A chimeric growth factor receptor (CSF1R/IR) was constructed by splicing cDNA sequences encoding the extracellular ligand binding domain of the human colony stimulating factor-1 (CSF-1) receptor to sequences encoding the transmembrane and cytoplasmic domains of the human insulin receptor. The addition of CSF-1 to cells transfected with the CSF1R/IR chimera cDNA stimulated the tyrosine phosphorylation of a protein that was immunoprecipitated by an antibody directed against the carboxyl terminus of the insulin receptor. Phosphopeptide maps of the 32P-labeled CSF1R/IR protein revealed the same pattern of phosphorylation observed in 32P-labeled insulin receptor β subunits. CSF-1 stimulated the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and Shc in cells expressing the CSF1R/IR chimera. Lipid accumulation and the expression of a differentiation-specific marker demonstrated that 3T3-L1 preadipocytes undergo CSF1R/IR-dependent differentiation when transfected with the CSF1R/IR chimera cDNA but not when transfected with the expression vector alone. A 12-amino acid deletion within the juxtamembrane region of the CSF1R/IR (CSF1R/IRα960) blocked CSF1R/IR-stimulated phosphorylation of IRS-1 and Shc but did not inhibit CSF1R/IR-mediated differentiation of 3T3-L1 preadipocytes. These observations indicate that adipocyte differentiation can be initiated by intracellular pathways that do not require tyrosine phosphorylation of IRS-1 or Shc.

A primary goal in the study of insulin action is the identification of the intracellular pathways that lead ultimately to changes in the rates of growth, development, and metabolism in primary target tissues of insulin (e.g. adipose, muscle, and liver). One strategy for the study of insulin-sensitive intracellular signaling has been to identify structural features within the insulin receptor that are important components of divergent signal transduction pathways. The goal of this strategy has been to create mutations within specific receptor sequences that result in the selective disruption of some insulin-regulated metabolic pathways while leaving others intact. Deletion mutagenesis of the insulin receptor cytoplasmic domain has generated insulin receptors with altered biological properties (1-4). Experiments with these receptor mutants have led to the suggestion that different regions of the insulin receptor cytoplasmic domain may play roles in modulating the distinct biological effects of insulin. Most mutations of the insulin receptor cytoplasmic domain have removed or altered autophosphorylation sites within the cytoplasmic domain (5-8). This approach has furthered the notion that tyrosine phosphorylation and the tyrosine kinase encoded within the receptor β subunit are essential components of normal insulin action. The majority of structure/function analyses of the insulin receptor, however, have been performed in cells expressing low levels of endogenous insulin receptors, e.g. Chinese hamster ovary (CHO)1 or Rat-1 fibroblasts cell lines (reviewed in Ref. 9). Although such cell lines readily express mutated insulin receptors following transfection, they have been less than ideal models for studying the anabolic actions of insulin because they are relatively insensitive to insulin treatment.

Although insulin-responsive cell types are excellent systems in which to study insulin action, the fact that they are highly responsive to insulin is an ironic impediment to the mutational analysis of insulin receptor function. Analysis of the ability of mutated insulin receptors to mediate insulin signaling in insulin-responsive cells is confounded by the significant background generated by endogenous insulin receptors. In an attempt to circumvent this concern, we have constructed a chimeric receptor (CSF1R/IR) consisting of the extracellular ligand binding domain of the human CSF-1 receptor (10) coupled to the transmembrane and cytoplasmic domains of the human insulin receptor (11-13). Chimeric receptors have proven useful for demonstrating that specific receptor structures transduce specific biological responses (14, 15).

In this report, we show that the CSF1R/IR chimera has CSF1R/IR-dependent enzymatic and biological properties expected of the insulin receptor tyrosine kinase in insulin-responsive cells. The CSF1R/IR permits the differentiation of 3T3-L1 cells when nanomolar concentrations of CSF-1 are substituted for insulin in the differentiation protocol. A mutation within the juxtamembrane domain of the CSF1R/IR that inhibits CSF1R/IR-stimulated phosphorylation of IRS-1 and Shc does not block the ability of CSF-1 to stimulate the differentiation of 3T3-L1 cells.

1 The abbreviations used are: CHO, Chinese hamster ovary; CSF, colony-stimulating factor; IR, insulin receptor; DMEM, Dulbecco’s modified Eagle’s medium; PCR, polymerase chain reaction; IGF, insulin-like growth factor.
into adipocytes. Thus, alternative intracellular pathways that do not require tyrosine phosphorylation of IRS-1 or Shc may be used by insulin or IG F I to stimulate differentiation of 3T3-L1 cells.

EXPERIMENTAL PROCEDURES

CHO K1 cells and CHO cells expressing the wild-type and mutated human insulin receptors were described previously (16, 17). 3T3-L1 preadipocytes were provided by Richard MacDonald, Omaha, NE. Antibody CT-1 directed against the carboxyl terminus of the human insulin receptor (18) was a gift from Ken Siddle, Cambridge, United Kingdom. Antibodies to IRS-1 were described previously (19). Anti-Shc and anti-phosphotyrosine (PY20) antibodies were from Transduction Laboratories. 32P-Orthophosphate was purchased from ICN. 35S-Methionine was purchased from Amersham. Dulbecco’s modified Eagle medium (DMEM), penicillin, streptomycin and neomycin antibiotics, G418, fetal bovine serum, restriction endonucleases, and Lipofectamine were purchased from Life Technologies, Inc. Tryptsin treated with tosylphenylalanylchloromethyl ketone was obtained from Worthington Biochemical Corporation. Porcine insulin was from Calbiochem. Dexamethasone and 3-isobutyl-1-methylxanthine were from Sigma, and thin-layer chromatography plates were obtained from American Bioanalytical. The mammalian expression plasmid pcdNA3 was purchased from Invitrogen. The plasmid pSP72 was from Promega. Human recombinant CSF-1 was a gift from Genetics Institute. The cDNA for the human CSF-1 receptor was a gift from Charles Sherr, Memphis, TN.

Construction of the CSF-1/IR Chimera—The CSF-1/IR chimera was constructed using a PCR strategy. Three PCR primers were designed for construction of the chimeric receptor. chi-1 was an upstream primer that hybridizes to nucleotides 1598–1622 of the human CSF1R (10). chi-2 was a chimeric oligonucleotide to act as an upstream primer for PCR using the human insulin receptor as template. The first 19 nucleotides of chi-2 included an overhang corresponding to nucleotides 2930–2957 of the human IR (13). chi-3 was a downstream primer corresponding to the complementary strand of base pairs 3028–3055 of the human IR.

The first PCR used primers chi-2 and chi-3 and the human IR cDNA as the template to produce the expected product of 172 base pairs. The second PCR used chi-1, the 172-base pair product of the first PCR, and chi-3. The template was the human CSF1R (10). The expected product was two base pairs and generated a DNA sequence with the expected attachment of sequences encoding the extracellular domain of the CSF1R to sequences encoding the transmembrane domain and a portion of the cytoplasmic domain of the insulin receptor. Restriction digestion of the 392-base pair product from the second PCR with Bsu36I and BglII yielded a 259-base pair fragment. Prior to restriction digestion, the PCR product was sequenced to confirm the integrity of the expected product.

A HindIII/SalI fragment of the CSF1R was subcloned into pUC18 to create pUC18CSF1041. pUC18CSF1041 was digested with Bsu36I and SalI, and a Bsu36I/SalI 2081-base pair fragment of the human insulin receptor cDNA was ligated to pUC18CSF1041. The resulting construct, pUC18CSF1041/IR2081, was partially digested with Bsu36I and a 5083-base pair fragment of pUC18CSF1041 was isolated. The 5083-base pair fragment of pUC18CSF1041 was digested with BglII, and the 259-base pair Bsu36I/BglII chimeric cDNA product was ligated to produce pUC18CSF1041/IR. pUC18CSF1041/IR was digested with KpnI and BamHI, and a 1966-base pair fragment was isolated.

The 3384-base pair CSF1R cDNA (10) was subcloned into EcoRI/BamHI cut pSP72 to create pSP72CSF1R. pSP72CSF1R was digested with KpnI and BamHI, and the resulting 4228-base pair fragment was isolated. The 4028-base pair KpnI/BamHI fragment of pSP72CSF1R was ligated to the 1966-base pair KpnI/BamHI fragment isolated from pUC18CSF1041/IR to yield the pSP72CSF1R/IR chimera. The CSF1R/IR chimeric cDNA was isolated by EcoRI and XbaI digestion and subcloned into pcdNA3. For stable expression of the CSF1R/IR in 3T3-L1 fibroblasts and adipocytes, the vector pEF-1 was prepared by replacing the mouse elongation factor-1 fragment from the CSF1R/IR with the corresponding 1367-base pair fragment from pSV1960 (17).

Cell Culture—3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% fetal bovine serum and antibiotics. CHO cells were maintained in F12 medium supplemented with 10% fetal bovine serum and antibiotics. All cells were incubated at 37 °C in 5% CO2. For differentiation, 2 × 103 3T3-L1 preadipocytes were plated in 35-mm dish, and the medium was replaced every other day after plating until the cells became confluent. CHO cells were maintained an additional 2 days after reaching confluence without being fed to induce quiescence. After the 3T3-L1 cells had become quiescent, differentiation was induced with DMEM containing 10% fetal bovine serum and supplemented with 1 μg insulin, 0.25 μg dexamethasone, and 0.5 μg 3-isobutyl-1-methylxanthine as described previously (21). CSF-1 was substituted for insulin at the concentrations indicated. Cells were maintained in the differentiation media for 2 days, at which time the medium was replaced with DMEM containing 10% fetal bovine serum and either insulin or CSF-1 at the same concentration used to induce differentiation. This media was replaced 2 days later with DMEM containing 10% fetal bovine serum alone. The cells were fed every 2 days thereafter with DMEM containing 10% fetal bovine serum used until used.

Transfection of Plasmid DNA into Mammalian Cells and Isolation of Clonal Cell Lines—Transfection of CHO K1 cells and 3T3-L1 preadipocytes was performed with Lipofectamine according to the manufacturer’s instructions. Cells expressing the CSF1R/IR were selected with G418 and isolated by flow cytometry with a monoclonal antibody against the extracellular domain of the human CSF-1 receptor (22) (Oncogene Science) and fluorescent secondary antibody. Flow cytometry was performed by the University of Nebraska Medical Center Flow Cytometry Core Laboratory.

Phosphorylation and Immunoprecipitation of Receptors—Metabolic labeling with 2 μCi/ml 32P-Orthophosphate was performed in phosphate-free DMEM for 2 h at 37 °C as described previously (23). Lysis of cells in 1% Triton X-100 in the presence of protease and phosphatase inhibitors was performed as described previously (23). Immunoprecipitations were performed with the indicated antibodies bound to agarose-conjugated protein G.

Phosphoamino Acid Analysis—Each portion of polyacrylamide gel containing 32P-labeled receptor was excised and washed successively three times with water and acetone to remove SDS. The 32P-labeled receptor protein was digested with 10 μg tosylphenylalanylchloromethyl ketone-trypsin in 300 μl of 0.25% ammonium bicarbonate, pH 8.0, in a clean 1.5-ml Eppendorf tube. The tryptic eluate was lyophilized to dryness, and the dried phosphopeptides were reconstituted in 0.2 ml of 6 M HCl. Partial hydrolysis of the phosphopeptides was performed at 110 °C for 1 h, after which the hydrolysate was diluted 6-fold with water, lyophilized, reconstituted with 100 μl of H2O, and lyophilized a second time. Electrophoretic separation of the phosphoamino acids was performed as described (24). The plates were dried, and phosphoamino acid standards were visualized with ninhydrin. Radiolabeled phosphoamino acids were identified by autoradiography.

HPLC Phosphopeptide Mapping—For phosphopeptide mapping, the phosphorylated proteins were separated on an 8% SDS-polyacrylamide gel. The CSF1R/IR and the insulin receptor β subunit were localized by autoradiography and digested twice with 100 μg/ml tosylphenylalaninylchloromethyl ketone-trypsin in 0.25% ammonium bicarbonate. Phosphopeptides were separated on a Brownlee Aqapore OD300 column with a gradient of 50% acetonitrile in 0.1% trifluoroacetic acid at 0.25 ml/min. Fractions were collected at 30-s intervals and counted for Cerenkov radiation.

Western Blot Analysis—Proteins were transferred to polyvinylidene difluoride or nitrocellulose membranes, and Western blots were developed using anti-Shc (1:250) or anti-phosphotyrosine (PY20 or RC-20) antibodies as specified by the manufacturer (Transduction Laboratories) or using CT-1 (1:5000) (18) and anti-IRS-1 (1:800) (25) antibodies. Blots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium using the appropriate secondary antibody conjugated to alkaline phosphatase or with the ECL chemiluminescence kit (Amersham Corp.).

RESULTS

The CSF1R/IR chimera was expressed stably in CHO cells to assess its structure and enzymatic activity. Treatment of cells expressing the CSF1R/IR with CSF-1 for 10 min resulted in the tyrosine phosphorylation of the 170-kDa chimeric receptor protein (Fig. 1). Furthermore, tyrosine-phosphorylated CSF1R/IR chimera was precipitated by an antibody directed against the extracellular ligand binding domain of the CSF1R or by monoclonal antibody CT-1, which is specific for the carboxyl termi-
nus of the human insulin receptor (18) (Fig. 1). No detectable tyrosine-phosphorylated proteins were immunoprecipitated from CSF-1-treated CHO cells transfected with the expression vector alone (Fig. 1). Insulin did not stimulate phosphorylation of the CSF1R/IR chimera (data not shown). These observations demonstrate the chimeric nature of the CSF1R/IR protein and that it has CSF-1-activated tyrosine kinase activity. The CSF-1 receptor has been placed in the subclass of the family of growth factor receptor tyrosine kinases that includes the platelet-derived growth factor receptor and c-kit (26). Activation of the insulin receptor tyrosine kinase in the chimera may result from CSF-1-induced dimerization of the CSF1R/IR protein that permits trans-phosphorylation of juxtaposed tyrosine kinase domains (27). These observations suggest that the proper orientation of juxtaposed tyrosine kinase domains is sufficient for the activation of tyrosine kinase activity.

CSF-1 caused a dose-dependent increase in tyrosine phosphorylation of the CSF1R/IR in intact cells (Fig. 2). Quantitative analysis of the anti-phosphotyrosine Western blot indicated that stimulation of the CSF1R/IR tyrosine autophosphorylation was half-maximal at 1 nM CSF-1 and maximal at 10 nM CSF-1. The pattern of phosphorylation of the CSF1R/IR chimera was compared with the pattern of phosphorylation on the insulin receptor β subunit produced by insulin-stimulated autophosphorylation of the heterotetrameric insulin receptor (Fig. 3). CSF1R/IR chimeras were immunoprecipitated with antibody CT-1 from $^{32}$P-labeled and CSF-1-treated CHO cells. Immunoprecipitated CSF1R/IR protein was isolated by electrophoresis on a polyacrylamide gel and visualized by autoradiography. Human insulin receptors were similarly isolated from $^{32}$P-labeled and insulin-treated CHO cells. Analysis of CSF1R/IR and insulin receptor β subunit phosphopeptide maps revealed a nearly identical distribution of phosphopeptides between the two receptors (Fig. 3A). Phosphoamino acid analysis of the $^{32}$P-labeled CSF1R/IR chimera revealed the presence of phosphoserine, phosphotyrosine, and only a trace of phosphothreonine (Fig. 3B).

The murine 3T3-L1 preadipocyte is a model system for the process of adipocyte differentiation (28). Differentiation results in the increased expression of insulin receptors (29), insulin-responsive glucose transporters (30, 31), and enzymes required for fatty acid synthesis (32–34). The differentiated 3T3-L1 adipocyte responds to insulin in a manner that resembles the normal adipocyte, a primary target of insulin action. The ability of the CSF1R/IR chimera to stimulate biological effects in cells was examined further by transfection of the plasmid pEFCSF1R/IR into 3T3-L1 preadipocytes and selection for resistance to G418. Pools consisting of at least seven independent clones expressing the CSF1R/IR were isolated by flow cytometry. 3T3-L1 preadipocytes expressing the CSF1R/IR (Fig. 4) were grown to confluence and quiescence. Differentiation was initiated with dexamethasone, isobutylmethylxanthine, and 1 μM insulin as described previously (21). Subjecting each 3T3-L1

![FIG. 1. CSF-1-dependent phosphorylation of the CSF1R/IR chimera. CHO cells expressing the CSF1R/IR chimeric receptor (CHO-chi.10) or CHO cells transfected with the expression vector alone (CHO-neo) were left untreated or treated with 10 nM CSF-1 for 10 min at 37 °C. Proteins were immunoprecipitated with antisera specific to the carboxyl terminus of the insulin receptor (CT-1), with an antibody directed against the extracellular ligand binding domain of the CSF-1 receptor (aCSF1R), or with non-immune antisera (NI). Immunoprecipitates were resolved by SDS-PAGE and immobilized to nitrocellulose by Western transfer. Tyrosine-phosphorylated proteins were identified by blotting with anti-phosphotyrosine antibodies.](http://www.jbc.org/)

![FIG. 2. CSF-1 dose-dependent phosphorylation of the CSF1R/IR chimera. A, 3T3-L1 cells were labeled for 3 h at 37 °C with $[^{35}]$S)methionine and then treated with CSF-1 at the concentrations indicated for 10 min at 37 °C. The cells were lysed and immunoprecipitated with antibody CT-1. Tyrosine phosphorylation of the CSF1R/IR was determined by Western blot with anti-phosphotyrosine antibody (PY20). B, autoradiography was performed on the Western blot shown in A to identify the amount of CSF-1/IR present in each immunoprecipitate. C, the relative amount of tyrosine phosphorylation on the CSF1R/IR shown in A was quantified with a Molecular Dynamics densitometer.](http://www.jbc.org/)
The ability of CSF-1 and insulin to differentiate cells expressing a mutated CSF1R/IR lacking 12 amino acids within the juxtamembrane region of the cytoplasmic domain (CSF1R/IRΔ960) was also tested. 3T3-L1 cells expressing the CSF1R/IRΔ960 also accumulated lipid when subjected to the differentiation protocol containing dexamethasone and isobutylmethylxanthine in combination with 1 μM insulin or 25 nM CSF-1 (Fig. 4, lower panels). Lipid vesicle formation in CSF-1-treated 3T3-L1 cell adipocytes expressing the CSF1R/IRΔ960 was more closely akin to that observed in insulin-treated cells (Fig. 4).

Differentiation of 3T3-L1 cells expressing the CSF1R/IR and CSF1R/IRΔ960 was confirmed by detection of the fatty acid binding protein aP2/422 following CSF-1 treatment (Fig. 5). Relative expression of the fatty acid binding protein was stronger in cells expressing the CSF1R/IR or the CSF1R/IRΔ960 than in cells expressing a comparable amount of the intact CSF1R/IR and are closer in magnitude to that observed with insulin treatment of the same cells. These observations are consistent with the relative amount of lipid accumulation in CSF-1-treated 3T3-L1 cells expressing the CSF1R/IRΔ960 as compared with cells expressing the intact CSF1R/IR (Fig. 4).

The juxtamembrane region of the insulin receptor cytoplasmic domain encodes structures that are important for the recognition and phosphorylation of the intracellular mediators IRS-1 and Shc by the insulin receptor tyrosine kinase (1, 4, 35). We tested the ability of CSF-1 to stimulate the tyrosine phosphorylation of IRS-1 and Shc in 3T3-L1 cells expressing the CSF1R/IR or the CSF1R/IRΔ960. CSF-1 stimulated phosphorylation of IRS-1 and Shc (Figs. 6 and 7) in 3T3-L1 cells expressing the CSF1R/IR. However, CSF-1 was unable to stimulate the tyrosine phosphorylation of IRS-1 or Shc in cells expressing the CSF1R/IRΔ960 (Figs. 6 and 7). Comparable amounts of IRS-1 and Shc proteins were precipitated from 3T3-L1 cells expressing either the CSF1R/IR or the CSF1R/IRΔ960 (Figs. 6 and 7). CSF-1 was equipotent to IGF I in its ability to stimulate the phosphorylation of IRS-1 in cells expressing the CSF1R/IR (Fig. 6). Interestingly, insulin consistently failed to stimulate the tyrosine phosphorylation of Shc in 3T3-L1 cells expressing the CSF1R/IR, despite the fact that insulin readily induced differentiation of these cells (Fig. 7). In contrast, insulin stimulated the phosphorylation of Shc strongly in 3T3-L1 adipocytes expressing the CSF1R/IRΔ960 (Fig. 7).

The ability of the CSF-1/IR to transactivate endogenous insulin receptors. This possibility was tested in CHO cells expressing both the human insulin receptor and the CSF1R/IR and in differentiated 3T3-L1 adipocytes. Fig. 8 demonstrates that the CSF-1 does not stimulate the tyrosine phosphorylation of the insulin receptor β subunit in either transfected CHO cells (A) or in differentiated 3T3-L1 adipocytes (B) despite the fact that the insulin receptor and the CSF1R/IR are detected in similar amounts in both cell lines. Similarly, insulin does not stimulate the tyrosine phosphorylation of the CSF1R/IR in these cells.

**DISCUSSION**

We have constructed a chimeric growth factor receptor consisting of the extracellular ligand binding domain of the human CSF-1 receptor spliced to the transmembrane and cytoplasmic domains of the human insulin receptor. CSF-1 activates the CSF1R/IR tyrosine kinase to autophosphorylate and phosphorylate endogenous proteins in a manner characteristic of the normal insulin receptor. Furthermore, the CSF1R/IR is capable of mediating the CSF1-1-dependent differentiation of 3T3-L1 adipocytes. Deletions within the juxtamembrane domain of the CSF1R/IR that abrogate its ability to phosphorylate IRS-1 and...
Fig. 4. Insulin- and CSF-1-dependent differentiation of 3T3-L1 preadipocytes. Parental 3T3-L1 cells (top panels), 3T3-L1 cells expressing the CSF1R/IR (middle panels), and 3T3-L1 cells expressing the CSF1R/IRΔ960 (bottom panels) were subjected to the adipocyte differentiation protocol as described under “Experimental Procedures” using dexamethasone and isobutylmethylxanthine alone (left panels), or in combination with 1 μM insulin (middle panels), or with 25 nM CSF-1 (right panels). Cells were fixed and stained for the presence of lipid with Oil Red O prior to photomicroscopy.

Fig. 5. CSF-1-dependent expression of aP2/422a in 3T3-L1 adipocytes expressing the CSF-1R/IR and CSF1R/IRΔ960. Parental 3T3-L1 cells (left panel), 3T3-L1 cells expressing the intact CSF1R/IR (center panel), or 3T3-L1 cells expressing the CSF1R/IRΔ960 (right panel) were treated with or without dexamethasone and isobutylmethylxanthine in the presence or absence of CSF-1 or insulin as described under “Experimental Procedures.” Cells were lysed 6 days after the initiation of the differentiation protocol, and aliquots of each lysate were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with antisera against the fatty acid binding protein aP2/422a.

Blot: anti-aP2

3T3-L1 preadipocytes express low levels of high-affinity insulin receptor binding sites prior to differentiation (29). High concentrations of insulin (1 μM) are thought to induce differentiation of 3T3-L1 preadipocytes through activation of the IGF I receptor (36). However, gene targeting of one insulin receptor allele in 3T3-L1 preadipocytes impaired the ability of these cells to differentiate into adipocytes (37), suggesting that insulin can play a direct role in the differentiation of the 3T3-L1 cells. These previous studies support the notion that the insulin receptor cytoplasmic domain is also capable of initiating the intracellular signals sufficient for adipocyte differentiation. The insulin receptor tyrosine kinase domain, which is essential to receptor signaling, shares 84% identity with the tyrosine kinase domain of the IGF I receptor (38). The juxtamembrane domains of the insulin and IGF I receptors are 61% identical (38). Furthermore, five of the six known tyrosine autophosphorylation sites in the insulin receptor cytoplasmic domain (5–8) are conserved within the IGF I receptor (38). Thus, it is likely that structures conserved between the cytoplasmic domains of the insulin receptor and IGF I receptor are essential components of intracellular signaling pathways that result in adipocyte differentiation.

IRS-1 and Shc are substrates for the insulin receptor tyrosine kinase and contain structural domains that interact directly with the juxtamembrane region of the insulin receptor cytoplasmic domain (39–42). Mutations within the insulin receptor juxtamembrane domain abrogate the ability of the insulin receptor to phosphorylate IRS-1 and Shc and couple to intracellular signaling pathways (1, 4, 35). The ability of juxtamembrane mutations to block IRS-1 and Shc phosphorylation by the insulin receptor kinase is likely due to the loss of phosphorylation at Tyr960. Tyr960 has been shown to be essential for the interaction of the insulin receptor with IRS-1 and Shc (39, 41–43). These intracellular substrates are believed to be important intermediates in the activation of Ras in response to insulin addition (4, 44–46). Phosphatidylinositol 3′-kinase activity is also stimulated primarily through the phosphorylation of IRS-1 (47, 48), although a small amount of phosphatidylinositol 3′-kinase activity occurs through association of p85 with the carboxyl-terminal tail of the insulin receptor (49, 50). The observation that the juxtamembrane deletion within the CSF1R/IRΔ960 inhibits CSF-1-stimulated phosphorylation of IRS-1 and Shc (Figs. 6 and 7) in 3T3-L1 fibroblasts suggests that alternative pathways may be able to mediate the ability of the receptor to stimulate adipocyte differentiation. We cannot exclude the possibility that IRS-1 or Shc proteins might be phosphorylated in 3T3-L1 cells by the CSF1R/IRΔ960 to a small extent during the prolonged period of exposure of 3T3-L1 cells to differentiation medium containing CSF-1. Slow or imperceptible amounts of phosphorylation might not be detected by assay of IRS-1 and Shc phosphorylation following an acute exposure of cells to CSF-1. However, deletion of the juxtamembrane domain from the CSF1R/IR facilitated the accumulation of lipid (Fig. 4) and the expression of the adipocyte-specific fatty acid binding protein aP2/422 (Fig. 5) in cells treated with CSF-1. These observations suggest that IRS-1 or Shc-mediated signaling may not facilitate the accumulation of lipid or the fusion of lipid-containing vesicles. The amount of CSF-1 used to
induce adipocyte differentiation (25 nM) is sufficient to maximally activate the CSF1R/IR tyrosine kinase (Fig. 3). The limited degree of CSF-1-induced differentiation we observe in 3T3-L1 cells expressing the intact CSF1R/IR appears consistent with the description of fat cells formed in transgenic mice overexpressing normal Ras protein (51). Because IRS and Shc proteins appear to link insulin receptor tyrosine kinase activity to Ras, it is interesting to speculate that increased sensitivity to Ras may limit the extent of differentiation.

These data suggest that alternative signaling intermediates may be used for transmission of signals from the insulin receptor cytoplasmic domain to promote adipocyte differentiation. Additional substrates for the insulin receptor kinase have been identified, including IRS-2 (52), Gab-1 (53), a 115-kDa protein that associates with the protein-tyrosine phosphatase SHPTP2 (54), and three distinct substrates of molecular weight 53,000–60,000 (55–57). IRS-2 contains a PTB domain and has been shown to interact with the NPEY motif within the juxtamembrane domain of the insulin and IGF I receptors (58, 59). Yeast two-hybrid analysis indicates that additional sequences within
IRIS-2 may also interact with the insulin receptor (58, 59). Gab-1 shares homology with IRS-1 and IRS-2 but does not encode a PTB domain (53). Thus, the juxtamembrane domain of the insulin receptor may not be essential for the phosphorylation of Gab-1 by the tyrosine kinase of the insulin receptor. The potential role of these substrates in regulating insulin-induced adipocyte differentiation is not yet known.

The CSFIR/IR chimera provides an opportunity to explore the role of specific cytoplasmic structures in the induction of adipocyte differentiation. Additional autophosphorylation sites capable of associating with SH2 domain-containing proteins reside in the carboxyl-terminal tail and within the catalytic domain. The role of the carboxyl-terminal tail of the insulin receptor cytoplasmic domain in regulating metabolic and mitogenic signaling (2, 3, 60, 61), as well as differentiation, can be similarly explored in insulin-responsive cell types with the CSFIR/IR chimera. Additional alterations of the CSFIR/IR cytoplasmic domain will provide the opportunity to probe for structures within the insulin receptor cytoplasmic domain that may be necessary for the induction of differentiation and other biological events.

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CSF-1 Receptor/Insulin Receptor Chimera Permits CSF-1-dependent Differentiation of 3T3-L1 Preadipocytes

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