c-Crk, a Substrate of the Insulin-like Growth Factor-1 Receptor Tyrosine Kinase, Functions as an Early Signal Mediator in the Adipocyte Differentiation Process*

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Determination of 3T3-L1 preadipocytes into adipocytes is induced by a combination of inducers, including a glucocorticoid, an agent that elevates cellular cAMP, and a ligand of the insulin-like growth factor-1 receptor. Previous studies have implicated protein-tyrosine phosphatase (PTPase) HA2, a homologue of PTPase 1B, in the signaling cascade initiated by the differentiation inducers. Vanadate, a potent PTPase inhibitor, blocks adipocyte differentiation at an early stage in the program, but has no effect on the mitotic clonal expansion required for differentiation. Exposure of preadipocytes to vanadate along with the inducing agents led to the accumulation of pp35, a phosphotyrosyl protein that is a substrate for PTPase HA2. pp35 was purified to homogeneity and shown by amino acid sequence and mass analyses of tryptic peptides to be c-Crk, a known cytoplasmic target of the insulin-like growth factor-1 receptor tyrosine kinase. Transfection of 3T3-L1 preadipocytes with a c-Crk antisense RNA expression vector markedly reduced c-Crk levels and prevented differentiation into adipocytes. Studies with C3G, a protein that binds to the SH3 domain in c-Crk, showed that phosphorylation of c-Crk rendered the SH3 domain inaccessible to C3G. Taken together, these findings indicate that locking c-Crk in the phosphorylated state with vanadate prevents its participation in the signaling system that initiates adipocyte differentiation.

Adipocytes serve an important function in the energy economy of higher organisms, providing a large energy reserve that can be mobilized when needed. Thus, when caloric intake exceeds expenditure, metabolite flux is diverted into triglyceride synthesis for storage in adipocytes. Conversely, when caloric expenditure exceeds intake, this triglyceride reserve is mobilized as free fatty acids to provide physiological fuel for use by other tissue/cell types. The need for an energy reserve begins at birth when the newborn must be prepared to survive periods of energy deprivation. Adipocytes, which provide this reserve, develop late in embryonic life, with major expansion of this cell population occurring after birth. The adipose lineage arises from the same multipotent stem cells of mesodermal origin that give rise to the muscle and cartilage lineages.

Established preadipocyte cell lines, e.g., the 3T3-L1 preadipocytes, which can be induced to differentiate into adipocytes in cell culture, provide faithful models with which to investigate the adipocyte differentiation program (1–6). When exposed to the appropriate differentiation inducers, including IGF-1 (or insulin at a non-physiologically high concentration), dexamethasone (a glucocorticoid), and 1-methyl-3-isobutylxanthine (MIX; a CAMP phosphodiesterase inhibitor that increases intracellular cAMP), 3T3-L1 preadipocytes differentiate into cells that express the adipocyte phenotype (6). Induction of the adipocyte differentiation program involves at least three different signal transduction systems, including those mediated by the glucocorticoid receptor, the cAMP-dependent protein kinase, and the IGF-1 receptor (6–10). Activation of these pathways triggers the sequential expression of a group of transcription factors (9–16), including members of the C/EBP family (C/EBPα, -β, and -δ) and peroxisomal proliferator-activated receptor-γ, leading to the coordinate transcriptional activation of a large number of adipocyte genes that produce the differentiated phenotype. Once adipocyte gene expression has been initiated, further stimulation with differentiation inducers is no longer required.

Although the transcriptional activation of adipocyte-specific genes during differentiation has been studied intensively (17–24), far fewer studies have been conducted on the signal transduction pathways by which the differentiation inducers act. Upon exposure to these inducers, confluent growth-arrested preadipocytes synchronously reenter the cell cycle and undergo approximately two rounds of mitosis (25), referred to as “mitotic clonal expansion.” Mitotic clonal expansion is required for progression through subsequent steps in the differentiation program (26, 27). It appears that DNA replication and chromatin remodeling during mitotic clonal expansion render cis-ele-

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1 The abbreviations used are: IGF-1, insulin-like growth factor-1; MIX, 1-methyl-3-isobutylxanthine; C/EBP, CCAAT/enhancer-binding protein; PTPase, protein-tyrosine phosphatase; DMEM, Dulbecco’s modified Eagle’s medium; FPLC, fast protein liquid chromatography; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PAO, phenylarsine oxide; PIF, protexase inhibitor mixture; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
ments accessible to the trans-acting factors that activate (or derepress) transcription of genes critical to progression of the differentiation program (9).

The requirement of IGF-1 (or a high level of insulin, which can also activate the IGF-1 receptor) as a differentiation inducer for 3T3-L1 preadipocytes implicated tyrosine phosphorylation in the induction process, as the IGF-1 receptor is a ligand-activated tyrosine kinase (8). Previously, we showed that the expression of PTPase HA2 is both regulated during and required for differentiation of 3T3-L1 preadipocytes (28). The expression of PTPase HA2 increases dramatically for 2 days following induction of differentiation and then decreases (28). Furthermore, constitutive overexpression of PTPase HA2 by 3T3-L1 preadipocytes transfected with a PTPase HA2 expression vector blocks differentiation. Importantly, however, exposure of the transfected preadipocytes to vanadate (a potent PTPase inhibitor) at the time when the endogenous PTPase is normally down-regulated during differentiation, i.e. following clonal expansion, fully restores their capacity to differentiate into adipocytes (28). Exposure to vanadate at any other time during the differentiation program, however, fails to restore differentiation. Moreover, inhibition of PTPase HA2 activity with vanadate in untransfected 3T3-L1 cells (between days 0 and 2 of the standard differentiation protocol) also blocks differentiation (28). Taken together, these findings suggest that fluctuation of PTPase HA2 activity early in the differentiation program is both regulated during and required for adipocyte differentiation. Thus, it appears that a protein, phosphorylated by the IGF-1 receptor, is generated early in the program and is subsequently dephosphorylated by PTPase HA2. Conceivably, the coordinated sequential actions of the IGF-1 receptor tyrosine kinase and PTPase HA2 generate a signal required for the induction process.

In this work, we provide evidence that c-Crk (cellular CT10 regulator of kinase), a bona fide substrate of the IGF-1 receptor tyrosine kinase, functions “early” in the induction of adipocyte differentiation. Our results suggest that signaling, initiated by IGF-1 and mediated by c-Crk, involves tyrosine phosphorylation, followed by dephosphorylation. It appears that in the phosphorylated state, the SH3 domain of c-Crk is blocked due to an intramolecular interaction of the SH2 domain with the phosphotyrosyl group, thereby preventing its interaction with a putative downstream signaling molecule. Presumably, dephosphorylation by PTPase HA2 would allow this interaction to occur at the appropriate time in the differentiation program.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-phosphotyrosine antibody was purchased from Upstate Biotechnology, Inc. Anti-Crk antibody was from Transduction Laboratories. Anti-CT10 antibody was from Santa Cruz Biotechnology, Inc. Horseradish peroxidase-conjugated secondary antibody, tetramethylrhodamine B isothiocyanate-conjugated secondary antibody, sodium vanadate, dexamethasone, 1-methyl-3-isobutylxanthine, and insulin were purchased from Sigma. Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Life Technologies, Inc., and the Bio-Scale DEAE 10 column was from Bio-Rad. The HitTrap heparin column (1 ml), the Resource-Q column (1 ml), the Superose 12 column (HR 10/30), and the FPLC unit were from Amersham Pharmacia Biotech.

**Cell Culture, Differentiation, and Vanadate Treatment of 3T3-L1 Preadipocytes—**3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% calf serum and allowed to reach confluence. Differentiation of 2-day post-confluent preadipocytes (designated as day 0) was initiated with 1 µg/ml insulin, 1 µM dexamethasone, and 0.5 mM MIX in DMEM supplemented with 10% fetal bovine serum (6, 29). After 48 h (day 2), the culture medium was replaced with DMEM supplemented with 10% fetal bovine serum and 1 µg/ml insulin, and the cells were then fed every other day with DMEM containing 10% fetal bovine serum. Cytoplasmic triglyceride droplets were visible by day 4, and cells were fully differentiated by day 8.

For vanadate treatment, 20 mM sodium vanadate was added to the culture medium along with MIX, dexamethasone, and insulin on day 0.

For analysis, cells (usually containing 10–50 µg of protein) were subjected to SDS-PAGE and then transferred to Immobilon-P membrane (Millipore Corp.). After blocking with 5% nonfat dried milk in 1× Tween/Tris-buffered saline containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Tween, and 0.01% sodium azide, membranes were incubated with primary antibody for 2 h at room temperature. Membranes were washed with cold PBS supplemented with 0.1% Tween and 0.1% sodium dodecyl sulfate and then exposed to horseradish peroxidase-conjugated secondary antibody for 45 min. Target proteins were visualized by enhanced chemiluminescence (ECL).

**Dephosphorylation of the Phosphotyrosyl Protein pp35 by Protein-Tyrosine Phosphatase HA2 from 3T3-L1 Cell—**Unless otherwise indicated, all operations were carried out at 4 °C. 3T3-L1 preadipocyte extract containing PTPase HA2, a PTPase inhibitor (28), was prepared as described previously (33). In brief, 3T3-L1 preadipocyte monolayers were washed twice with cold PBS; scraped from the culture dishes; and then fixed for 2 min with 3.7% formaldehyde in PBS. Oil red O (0.5%) in isopropyl alcohol was diluted with 1.5 volumes of water, filtered, and added to the fixed cell monolayers for 1 h at room temperature. Cell monolayers were then washed with water, and the stained triglyceride droplets in the cells were visualized and/or photographed.

**Preparation of Cell Extracts and SDS-PAGE—**For analysis of tyrosine-phosphorylated proteins, cell monolayers from cells treated as described in the figure legends were washed three times with cold PBS supplemented with 0.1 mM sodium vanadate. Cells were then scraped from the plates into hypotonic buffer containing 10 mM Hepes (pH 7.0), 2 mM MgCl2, 15 mM KCl, 0.1 mM phenylarsine oxide (PAO; a PTPase inhibitor) (34), 2 mM sodium vanadate, 5 mM sodium pyrophosphate, 0.5 mM sodium metaphosphate, 0.1 mM Na3VO4, and then homogenized with a glass homogenizer. Cellular membranes and cytosol were separated by centrifugation of the cell lysate at 150,000 × g for 45 min at 4 °C. Membranes were then extracted with 10 mM Hepes (pH 7.0), 0.5 mM DTT, 0.1 M NaCl, 0.1 mM sodium vanadate, 1 mM PMSF, and 2 µl/ml PIC1 and PIC2 for 1 h at 4 °C. The final extract was centrifuged at 150,000 × g for 45 min at 4 °C. The supernatant is referred to as the solubilized membrane extract, and the pellet as the insoluble membrane fraction. The total cell extract was prepared by washing the cell monolayers with cold PBS as described above, followed by lysis with 1× boiling Laemmli SDS sample buffer (36) containing 20 mM dithiothreitol, 0.1 mM PMSF, and 1 mM sodium vanadate. The cell lysate was then heated at 100 °C for 5 min.

For analysis, cells (usually containing 10–50 µg of protein) were subjected to SDS-PAGE and then transferred to Immobilon-P membrane (Millipore Corp.). After blocking with 2% nonfat dried milk in 1× Tween/Tris-buffered saline containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween, and 0.001% Merthiolate for 2 h at room temperature, membranes were incubated with primary antibody for 2 h at room temperature, followed by horseradish peroxidase-conjugated secondary antibody for 45 min. Target proteins were visualized by enhanced chemiluminescence (ECL).
buffer and extracted with 20 ml Heps (pH 7.0), 1% Triton X-100, 0.3 M NaCl, 0.1 mM EDTA, 2 mM DTT, 0.1 mM PMSF, and 2 l/ml PIC1 and PIC2. After rotating for 1 h, the membrane suspension was centrifuged at 150,000 × g for 45 min, and the supernatant containing the PT-Pase HA2 activity was collected. Partial purification of PT-Pase HA2 was achieved by hydroxylapatite and DEAE ion-exchange chromatography (33).

The pp35 protein for use as substrate was prepared from day 2 cells after hormonal stimulation and vanadate treatment. In brief, 2-day post-confluent 3T3-L1 preadipocytes were treated with 1 μg/ml insulin, 1 μM dexamethasone, 0.5 mM MIX, and 20 μM sodium vanadate for 2 days, during which cell monolayers were washed twice with PBS, scraped from the culture dishes into hypotonic buffer containing 10 ml Heps (pH 7.0), 15 mM KCl, 2 mM MgCl2, 0.1 mM PAO, 1 mM PMSF, and 2 μM/ml PIC1 and PIC2; and homogenized. The cytosolic fraction was separated by centrifugation at 150,000 × g for 45 min. Vanadate was omitted from the hypotonic buffer since it interferes with the PT-Pase assay, the high concentration of DTT in the dephosphorylation assay neutralized the PAI.

Dephosphorylation of pp35 by PT-Pase HA2 was followed using the PT-Pase HA2 activity assay described previously (33). The PT-Pase HA2 preparation was incubated with pp35 for the indicated times at 30 °C in a reaction mixture containing 50 mM PIPES (pH 6.5), 1 mM EDTA, and 5 mM DTT. The reaction was terminated by adding 3-fold concentrated Laemmli SDS sample buffer. The amount of pp35 protein was quantitated by SDS-PAGE and Western immunoblotting with anti-phosphotyrosine antibody as described above.

Purification of pp35 from 3T3-L1 Cells—650 10-cm cell monolayers, treated with 1 μg/ml insulin, 1 μM dexamethasone, 0.5 mM MIX, and vanadate (20 μM on day 1 and an additional 15 μM on day 2 to maximize pp35 accumulation) for 2 days, were washed twice with cold PBS containing 0.1 mM vanadate; scraped from the culture dishes; resuspended in hypotonic buffer containing 10 ml Heps (pH 7.0), 2 mM MgCl2, 15 mM KCl, 0.1 mM PAO, 1 mM PMSF, and 2 μM/ml PIC1 and PIC2; and homogenized using a glass homogenizer. After centrifugation at 150,000 × g for 45 min, the supernatant (~500 ml) was retained, and proteins were precipitated by bringing the solution to 60% saturation with solid ammonium sulfate. After stirring for 15 min, and the supernatant was discarded. The protein pellet was redissolved in 80 ml of 20 ml Tris-HCl (pH 7.5), 10 mM NaCl, 0.1 mM vanadate, 0.1 mM PAO, 0.1 mM PMSF, 1 mM EDTA, and 2 μM/ml PIC1 and PIC2 (Buffer A). The solubilized proteins were then dialyzed against the same buffer for 3 h at 4 °C. After removal of undissolved proteins by centrifugation, in 5 min, 90% Buffer A and 10% Buffer B (0.052% trifluoroacetic acid and 80% acetonitrile) was added, and the mixture was centrifuged at 12,000 × g for 15 min, and the supernatant was discarded. The protein pellet was redissolved in 80 ml of 20 ml Tris-HCl (pH 7.5), 10 mM NaCl, 0.1 mM vanadate, 0.1 mM PAO, 0.1 mM PMSF, and 1 mM EDTA (Buffer A), were added to a two-step elution: 15 ml of 200 mM NaCl in Buffer A, followed by a linear gradient of 200–500 mM NaCl (85 ml) in the same buffer at a flow rate of 2 ml/min. Twenty-five fractions (4 ml/fraction) were collected. The eluted fractions were monitored for pp35 by Western blotting with anti-phosphotyrosine antibody as described above. Protein concentration was determined using the Lowry method. After this step, proteins were reduced to ~3.6 mg, with a pp35 yield of ~23%.

Following Bio-Scale DEAE 10 FPLC, HiTrap heparin FPLC and Resource-Q FPLC were used to further separate pp35. pp35-containing fractions from the Bio-Scale DEAE 10 chromatography were pooled; buffer-exchanged to a column loading buffer containing 20 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.1 mM vanadate, 0.1 mM PAO, 0.1 mM PMSF, and 2 μM/ml PIC1 and PIC2; and then applied to a HiTrap heparin affinity column pre-equilibrated with the same buffer at flow rate of 0.25 ml/min. The flow-through fraction containing all of the pp35 was immediately applied to a Resource-Q column equilibrated with the same buffer. After washing the column with 5 ml of the equilibration buffer, proteins were eluted with a 20-ml linear gradient of 200–500 mM NaCl in Buffer B, followed by a linear gradient of 200–500 mM NaCl (85 ml) in the same buffer at a flow rate of 2 ml/min. Twenty-five fractions (4 ml/fraction) were collected. The eluted fractions were monitored for pp35 by Western blotting with anti-phosphotyrosine antibody as described above. After this step, proteins were reduced to ~3.6 mg, with a pp35 yield of ~23%.

The first chromatographic step was Bio-Scale DEAE 10 FPLC. The above solubilized proteins (~90 mg of proteins were loaded onto the column each time) were applied to a 10-ml column equilibrated with Buffer A (0.5 mM NaCl, 0.5 mM PIC1 and PIC2; and then applied to a HiTrap heparin column loading buffer containing 20 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.1 mM vanadate, 0.1 mM PAO, 0.1 mM PMSF, and 2 μM/ml PIC1 and PIC2; and then applied to a HiTrap heparin column. The protein in the column was reduced to 20% Buffer A and 80% Buffer B, and eluted at a flow rate of 0.2 ml/min. Peptides were eluted at a flow rate of 0.2 ml/min with a two-step gradient: first, 100% Buffer A to 90% Buffer A and 10% Buffer B (0.052% trifluoroacetic acid and 80% acetonitrile); second, 90% Buffer A and 10% Buffer B to 20% Buffer A and 80% Buffer B in 100 min. The effluent was monitored for UV absorbency at 214 nm, and the fractions were collected manually. Three peptide peaks (eluting at 42, 52, and 56 min) were subjected to matrix-assisted laser desorption ionization mass spectral analysis and Edman amino acid sequencing.

Four tryptic peptide sequences were obtained from these three peptide peaks: peptide 1, SSWSQDQWVWNNESDGK; peptide 3, GMIPVPYVEK; and peptide 4, LLDQQNPDEDFS. Peptides 2 and 4 were each from a single peak. The amino acid assignments for these two peptides were verified by the mass spectral analysis. Peptides 1 and 3 were from one reverse-phase chromatographic peak. Owing to the large differences in the amounts of these two peptides, the amino acid sequences for both peptides could be readily deduced from each Edman degradation cycle. The amino acid sequences of the four peptides were used to search the GenBankTM protein sequence data base. The computer search for sequence similarities of these four peptides revealed a 100% amino acid sequence match with the mouse proto-oncogene product c-Crk, an adapter molecule. The results from matrix-assisted laser desorption ionization mass spectral analysis of these four tryptic peptides corresponded exactly with the masses of predicted tryptic cleavage products of mouse c-Crk. The sequence of peptide 4 matched that of the C terminus of c-Crk, explaining why this peptide did not end with Arg or Lys. This purified pp35 was also immunoblotted by anti-Crk antibody on a Western blot (see Fig. 7C, panel I). Taken together, these results provide strong evidence that pp35 is the tyrosine-phosphorylated form of mouse c-Crk, a known substrate of the IGF-1 receptor tyrosine kinase (39, 40).

**Construction of and Transfection with Crk Antisense and Sense RNA Expression Vectors**—Two pBCMGneo expression vectors (18, 28) were constructed: pBCMGneo-Crk/Antisense, with a 200-base pair Crk antisense fragment (from the BamHI site in the 5’-untranslated region to the XhoI site in the coding region) (37) inserted in the antisense orientation, and pBCMGneo-Crk/Sense, with the same fragment inserted in...
the sense orientation. These vectors were transfected into 30% confluent low-passage 3T3-L1 preadipocytes using the calcium phosphate precipitation method (38) to generate stably transfected cell lines. Briefly, 20 μg of pBGMneo-Crk/Antisense or pBGMneo-Crk/Sense vector DNA in a 250 mM CaCl2 solution were added to an equal volume solution containing 250 μM NaCl, 50 mM Hepes (pH 7.12), and 1.5 mM Na2HPO4 to form DNA/calcium phosphate coprecipitates, and the mixture was then added directly to the culture medium. After 8 h at 37 °C in the CO2 incubator, cells were shocked with 10% dimethyl sulfoxide and PBS for 3 min and then returned to the incubator for 24 h in fresh DMEM containing 10% calf serum. G418 was added to select the neomycin-resistant cells. The antibiotic-resistant foci were isolated and propagated to generate stable cell lines for further analysis.

**Immunoprecipitation**—3T3-L1 cell monolayers (10-cm plate) treated as described in the figure legends were washed twice with ice-cold PBS and lysed in 1 ml of 1% Triton X-100 buffer containing 50 mM Hepes (pH 7.4), 2.5 mM EDTA, 150 mM NaCl, 30 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and 2 μg/ml PCh and PCl2. The lysed cells were homogenized and extracted at 4 °C for 1 h. Insoluble material was removed by centrifugation at 12,000 × g for 15 min at 4 °C. Cell extract (1000 μg of total protein) was mixed with 1 μg of anti-Crk antibody for 2 h at 4 °C, and then protein A-agarose was added to the mixture at 4 °C overnight. After incubation, the agarose beads were collected by centrifugation at 1000 × g for 5 min at 4 °C. The pellet was then washed four times with the Triton X-100 lysis buffer and resuspended in 1 × Laemmli SDS sample buffer with 20 mM dithiothreitol. After heating at 100 °C for 5 min, the sample was subjected to SDS-PAGE and Western immunoblot analysis.

**Immunofluorescence**—3T3-L1 cells were cultured and induced on glass coverslips. At the indicated times, the coverslips were rinsed with PBS and fixed for 10 min in 3.7% formaldehyde and 0.18% Triton X-100 in PBS solution. The fixed cells were incubated in blocking buffer (1% bovine serum albumin in Tween/Tris-buffered saline) for 2 h at room temperature, with primary anti-Crk antibody at 4 °C overnight, and with tetramethylrhodamine B isothiocyanate-conjugated secondary antibody for 1 h at room temperature, after which the cells were visualized by fluorescence microscopy.

**RESULTS**

**Vanadate Inhibits an Early Step in Adipocyte Differentiation without Blocking Mitotic Clonal Expansion**—Mitotic clonal expansion is an essential step that occurs early in the differentiation program (7, 9, 10). A previous study (28) showed that vanadate, a potent PT-Pase inhibitor, blocks differentiation of 3T3-L1 preadipocytes when added along with differentiation inducers during the first 48 h, during which mitotic clonal expansion occurs. To locate the time window during this period when vanadate exerts its inhibitory effect, preadipocytes were induced to differentiate in the presence of vanadate. Cytoplasmic triglyceride was visualized by staining with oil red O on day 8.

During the first 48 h after induction of differentiation, however, the preadipocytes differentiated normally. When vanadate addition was delayed for 24 h after the induction of differentiation, the cells treated with 20 μM sodium vanadate during the period of hormonal stimulation (first 48 h); +vanadate delayed, cells treated with 20 μM sodium vanadate after they were induced 24 h after induction of differentiation was initiated; +vanadate re-induced, cells treated as described for +vanadate, after which the medium was replaced on day 2 following the normal differentiation protocol, and the cells were cultured to day 6, when cells were re-induced, i.e., subjected again on day 6 to the differentiation protocol. B, total cellular RNA was isolated from the cells on the days indicated (after induction of differentiation) and subjected to Northern blot analysis using C/EBPα and 422/aP2 cDNA probes. Contr. +Van. are as described for A. Numbers refer to days after induction of differentiation. C, 3T3-L1 preadipocytes were induced to differentiate on day 0; 20 μM sodium vanadate was added to the medium at the indicated times after the addition of the differentiation inducers, including the 422/aP2 gene (17). As illustrated in Fig. 1B, vanadate inhibited the expression of both of these marker genes.

During the first 48 h after induction of differentiation, 3T3-L1 preadipocytes synchronously undergo mitotic clonal expansion progressing through approximately two rounds of cell division. This process does not appear to be affected by vanadate; thus, the increase in cell number following induction of differentiation was the same whether vanadate was present or not (Fig. 2). To ascertain whether vanadate might cause a more subtle change in cell cycling during mitotic clonal expansion,
fluorescence-activated cell sorter analysis was conducted on 3T3-L1 cells during the first round of differentiation-induced mitotic clonal expansion. Based on the fluorescence-activated cell sorter analysis (data not shown), the first round of the cell cycle was completed ~28 h after induction. Moreover, vanadate had no detectable effect on progression through any phase of the cell cycle. It can be concluded that vanadate blocks the induction of differentiation without affecting mitotic clonal expansion and that the blockade is reversible.

Accumulation of a 35-kDa Phosphotyrosyl Protein during Induction of Differentiation in the Presence of Vanadate—Since vanadate appears to block a PTPase-dependent signaling event required for induction of adipocyte differentiation, the possibility was considered that this blockade might cause accumulation of a phosphotyrosyl intermediate in the signaling pathway. To test this possibility, cell lysates from 3T3-L1 preadipocytes induced to differentiate in the presence or absence of vanadate were subjected to Western blot analysis with anti-phosphotyrosine antibody. As illustrated in Fig. 3, several phosphotyrosyl proteins were detected. The most prominent of the vanadate-dependent phosphoproteins, whose level was markedly decreased when exposure to vanadate was delayed for 24 h, was a 35-kDa phosphotyrosyl protein (pp35). This phosphoprotein did not accumulate in cells that had not been induced to differentiate, but that had been treated with vanadate (data not shown), and was particularly prominent in the short ECL exposure shown in Fig. 3C.

Cell fractionation revealed that pp35 is located primarily (>95%) in the cytosol (Fig. 3B). The slightly faster moving phosphoprotein of ~33 kDa evident in Fig. 3B appears to be a proteolytic fragment of pp35 and was not always detected (data not shown). pp35 is not mitogen-activated protein kinase or Rab3, which also have molecular masses in the 35-kDa range, as pp35 was not immunoprecipitated by antibodies directed against either of these proteins (data not shown). A time course study showed that during the normal differentiation process (days 0–6) in the absence of vanadate, pp35 did not accumulate (Fig. 3D). Moreover, the accumulation of pp35 was transient and present only on days 1 and 2. When added on days 3 and 4 after the hormonal induction, vanadate did not cause the accumulation of pp35 (Fig. 3D), nor did it block differentiation (28). Maximal accumulation of pp35 (in the presence of vanadate with preadipocytes that had been induced to differentiate) occurred between days 1 and 2; by day 3, virtually no pp35 remained. This was likely due to dephosphorylation after the removal of vanadate by the medium change on day 2 because there was a higher amount of the non-phosphorylated form of pp35 (c-Crk) in day 3 cells than in day 2 cells (data not shown).

Dephosphorylation of pp35 by PTPase HA2 in Vitro—The time window during which vanadate is capable of blocking adipocyte differentiation (Fig. 1C) and causing accumulation of pp35 (Fig. 3) coincides with the time of maximal PTPase HA2 expression (28). It was therefore of interest to verify that the PTPase activity in preadipocytes is sufficient to hydrolyze in vitro the maximal amount of pp35 contained in vanadate-treated cells. It should be noted that in previous studies, it was shown that the only PTPase present in preadipocytes at a significant level is PTPase HA2, a homologue of PTPase 1B (33). Thus, pp35 was partially purified and used as substrate for PTPase present in lysates of “induced” 3T3-L1 preadipocytes. As shown in Fig. 4, ~10 min was required for 50% hydrolysis of pp35 by pp35 by a cell equivalent amount of cell lysate PTPase (measured at 30 °C). Consistent with PTPase HA2 being the responsible phosphatase activity, this enzymatic activity was inhibited by phosphotyrosine, vanadate, and ZnCl₂, with vanadate being the most potent inhibitor (data not shown). Similar results were obtained with pp35 and PTPase HA2 purified from 3T3-L1 preadipocytes by methods described previously, i.e. hydroxylapatite and DEAE-cellulose chromatography (33).

Effect of Expressing c-Crk Antisense RNA on Adipocyte Differentiation—Activation of the IGF-1 receptor by IGF-1 or a high concentration of insulin was previously shown to be involved in the induction of differentiation of 3T3-L1 preadipocytes (8). Since pp35 was identified to be the tyrosine-phosphorylated form of mouse c-Crk (see “Experimental Procedures”), a known substrate of the IGF-1 receptor tyrosine kinase (39, 40), it became important to verify that c-Crk plays an essential role in the differentiation process. Thus, the role of c-Crk in this
The PTPase HA2 preparation (about one-twentieth plate equivalent) was incubated with the pp35 preparation (about one-twenty-fifth plate equivalent) in vitro as described under "Experimental Procedures." The amount of pp35 remaining was determined by Western blotting with anti-phosphotyrosine antibody.

Phosphorylation of c-Crk at Tyrosine Prevents Its Interaction with C3G, a Downstream Signaling Molecule—It has been demonstrated that the SH2 domain of c-Crk can interact intramolecularly with Tyr(P)221, which is phosphorylated by IGF-1 receptor tyrosine kinase (39–42). This interaction might be expected to obscure the SH3 domain of c-Crk because it lies between the SH2 domain and Tyr221 (39, 40). The fact that vanadate, a PTPase inhibitor, causes accumulation of phospho-c-Crk (pp35, presumably at Tyr221 because it is phosphorylated by IGF-1 receptor kinase in 3T3-L1 preadipocytes) and blocks the induction of differentiation suggested that phosphorylation and dephosphorylation of c-Crk are involved in the induction mechanism essential for signal transduction. Thus, by locking c-Crk in the phosphorylated state with vanadate, the SH3 domain–binding proline-rich motif that possesses an SH3 domain–binding proline-rich motif that has been shown to bind to the SH3 domain in c-Crk.

To test the hypothesis that interaction of c-Crk with C3G occurs during differentiation induction and is prevented by phosphorylation of c-Crk, we determined whether C3G could be coprecipitated with antibody against c-Crk at different times following induction of differentiation when c-Crk was in either the phospho or dephospho state. Fig. 7A shows that both c-Crk and C3G were expressed by 3T3-L1 preadipocytes at an early stage of the differentiation program. Following induction of differentiation, C3G protein was coprecipitated with antibody directed against c-Crk (Fig. 7B). Within 15 min after induction, a substantial amount of C3G was co-immunoprecipitated with anti-Crk antibody. Even after 24 h, there was still some C3G binding to c-Crk. However, in non-induced 3T3-L1 preadipocytes, c-Crk did not associate with C3G, i.e. C3G was not co-immunoprecipitated with c-Crk prior to hormonal induction of differentiation (Fig. 7B). Thus, induction of differentiation led to rapid association of C3G with c-Crk. At this moment, it is not clear whether the activation of c-Crk occurs before...
phosphorylation of c-Crk or after dephosphorylation of phospho-c-Crk.

Since vanadate causes the accumulation of phospho-c-Crk, and phosphorylation may shield the SH3 domain from C3G (see above), we investigated the effect of vanadate on the interaction of c-Crk, i.e. phospho-c-Crk, with C3G. Immunoprecipitation of C-Crk from lysates of day 1 cells (treated or not with vanadate) revealed virtually no association between phospho-c-Crk and C3G in vanadate-treated cells (Fig. 7C). Significant association of C3G with c-Crk did occur, however, in day 1 cells not treated with vanadate, at which time almost all of the c-Crk was in the dephospho form. Moreover, there was little change in the amounts of either c-Crk or C3G protein per se in cells that were treated or not with vanadate (Fig. 7C). Taken together, these results indicate that C3G associates only with differentiation inducer-activated (presumably through the IGF-1 receptor) c-Crk and does not associate with either phospho-c-Crk or “unactivated” c-Crk. Vanadate treatment prevents the turnover of phospho-c-Crk to c-Crk, thereby preventing downstream signaling molecules from binding to the SH3 domain of Crk.

To prevent c-Crk signaling with vanadate, a significant fraction of c-Crk would have to be locked in the phosphorylated form. As illustrated in Fig. 7C, most of the c-Crk from vanadate-treated preadipocytes migrated more slowly upon SDS-PAGE than c-Crk from control preadipocytes. Since vanadate blocked differentiation only when added during the first 24 h after induction (Fig. 1), we focused our efforts on this time interval even though there was more phospho-c-Crk in day 2 cells (Fig. 3D). To verify that the slow-moving form of c-Crk is indeed phospho-c-Crk, purified phospho-c-Crk (pp35) was treated with (+) or without (−) alkaline phosphatase (AP). An equal amount of purified protein was loaded onto the gel. Anti-Crk antibody (α-Crk) was used to immunoblot the transferred membrane. Panel III, cell extracts prepared from day 1 3T3-L1 preadipocytes induced in the presence (+) or absence (−) of vanadate, followed by Western immunoblotting with anti-Crk antibody. Panel II, purified phospho-c-Crk protein (pp35) was treated with (+) or without (−) alkaline phosphatase (AP). An equal amount of purified protein was loaded onto the gel. Anti-Crk antibody (α-Crk) was used to immunoblot the transferred membrane. Panel III, cell extracts prepared from day 1 3T3-L1 preadipocytes induced in the presence (+) or absence (−) of vanadate, followed by Western immunoblotting with anti-Crk antibody, and then the immunoprecipitated samples were subjected to Western blotting with anti-C3G antibody (α-C3G). Panel IV, aliquots of cell extracts used for immunoprecipitation in panel III were subjected directly to Western blotting with anti-C3G antibody.

expression of c-Crk occurs after 3T3-L1 preadipocytes achieve confluence. A, immunofluorescence of 3T3-L1 preadipocytes stained with anti-Crk antibody. proliferating refers to 3T3-L1 preadipocytes prior to reaching confluence; confluent refers to 3T3-L1 preadipocytes just reaching confluence. 0h, 2h, 24h, and 48h indicate the time after induction of differentiation. The yellow bar represents 50 µm. B, Western blot of c-Crk in 3T3-L1 preadipocyte lysates before induction and in the early stages of differentiation. C refers to 3T3-L1 preadipocytes just reaching confluence; 1 is 1 day post-confluent; 0 is 2 days post-confluent; and 1 and 2 refer to 1 and 2 days after induction of differentiation, respectively.
increased. Thus, consistent with its putative role as signal mediator, c-Crk was present at the time of exposure of preadipocytes to the differentiation inducers.

**DISCUSSION**

When treated with differentiation inducers (on day 0 of the differentiation protocol), confluent growth-arrested 3T3-L1 preadipocytes synchronously reenter the cell cycle, undergo mitotic clonal expansion (days 0–2), and then coordinately express genes that produce the terminally differentiated adipocyte phenotype (days 3–6) (7, 9, 47). Immediately following induction, PTPase HA2 (a homologue of PTPase 1B) is expressed, reaching a maximal level during clonal expansion and then declining (28). If vanadate, a potent PTPase inhibitor, is added to the cells during this period of maximal PTPase level, differentiation induction is blocked (28). The time window during which vanadate can block differentiation is relatively short, i.e. between 0 and 20 h following induction (Fig. 1C). After that, exposure to vanadate has no effect on differentiation (Fig. 1C) (28). Thus, vanadate blocks a tyrosine dephosphorylation event required for terminal differentiation, but has no effect on clonal expansion per se (Figs. 1–3). Since the PTPase-catalyzed tyrosine dephosphorylation process appears to be required at an early stage of adipocyte differentiation, the phosphotyrosine protein will be an important intermediate in the induction of adipocyte differentiation. In this investigation, we have identified phosphotyrosyl-c-Crk, a known substrate of IGF-1 receptor kinase (39, 40), as the intermediate for the PTPase-catalyzed tyrosine dephosphorylation process during 3T3-L1 preadipocyte differentiation.

That the IGF-1 receptor tyrosine kinase is involved in the induction process was first recognized by Rubin and co-workers (8). IGF-1, rather than insulin, was found to be the physiological inducer of adipocyte differentiation, along with cAMP and glucocorticoid. Because of its low binding affinity for the IGF-1 receptor, insulin can be used to replace IGF-1 as a differentiating inducer only at non-physiologically high concentrations (5, 6). In contrast, IGF-1 induces differentiation at in vivo concentrations. It should also be noted that preadipocytes possess numerous IGF-1 receptors, ~30,000/cell (48), but only a small number of insulin receptors. The number of insulin receptors begins to increase only after induction of differentiation (49). Thus, activation of the IGF-1 receptor tyrosine kinase by IGF-1 is one of the signals (along with activation of protein kinase A by cAMP and of the glucocorticoid receptor by glucocorticoid) that triggers adipocyte differentiation. Thus, taken together with the findings that vanadate, a potent PTPase inhibitor, blocks hormone (insulin through the IGF-1 receptor)-induced 3T3-L1 preadipocyte differentiation and PTPase HA2-catalyzed dephosphorylation of phospho-c-Crk (pp35) both ex vivo and in vitro (28, Fig. 3 and 4), c-Crk serves both as a substrate of the IGF-1 receptor tyrosine kinase and as a substrate of PTPase HA2. These results support the view that the IGF-1 receptor tyrosine kinase, c-Crk, PTPase HA2, and, most likely, C3G (see below) function in a signaling cascade early in the adipocyte differentiation program.

In view of the apparent role of c-Crk in the signaling cascade, it became important to verify that it is actually required for adipocyte differentiation. Results of the antisense RNA experiments (Figs. 5 and 6) confirmed our observation. As an adapter molecule having both SH2 and SH3 domains, c-Crk has been suggested to be involved in growth factor-mediated tyrosine phosphorylation signaling cascades (50–52). Based on our findings and those of others, we postulate the following sequence of events summarized in Fig. 9. The interaction of IGF-1 with cell-surface IGF-1 receptors activates receptor autophosphorylation and thereby activation of the receptor tyrosine kinase.

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