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Csk Enhances Insulin-Stimulated Dephosphorylation of Focal Adhesion Proteins

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Insulin has pleiotropic effects on the regulation of cell physiology through binding to its receptor. The wide variety of tyrosine phosphorylation motifs of insulin receptor substrate 1 (IRS-1), a substrate for the activated insulin receptor tyrosine kinase, may account for the multiple functions of insulin. Recent studies have shown that activation of the insulin receptor leads to the regulation of focal adhesion proteins, such as a dephosphorylation of focal adhesion kinase (pp125^{FAK}). We show here that C-terminal Src kinase (Csk), which phosphorylates C-terminal tyrosine residues of Src family protein tyrosine kinases and suppresses their kinase activities, is involved in this insulin-stimulated dephosphorylation of focal adhesion proteins. We demonstrated that the overexpression of Csk enhanced and prolonged the insulin-induced dephosphorylation of pp125^{FAK}. Another focal adhesion protein, paxillin, was also dephosphorylated upon insulin stimulation, and a kinase-negative mutant of Csk was able to inhibit the insulin-induced dephosphorylation of pp125^{FAK} and paxillin. Although we have shown that the Csk Src homology 2 domain can bind to several tyrosine-phosphorylated proteins, including pp125^{FAK} and paxillin, a majority of protein which bound to Csk was IRS-1 when cells were stimulated by insulin. Our data also indicated that tyrosine phosphorylation levels of IRS-1 appear to be paralleled by the dephosphorylation of the focal adhesion proteins. We therefore propose that the kinase activity of Csk, through the insulin-induced complex formation of Csk with IRS-1, is involved in insulin's regulation of the phosphorylation levels of the focal adhesion proteins, possibly through inactivation of the kinase activity of c-Src family kinases.

Among the earliest cellular responses following insulin stimulation are the tyrosine phosphorylations of insulin receptor (IR) β subunit as well as IR substrate 1 (IRS-1) (pp185) by the activated IR kinase activity (22, 23, 52, 64; for a review, see reference 62). IRS-1, a cytoplasmic protein found in most cells and tissues, contains more than 20 potential tyrosine phosphorylation sites; several of these sites have been shown to bind specifically to each of the Src homology 2 (SH2) domains of various signaling proteins such as phosphatidylinositol (PI) 3′-kinase, Ash/Grb2, Syp, and Nck (3, 4, 26, 27, 29, 50, 52, 58). It is possible that the divergent structures and functions of the SH2 proteins which bind to IRS-1 mediate the pleiotropic responses of insulin; tyrosine phosphorylation of IRS-1 may act as a switch to control the interaction of IRS-1 with these signaling proteins (52). In addition to IRS-1, another substrate, pp190 (IRS-2), which was found in IRS-1-deficient mice and can substitute, at least in part, for the function of IRS-1, has been reported. IRS-2 also binds to PI 3′-kinase (2, 53, 55, 59) and Ash/Grb2 (53, 59), suggesting that it too serves as an SH2-docking protein.

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C-terminal Src kinase (Csk) was first purified as a kinase able to phosphorylate the carboxyl-terminal tyrosine (Tyr-527) of c-Src, suppressing c-Src kinase activity (34), and was subsequently shown to phosphorylate several other Src family kinases such as Lyn, Fyn, Yes, and Lck at their carboxyl-terminal tyrosine residues in vitro (33; for a review, see reference 14). Csk has been shown to be involved in the negative regulation of the kinase activities of Src family kinases in vivo (10, 20, 31, 39, 41). The SH2 domain of Csk exhibits binding to several tyrosine-phosphorylated proteins such as the focal adhesion proteins pp125^{FAK} (focal adhesion kinase) and paxillin (40). Both SH2 and SH3 domains of Csk are required in its suppression of c-Src kinase activity (5, 19, 32, 40, 54).

pp125^{FAK} appears to be one of the important integration sites of various extracellular signals, including those via integrin receptors, providing docking sites for several SH2 proteins such as Src, Fyn, PI 3′-kinase p85, and Csk (for reviews, see references 8, 9, 12, 17, 18, 30, 40, 43, 45, 47, and 68). Activation and tyrosine phosphorylation of pp125^{FAK} may modulate the phosphorylation levels of several components of focal adhesion plaques and thereby may participate in the interaction between cytoskeletal structure and integrins, which in turn would affect the binding of integrins to the extracellular matrix (ECM) (for reviews, see references 11 and 45).

Tyrosine phosphorylation of paxillin, a 68-kDa focal adhe-

sion protein, is also regulated by cell adhesion to the ECM (7), platelet-derived growth factor stimulation, or stimulation with several hormones (69). Paxillin is capable of binding to vinculin (65) and the signaling proteins Csk, Crk, Src, and pp125^{FAK} (6, 40, 46). Inspection of the primary structure of paxillin reveals the presence of (i) three tyrosine residues within a binding motif for the Crk SH2 domain (YXXP), (ii) a proline-rich motif that could serve as an SH3 binding domain, and (iii) four motifs identical or very closely related to LIM domains (42, 60). Thus, paxillin appears to be closely associated with cell adhesions to the ECM as well as with the architecture of the cytoskeleton.

A number of observations suggest that pp125^{FAK} and paxillin become coordinately phosphorylated on tyrosine in response to various stimuli, including bombesin, lysophosphatidic acid, platelet-derived growth factor, and ECM binding to the integrins (7, 28, 37, 44, 48, 49). These signals appear to increase pp125^{FAK} and paxillin tyrosine phosphorylation and to enhance the formation of actin stress fibers. In contrast, insulin has been shown to decrease the tyrosine phosphorylation of pp125^{FAK} (25, 36, 67) and to decrease the cellular contents of actin stress fibers (25). Thus, the effect of insulin on both the dephosphorylation of pp125^{FAK} and the decreased number of focal adhesion plaques is concomitant with the decrease in the amounts and lengths of actin stress fibers. Our study has revealed that a fraction of Csk functions at focal adhesion plaques: major proteins to which the Csk SH2 domain binds include pp125^{FAK} and paxillin in fibroblasts (40), and Csk appears to phosphorylate paxillin directly (38). The Src family kinases such as c-Src and c-Fyn have also been implicated in playing a role in focal adhesion plaques (for reviews, see references 13 and 35) and are the potential substrates for Csk. Thus, we examined the possible involvement of Csk in insulin signaling at focal adhesions. Here, we show evidence that the kinase activity of Csk is involved in the regulation of the insulin-stimulated dephosphorylation of the focal adhesion proteins pp125^{FAK} and paxillin. We also show that Csk becomes associated via its SH2 domain with tyrosine-phosphorylated IRS-1 when stimulated by insulin. A possible mechanism by which Csk kinase activity is involved in the dephosphorylation of focal adhesion proteins is discussed.

MATERIALS AND METHODS

Materials. PI (bovine liver) was purchased from Avanti Polar Lipids Inc. Protein A-Sepharose, pGEX-2T, and glutathione-Sepharose beads were from Pharmacia-LKB (Uppsala, Sweden). [γ-^{32}P]ATP (6,000 Ci/mmol) was from Du Pont-New England Nuclear. Prestained molecular weight markers for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) were from Bio-Rad. Porcine insulin was a gift from Eli Lilly Co. (Indianapolis, Ind.).

Buffer. Buffer A contains 25 mM Tris-HCl (pH 7.4), 2 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium PP_i, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and 1 mM EDTA; buffer B is buffer A plus 1% Nonidet P-40.

Antibodies. Using the glutathione *S*-transferase (GST) fusion protein GST-Csk SH3/2 as an antigen, a rabbit polyclonal antibody against Csk (dCsk) was raised by a conventional method. This antibody did not recognize IRS-1, insulin receptor, FAK, or paxillin. The anti-IRS-1 antibody [αIRS-1 (1-6)] was a kind gift from Masaki Nishiyama (Jikei University School of Medicine, Tokyo, Japan) (16). Immunoglobulin G fraction of αIRS-1 was prepared by using protein G-Sepharose (Pharmacia) and covalently coupled to Affi-Gel 10 agarose. An antiphosphotyrosine antibody (αPY) was prepared as described previously (57). Another αPY, PY20 (ICN Biomedicals, Irvine, Calif.), was also used in some of the experiments. An antibody against pp125^{FAK} (αFAK) was prepared as described elsewhere (24). Briefly, a cDNA fragment encompassing amino acids 756 to 1053 of the mouse FAK sequence was inserted into pGEX-2T expression vector (15). The fusion protein was expressed in *Escherichia coli* and purified by using glutathione-Sepharose (Pharmacia-LKB). Polyclonal antibodies against the fusion protein were then raised in New Zealand White rabbits. An antibody against paxillin (αpaxillin) was from Zymed.

Transfection and establishment of cell lines. pcDNA-I csk, pcDNA-I csk (K222M), in which the Lys-222 ATP-binding site was changed to Met, and pcDNA-I csk (S108C), in which the Ser-108 in the SH2 domain was changed to Cys, were prepared as previously described (39–41). CHO-HIR cells (5×10^5) were transfected by calcium phosphate precipitation with 10 μg of the expression vectors (41) and 0.3 μg of pSV2 *bsr*. After 72 h, blasticidin S hydrochloride (5 μg/ml; Kaken Pharmaceutical Co.) was added to the medium to select for blasticidin S-resistant cells. Cells expressing high levels of chicken Csk and Csk (K222M) were identified by immunoblotting with αCsk. We obtained four and five independent clones, respectively, referred to as CHO-HIR-Csk 1-1, 1-2, 2-3, and 2-5 and CHO-HIR-Csk (K222M) 1, 3, 17, 19, and 21. We usually used CHO-HIR-Csk 1-1, 1-2, 2-3, and 2-5 and CHO-HIR-Csk (K222M) 3, 17, and 19. The experimental data described below were reproducible in at least two separate clones of transfected cells.

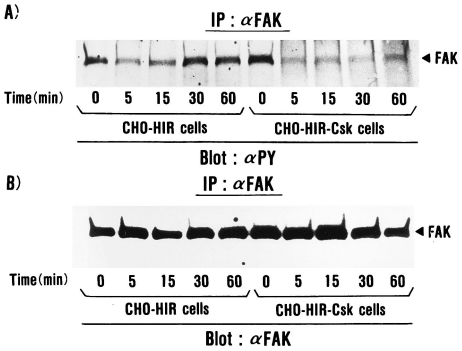

FIG. 1. Insulin stimulates dephosphorylation of pp125^{FAK} more significantly in CHO-HIR-Csk cells than in CHO-HIR cells. CHO-HIR or CHO-HIR-Csk cells were left untreated or stimulated with 10^{-7} M insulin for the indicated times and then lysed in buffer B, and the lysates were immunoprecipitated (IP) with αFAK; the immunoprecipitates were washed with buffer B three times and then subjected to SDS-PAGE followed by immunoblotting with αPY (A) or αFAK (B).

Transfection of IR cDNA and IRS-1 cDNA into Cos cells. Cos cells were transfected by calcium phosphate precipitation with 3 μg each of IR cDNA and IRS-1 cDNA inserted in an expression vector. IR cDNA was subcloned into pSV2-Neo expression vector; IRS-1 cDNA was subcloned into pRC-CMV expression vector. After 48 h, cells were stimulated with 10^{-7} M insulin, and cell lysates were prepared.

Immunoblotting. CHO cells overexpressing human IR (CHO-HIR cells) were usually used. The cells were lysed in buffer B (see above), and lysates of CHO-HIR (or CHO-HIR-Csk) cells with or without 10^{-7} M insulin treatment were incubated with αCsk-bound protein A-Sepharose. The immunoprecipitates were washed with buffer B three times and subjected to SDS-PAGE followed by Western blotting (immunoblotting) with RC20 (Transduction Laboratories) or PY20 and detection using an anti-mouse immunoglobulin G-alkaline phosphatase conjugate system (Sigma).

Bacterial fusion proteins. GST-Csk SH3/2 and GST fused to Csk with a mutation in the SH2 domain [GST-Csk SH3/2 (S108C)], encoding amino acids 9 to 189 (SH3 and SH2 domains), were prepared as described previously (40, 51).

In vitro binding of GST-Csk SH3/2 fusion proteins to phosphotyrosine-containing proteins. GST-Csk SH3/2 fusion proteins on glutathione-agarose beads were incubated with cell lysates from CHO-HIR cells treated or not treated with insulin. The fusion proteins on the beads were washed with buffer B three times and subjected to SDS-PAGE followed by Western blotting with αPY.

CHO cells expressing mutant IRs. CHO cells expressing mutant IRs were prepared as described above. We used three mutant IRs: (i) YF960IR, in which phenylalanine was substituted for Tyr-960 (21), (ii) YF1316,1322IR, in which phenylalanine was substituted for both Tyr-1316 and Tyr-1322 (1), and (iii) Δ82IR, in which the carboxyl-terminal 82 amino acids were deleted (66). All CHO cell lines expressing mutant IRs have almost the same number of receptors as CHO-HIR cells.

RESULTS

Csk enhances insulin-induced dephosphorylation of pp125^{FAK}. Since Csk has been shown to bind two major tyrosine-phosphorylated focal adhesion proteins, pp125^{FAK} and paxillin (40), we initiated our study to examine whether Csk affects the insulin-induced dephosphorylation of pp125^{FAK}. We established Csk-overexpressing CHO-HIR cells (CHO-HIR-Csk cells), which already expressed ectopic human IR (21). In CHO-HIR cells, insulin treatment resulted in pp125^{FAK} dephosphorylation that reached a maximum at 5 to 15 min (Fig.

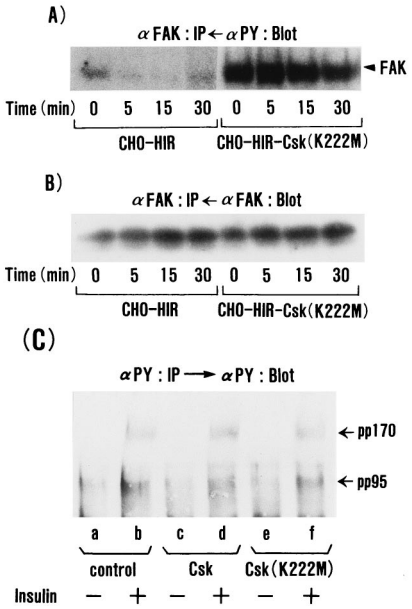

FIG. 2. Insulin-induced dephosphorylation of pp125^{FAK} is not observed in CHO-HIR-Csk (K222M) cells. CHO-HIR or CHO-HIR-Csk (K222M) cells without or with 10^{-7} M insulin treatment for the indicated time periods were lysed. The lysates were subjected to immunoprecipitation (IP) with αFAK followed by immunoblotting with αPY (A) or αFAK (B). (C) CHO-HIR, CHO-HIR-Csk, or CHO-HIR-Csk (K222M) cells without or with 10^{-7} M insulin treatment for 5 min were lysed with buffer B. The lysates were immunoprecipitated with PY20, and the immunoprecipitates were washed with buffer B three times and subjected to immunoblotting with αPY.

1A), as previously shown (36). However, the tyrosine phosphorylation of pp125^{FAK} at 30 min after the insulin treatment returned to almost the same level as that in untreated cells (Fig. 1A). In contrast, in CHO-HIR-Csk cells, the insulin-induced tyrosine dephosphorylation of pp125^{FAK} seemed to be more profound and prolonged; a maximal decrease in tyrosine phosphorylation was observed with 5 to 30 min of insulin treatment and did not return to the original level even 60 min after the treatment (Fig. 1A). The amounts of pp125^{FAK} were unchanged during the assay (Fig. 1B). These results were reproducible in at least two independent clones.

To further examine the role of Csk in insulin-induced pp125^{FAK} dephosphorylation, the effect of the expression of kinase-negative Csk (K222M) was examined (Fig. 2). Even in the absence of insulin stimulation, tyrosine phosphorylation of pp125^{FAK} in CHO-HIR-Csk (K222M) cells was much greater than that in the parental CHO-HIR cells (Fig. 2A). Moreover, insulin failed to dephosphorylate pp125^{FAK} in the CHO-HIR-Csk (K222M) cells (Fig. 2A). Even a longer treatment with insulin did not stimulate the dephosphorylation state of FAK in CHO-HIR-Csk (K222M) cells (Fig. 2A). The amounts of pp125^{FAK} immunoprecipitated were unchanged during the assay (Fig. 2B). Thus, the K222M mutant form of Csk appears to exhibit a dominant negative effect on the insulin-mediated dephosphorylation of pp125^{FAK}. These results were reproducible in at least two independent clones. We did not observe a significant change in insulin-induced tyrosine phosphorylation of IR β subunit and IRS-1 in CHO-HIR, CHO-HIR-Csk, and CHO-HIR-Csk (K222M) cells (Fig. 2C).

Csk enhances insulin-induced dephosphorylation of paxillin. Paxillin, another tyrosine-phosphorylated focal adhesion protein, has been implicated in the formation of focal adhesions and can bind Csk (40). We next examined the effect of

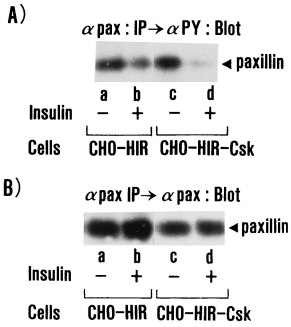

FIG. 3. Insulin stimulates dephosphorylation of paxillin more significantly in CHO-HIR-Csk cells than in CHO-HIR cells. CHO-HIR or CHO-HIR-Csk cells without or with 10^{-7} M insulin treatment for 5 min were lysed in buffer B, and the lysates were immunoprecipitated (IP) with αpaxillin. The immunoprecipitates were washed and subjected to SDS-PAGE followed by immunoblotting with αPY (A) or αpaxillin (B).

insulin treatment on the tyrosine phosphorylation of paxillin. In CHO-HIR cells, insulin dephosphorylated paxillin within 5 min, and it returned to its original level within 15 min (Fig. 3 and data not shown). In Csk-overexpressing cells, on the other hand, the decrease in phosphorylation was more significant and prolonged, as observed in pp125^{FAK} (Fig. 3 and data not shown). We also examined the insulin-induced dephosphorylation of paxillin in cells overexpressing kinase-negative Csk (K222M) (Fig. 4). As in the case of pp125^{FAK}, insulin-induced dephosphorylation was blocked by overexpression of Csk (K222M) (Fig. 4). During the assay, the amounts of paxillin immunoprecipitated were unchanged (Fig. 3B and 4B). These results were reproducible in at least two independent clones. This finding again suggested the involvement of Csk in the insulin-induced dephosphorylation of paxillin; insulin can dephosphorylate both pp125^{FAK} and paxillin in a Csk-dependent manner.

Tyrosine phosphorylation of IRS-1 parallels the insulin-induced tyrosine dephosphorylation of pp125^{FAK} and paxillin. We then explored the signaling pathway connecting the activation of IR kinase and the recruitment of Csk that leads to the modulation of focal adhesion proteins. CHO cells expressing three mutant forms of IRs were used (Fig. 5 and 6). YF960IR, in which a juxtamembrane tyrosine residue (Tyr-960) was changed to phenylalanine, can be autophosphorylated normally but fails to recognize IRS-1 (21, 63); YF1316,1322IR, in which carboxyl-terminal autophosphorylation sites (Tyr-1316

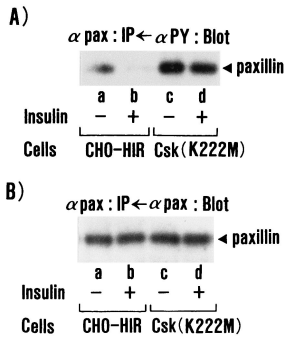

FIG. 4. Insulin-induced dephosphorylation of paxillin is not observed in CHO-HIR-Csk (K222M) cells. CHO-HIR or CHO-HIR-Csk cells without or with 5 min of 10^{-7} M insulin stimulation were lysed. The lysates were subjected to immunoprecipitation (IP) with αpaxillin followed by immunoblotting with αPY (A) or αpaxillin (B).

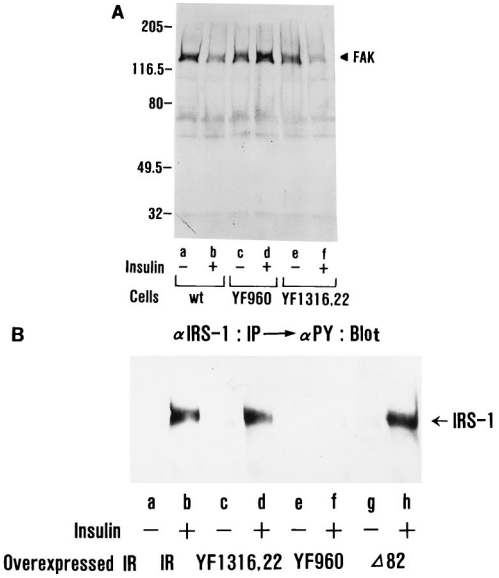

FIG. 5. Insulin stimulates dephosphorylation of pp125^{FAK} in CHO cells expressing wild-type (wt) human IRs or YF1316,1322IR but not in cells expressing YF960IR. (A) CHO-HIR (lanes a and b), CHO-HIR(YF960IR) (lanes c and d), or CHO-HIR(YF1316,1322IR) (lanes e and f) cells were stimulated with 10^{-7} M insulin or left unstimulated, and the lysates were subjected to immunoprecipitation with αFAK followed by immunoblotting with αPY. Sizes are indicated in kilodaltons. (B) CHO-HIR, CHO-HIR(YF960IR), CHO-HIR(YF1316,1322IR), and CHO-HIR(Δ82IR) cells were stimulated with 10^{-7} M insulin for 5 min or left unstimulated. The cells were lysed, and the lysates were subjected to immunoprecipitation (IP) with αIRS-1. The immunoprecipitates were washed and subjected to immunoblotting with αPY.

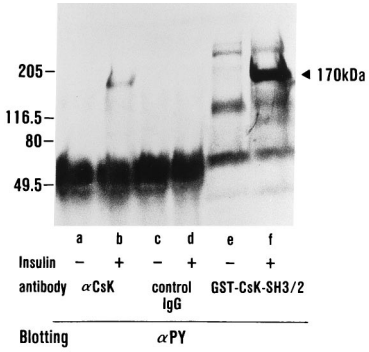

FIG. 7. Insulin stimulates association of IRS-1 with Csk in CHO-HIR cells. CHO-HIR cells without or with 10^{-7} M insulin treatment for 5 min were lysed, and the lysates were immunoprecipitated with control serum (lanes c and d) or αCsk (lanes a and b). The immunoprecipitates were subjected to immunoblotting with αPY. The lysates were incubated with the fusion protein GST-Csk SH3/2 bound to glutathione-agarose; the beads were washed with buffer B three times and subjected to SDS-PAGE followed by immunoblotting with αPY (lanes e and f). IgG, immunoglobulin G.

and Tyr-1322) were changed to phenylalanine, has a reduced autophosphorylation level but recognizes IRS-1 normally (1); Δ82IR, in which carboxyl-terminal 82 amino acids were deleted, lacks the ability to be autophosphorylated but recognizes IRS-1 normally (66). We observed an insulin-stimulated dephosphorylation of pp125^{FAK} (Fig. 5 and data not shown) as well as paxillin (Fig. 6) in CHO-HIR, CHO-HIR(YF1316, 1322IR), and CHO-HIR(Δ82IR) cells but not in CHO-HIR (YF960IR) cells. These results were reproducible in at least two independent clones. While tyrosine phosphorylation of IRS-1 by insulin was significantly increased in CHO-HIR, CHO-HIR(YF1316,1322IR), and CHO-HIR(Δ82) cells, it was not stimulated in CHO-HIR(YF960IR) cells (Fig. 5B). It therefore appears that IRS-1 may be an important downstream factor of the IR for the dephosphorylation of focal adhesion proteins. The autophosphorylation of IR may not be essential for this signaling.

Csk binds to tyrosine-phosphorylated IRS-1 in intact cells. Csk has an SH2 domain which can bind to various tyrosine-phosphorylated proteins. Since IRS-1 has multiple tyrosine phosphorylation motifs, we next examined the binding of Csk to IRS-1. We observed one substantial band of tyrosine-phosphorylated protein with a molecular mass of 170 kDa, which showed the same migration as IRS-1 on this SDS-polyacrylamide gel (data not shown), in αCsk immunoprecipitates from insulin-stimulated but not unstimulated CHO-HIR cell lysates (Fig. 7). Moreover, when Cos cells were transfected with both IR cDNA and IRS-1 cDNA and treated with insulin, a 170-kDa tyrosine-phosphorylated protein was also detected (Fig. 8). With αIRS-1 immunoprecipitates from CHO-HIR-Csk cell lysate, Csk was detected only after cells were stimulated with insulin (Fig. 9). These data showed a physical complex formation of Csk with IRS-1 in vivo only after insulin stimulation.

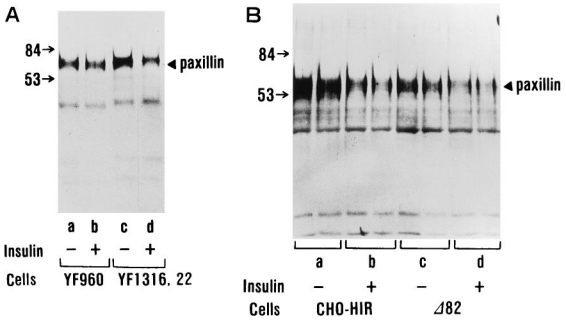

FIG. 6. Insulin stimulates dephosphorylation of paxillin in CHO cells expressing wild-type human IR, YF1316,1322IR, and Δ82IR but not in cells expressing YF960IR. CHO-HIR, CHO-HIR(YF960IR), CHO-HIR(YF1316, 1322IR), or CHO-HIR(Δ82IR) cells were stimulated with 10^{-7} M insulin or left unstimulated, and the lysates were subjected to immunoprecipitation with αpaxillin followed by immunoblotting with αPY. The duplicated samples were loaded in panel B. Sizes are indicated in kilodaltons.

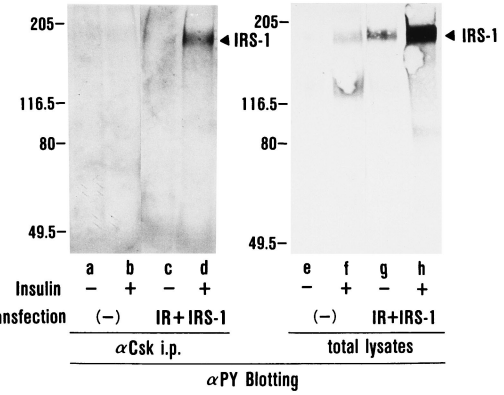

FIG. 8. Insulin stimulates association of IRS-1 with Csk in Cos cells transfected with IR cDNA and IRS-1 cDNA. Cos cells that had not (lanes a and b) or had (lanes c and d) been transfected with IR cDNA and IRS-1 cDNA were stimulated with 10^{-7} M insulin for 5 min or left unstimulated. The cells were lysed, and the lysates were immunoprecipitated (i.p.) with αCsk followed by immunoblotting with αPY. Sizes are indicated in kilodaltons.

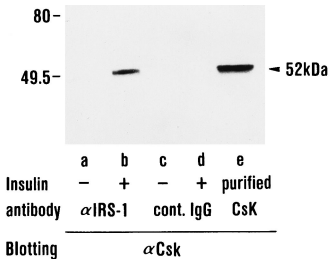

FIG. 9. Insulin stimulates IRS-1–Csk complex formation in CHO-HIR-Csk cells. Cell lysates from CHO-HIR-Csk cells not treated (lane a and c) or treated with 10^{-7} M insulin (lanes b and d) were subjected to immunoprecipitation with control immunoglobulin G serum (cont. IgG; lanes c and d) or αIRS-1 (lanes a and b) covalently coupled with Affi-Gel 10. The immunoprecipitates were subjected to immunoblotting with αCsk.

The SH2 domain of Csk is required for the association of IRS-1 with Csk. We also studied the tyrosine-phosphorylated proteins associated with Csk by using GST-Csk SH3/2 recombinant protein (Fig. 7, lanes e and f). Without insulin stimulation, 125- and 70-kDa tyrosine-phosphorylated proteins were observed to be associated with GST-Csk SH2/3 proteins, as previously found in assays using fibroblasts (40) (Fig. 7 and data not shown). After insulin stimulation, additional tyrosine-phosphorylated proteins, a protein with a molecular mass of 170 kDa, which is possibly IRS-1, and the 95-kDa protein, which was probably IR β subunit, were observed (Fig. 7, lanes e and f). It is noteworthy that tyrosine phosphorylation of those GST-Csk SH3/2-associated 125- and 70-kDa proteins was lower after insulin stimulation. The levels of all of these Csk SH2-associated proteins were diminished when GST-Csk SH3/2 (S108C) was used (Fig. 10), suggesting that these associations are primarily mediated by the Csk SH2 domain. The broad band migrating at 80 to 90 kDa may be nonspecific and may react with GST-SH2/3 Csk or GST-Csk SH3/2 (S108C) fusion protein.

DISCUSSION

It has recently been shown that insulin treatment of cells results in dephosphorylation of pp125^{FAK} (36, 67) that parallels the decrease in number and length of actin stress fibers (25). The mechanism by which insulin stimulates dephosphorylation of FAK, however, is not known. In this study, we showed that overexpression of wild-type Csk enhances insulin-stimulated dephosphorylation of pp125^{FAK} and paxillin, and expression of kinase-negative Csk (K222M) blocks insulin-stimulated dephosphorylation of these two proteins. Since overexpression of wild-type Csk or kinase-negative Csk (K222M) does not affect the insulin-stimulated tyrosine phosphorylation of IR or IRS-1 (Fig. 2C), Csk acts downstream of IRS-1 and may somehow regulate the tyrosine phosphorylation of FAK.

How is Csk involved in insulin-induced dephosphorylation of FAK lying downstream of IRS-1? We have demonstrated that Csk directly binds to tyrosine-phosphorylated IRS-1 in insulin-stimulated cells. Thus, a major tyrosine-phosphorylated protein observed in an αCsk immunoprecipitate was IRS-1 in insulin-stimulated cells, although it was shown that Csk complexed with FAK and paxillin (40); in fact, GST fusion protein containing an intact SH2 domain of Csk can bind these two focal adhesion proteins. In Cos cells transfected with IR cDNA and IRS-1 cDNA, we also demonstrated the presence of an IRS-1–Csk complex after insulin stimulation. Moreover, Csk was observed in an αIRS-1 immunoprecipitate after insulin stimulation. Finally, we demonstrated that an intact SH2 domain is required for the binding of Csk to IRS-1. These data suggested that insulin stimulates IRS-1–Csk complex formation in intact cells via SH2-phosphotyrosine binding. It seems that Csk may be involved in insulin-stimulated tyrosine dephosphorylation of FAK by complexing with IRS-1.

The importance of the IRS-1–Csk complex in insulin-induced dephosphorylation of FAK is supported by the analysis of cells expressing mutant IRs. We have shown that in cells expressing mutant IRs, tyrosine phosphorylation of IRS-1, rather than IR autophosphorylation itself, is more closely correlated with the ability to mediate the insulin-stimulated dephosphorylation of pp125^{FAK}. These data are consistent with our notion that the IRS-1–Csk complex plays an important role in regulating insulin-induced dephosphorylation of FAK. However, our findings differ from a previous report by Pillay et al. (36), who found that neither a mutant IR lacking the two C-terminal tyrosine phosphorylation sites nor a deletion mutant lacking the distal 43 amino acids of the β subunit (ΔCT cells), in which tyrosine phosphorylation of IRS-1 is almost normal despite the decrease in tyrosine autophosphorylation of IR, is able to mediate the insulin-stimulated dephosphorylation of FAK. It may be possible that if the amount of human IR expressed is much greater, and that of the endogenous IRS-1 expressed is much lower, in the Rat-1 fibroblasts that Pillay et al. used than in our cells, then the major tyrosine-phosphorylated protein complexed with Csk becomes the IR despite lower-affinity binding to Csk than to IRS-1. In that case, the Csk-insulin receptor complex may play some part in FAK dephosphorylation by insulin.

How is the IRS-1–Csk complex involved in insulin-induced dephosphorylation of FAK? It has been shown that the increase in phosphorylation of FAK is closely related to the activation of c-Src kinase activity at focal adhesions. It has been postulated that Src repression involves folding such that the SH2 domain and the phosphorylated tail interact. When c-Src is activated by biological stimuli and both SH2 and SH3 domains are available, c-Src is redistributed from the perinuclear region to adhesion plaques, probably through the binding of the SH2 domain of c-Src to the Tyr-397 autophosphorylation site of FAK in the YAEI motif (44). In turn, the binding of the SH2 domain of c-Src could either protect the FAK autophosphorylation site from the attack of a phosphotyrosine phosphatase(s) at focal adhesions or phosphorylate the other sites of FAK on tyrosine residues. Thus, phosphorylation of FAK seems to be closely related to the activity of c-Src kinase at

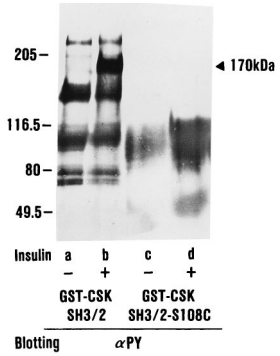

FIG. 10. Intact SH2 domain of Csk is required for Csk–IRS-1 complex formation. Cell lysates from CHO-HIR cells without or with 10^{-7} M insulin treatment were incubated with GST-Csk SH3/2 (lanes a and b) or GST-Csk SH3/2-S108C (lanes c and d) fusion protein on glutathione-Sepharose beads. The bound proteins on beads were washed and subjected to immunoblotting with αPY.

focal adhesions. The dephosphorylation of FAK by insulin may reflect the decrease in c-Src kinase activity at focal adhesions.

On the other hand, it has been shown that for Csk to negatively regulate c-Src, Csk should be located where active c-Src is located (19). It seems that the increase in the amount of functional Csk at focal adhesions is closely associated with the decrease in tyrosine phosphorylation of FAK via the regulation of c-Src kinase activity, and vice versa. In fact, we demonstrated that the increase in tyrosine phosphorylation of FAK and paxillin was observed when kinase-negative Csk (K222M) was overexpressed (Fig. 2 and 4), in which case FAK- or paxillin-bound endogenous functional Csk was replaced with kinase-negative Csk (K222M), thus leading to the activation of c-Src at focal adhesions. A recent study by Vuori et al. reported that insulin stimulated association of IRS-1 with the $\alpha_V\beta_3$ integrin or the vitronectin receptor (61), suggesting the possibility that tyrosine-phosphorylated IRS-1 is targeted to focal adhesions. If the IRS-1–Csk complex is involved in the dephosphorylation of FAK by insulin, this complex should be localized to focal adhesions; there, Csk on the tyrosine-phosphorylated IRS-1 could efficiently phosphorylate the carboxyl-terminal tail of c-Src which was bound to the FAK molecule, thereby down-regulating the c-Src kinase activity. However, we did not observe an insulin-induced decrease in c-Src kinase activity in either CHO-HIR or CHO-HIR-Csk cells, although c-Src kinase activity in the latter cells is 70% less than in the former cells (data not shown). This may be because the amount of c-Src localized in focal adhesions and regulated by the IRS-1–Csk complex is so small that we are not able to detect the decrease in c-Src kinase activity in the immunoprecipitation assay of total lysates. If we had been able to measure the activity of c-Src solely at focal adhesions, that activity could have been decreased.

We demonstrated that paxillin is also dephosphorylated by insulin in a Csk-dependent manner. Paxillin and FAK have been shown to become coordinately phosphorylated on tyrosine in response to a number of stimuli. Paxillin was tyrosine phosphorylated by c-Src kinase in vitro (40), and tyrosine phosphorylation of paxillin is FAK dependent both in vitro and in vivo (46). Furthermore, analysis of cell lines from Csk knockout mice and Csk/c-Src or Csk/c-Fyn double-knockout mice revealed that paxillin is a substrate for multiple Src family kinases at focal adhesions (56). Together with the previous reports, the data that insulin dephosphorylates FAK and paxillin in a coordinate fashion are consistent with our model that Csk on IRS-1 efficiently phosphorylates carboxyl-terminal tyrosines of c-Src family kinases bound to FAK at focal adhesions, thereby down-regulating the activities of these kinases and leading to a decrease in the tyrosine phosphorylation of substrates, including paxillin.

It is of note that the basal phosphorylation of FAK is dramatically enhanced when kinase-inactive Csk is overexpressed (Fig. 2A) but is not changed when YF960IR is overexpressed (Fig. 5), although both mutants inhibit insulin-induced dephosphorylation of FAK. There may be different mechanisms by which two different mutants inhibit insulin-induced dephosphorylation of FAK. The reason why overexpression of kinase-inactive Csk resulted in the dramatic increase in the basal phosphorylation of FAK may be as follows. When kinase-negative Csk is overexpressed, FAK- or paxillin-bound endogenous Csk that suppresses the kinase activity of c-Src family kinases at focal adhesions is replaced by kinase-negative Csk. Since kinase-negative Csk failed to suppress the c-Src kinase activity, the c-Src kinase activity was increased, thereby leading to the increase in FAK and paxillin phosphorylation under the basal conditions. Thus, it seems that FAK phosphorylation is negatively correlated with the amount of functional Csk at focal adhesions. After insulin stimulation, IRS-1 binds kinase-negative Csk that does not mediate the insulin-induced dephosphorylation of FAK. In contrast, the FAK phosphorylation state is not influenced by the overexpression of wild-type or mutant IR in the absence of insulin, as shown in Fig. 5. That may be because overexpression of any type of IR, either wild type or mutant, does not influence the amount of functional Csk at focal adhesions. That may be the reason why the basal phosphorylation state of FAK is not changed in cells overexpressing YF960IR. Since insulin fails to phosphorylate IRS-1 in cells overexpressing YF960IR and Csk–IRS-1 complex formation does not occur, insulin does not induce FAK dephosphorylation.

Syp (PTP1D, SHPTP2, PTP2C), a phosphotyrosine phosphatase that binds to tyrosine phosphorylated IRS-1, may be a candidate for a regulator of FAK dephosphorylation, although this is speculative. Yamauchi et al. reported that the expression of a dominant negative Syp/PTP1D does not inhibit the insulin-induced dephosphorylation of FAK, although the tyrosine phosphorylation of FAK is increased in cells overexpressing a dominant negative Syp/PTP1D (67). Thus, it seemed that Syp/PTP1D is not involved in insulin-induced FAK dephosphorylation.

We have been focusing on regulation of the kinase activity of c-Src, not c-Fyn, because of the unavailability of a good assay system with which to measure the c-Fyn kinase activity. However, it has been shown that the SH2 domain of c-Fyn has a higher affinity for the tyrosine autophosphorylation site of FAK than that of c-Src, and the analysis of Csk knockout mice and Csk/c-Src and Csk/c-Fyn double-knockout mice revealed that both c-Src and c-Fyn are involved in regulation of the phosphorylation of FAK and paxillin (56). The role of c-Fyn in this system should be further clarified.

The results of this study demonstrated that (i) overexpression of wild-type Csk resulted in the enhancement of insulin-stimulated dephosphorylation of FAK and paxillin, (ii) overexpression of kinase-negative Csk (K222M) inhibited the insulin-induced dephosphorylation of FAK and paxillin, and (iii) insulin stimulates the formation of the IRS-1–Csk complex and suggested that the IRS-1–Csk complex may play an important role in mediating the insulin-stimulated dephosphorylation of FAK and paxillin. Insulin has been thought to be a hormone that regulates carbohydrate, lipid, and protein metabolism. However, our data suggest an additional role: insulin may regulate cytoskeletal reorganization, cell attachment to an ECM, cell migration at the individual cell level, and morphogenesis at the organism level. Our data also suggested that growth factors may regulate the phosphorylation of FAK and c-Src kinase activity at focal adhesions by complex formation of Csk with growth factor receptor tyrosine kinases or their substrates.

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