulated in the absence of insulin. Truncation of the insulin receptor C terminus and more specifically, mutation of tyrosines 1328 and 1334 in this region abolished the insulin responsive dephosphorylation of pp125FAK, suggesting that the C terminus and in particular phosphorylation of these two tyrosine residues is critical for insulin effects on pp125FAK phosphorylation (19). In addition, this region of the receptor and in particular Tyr1328 and Tyr1334 has been linked to Syk activation by insulin (51), and also to Shc (52), p85 subunit of PI-3-kinase (53), and Grb10 (54) association. Previous studies have demonstrated that insulin binding to its receptor induces a conformational change in the receptor's cytoplasmic domain such that a signal transduction pathway involved in cell architecture is modulated in the absence of insulin. Truncation of the insulin receptor C terminus and more specifically, mutation of tyrosines 1328 and 1334 in this region abolished the insulin responsive dephosphorylation of pp125FAK, suggesting that the C terminus and in particular phosphorylation of these two tyrosine residues is critical for insulin effects on pp125FAK phosphorylation (19).
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