Leukocyte tyrosine kinase (LTK) is a receptor tyrosine kinase, which belongs to the insulin receptor family and is mainly expressed in pre-B cells and brain. In this study, we show that LTK utilizes insulin receptor substrate-1 (IRS-1) and Shc as major two substrates and possesses two NPXY motifs for them separately, tyrosine 485 of one NPXY motif at the juxtamembrane domain for IRS-1 and tyrosine 862 of another NPXY motif at the carboxyl-terminal domain for Shc. By using Ba/F3 cells expressing epidermal growth factor receptor-LTK chimeric receptors containing a mutation at each NPXY site, we showed that while both NPXY motifs equally contribute to activation of the Ras pathway and generation of mitogenic signals, only tyrosine 485 of LTK transmits cell survival signals. These data suggest that IRS-1 possesses anti-apoptotic function at least in LTK signaling. Moreover, our data indicate that the survival signaling pathway of LTK is distinct from the Ras pathway and the p70S6 kinase pathway. Our results provide a useful insight in understanding the distinctive roles of Shc and IRS-1 in the signal transduction system of the insulin receptor family, and this anti-apoptotic function of IRS-1 may explain the survival effects of insulin, IGF-1, and interleukin 4.

Although the intracellular signaling pathways of insulin receptor and insulin-like growth factor-1 (IGF-1) receptor have been well investigated, those of other members of insulin receptor family including leukocyte tyrosine kinase (LTK) are poorly understood. LTK is a receptor tyrosine kinase, which belongs to the insulin receptor family and is mainly expressed in pre-B cell, brain, placenta, and several hematopoietic cell lines (1–4). Recently ALK tyrosine kinase, which has 64% homology with LTK in the amino acid sequence, was cloned by using Ba/F3 cells expressing epidermal growth factor receptor-ALK chimeric receptors containing a mutation at each NPXY site. While the major binding site for Shc is tyrosine 862 at the carboxyl-terminal domain of LTK (6). In this study, we showed that LTK also utilizes IRS-1 as a substrate. Both of these tyrosines are located in the NPXY motifs that are recently identified as the consensus sequence of binding sites for the PTB domains of Shc and IRS-1.}

Many growth factors such as insulin (11), IGF-1 (12, 13), interleukin 4 (IL4) (14), IL9 (15), IL13 (16), and growth hormone (17, 18) have been shown to utilize insulin receptor substrate-1 (IRS-1) as a substrate. Although IRS-1-deficient mice showed mild growth retardation and glucose intolerance (19, 20), recently identified insulin receptor substrate 2 (IRS-2) is suggested to compensate the function of IRS-1 (21). Thus, it is revealed to be difficult to analyze the biological function of IRS-1 by using IRS-1-disrupted mice. Recent studies have shown that the phosphotyrosine-binding (PTB) domains of IRS-1 and Shc bind to the NPXY motif located at the juxtamembrane domain of insulin receptor β subunit (7). The mutation at tyrosine 960 in the NPXY motif abolishes phosphorylation of both IRS-1 and Shc (22, 23). In the insulin receptor signaling system, therefore, it is difficult to analyze the IRS-1-specific signals. In contrast, the binding sites of LTK for Shc and IRS-1 are separated; therefore, we have found that the LTK signaling system provides a useful tool to investigate the function of IRS-1 and Shc distinctively.

By comparing the growth of cells that express mutant chimeric receptors containing a mutation at each NPXY site, we showed that both NPXY sequences, which are binding sites for Shc and IRS-1, contribute equally to activation of the Ras pathway and generation of mitogenic signals, while only tyrosine 485, which is critical for IRS-1 phosphorylation, generates cell survival signal. In this respect, at least in LTK signaling, IRS-1 is suggested to play a critical role in preventing cells from apoptotic death. Our results provide a useful insight in

**References**

1. The abbreviations used are: IGF-1, insulin-like growth factor-1; LTK, leukocyte tyrosine kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; IRS-1, insulin receptor substrate-1; IL, interleukin; PAGF, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline-Triton X-100; MBP, myelin basic protein; PTB, phosphotyrosine-binding; FCS, fetal calf serum; MAP, mitogen-activated protein.
Materials and Methods

Antibodies and Cell Lines—Anti-LTK monoclonal antibody ID3-1, which recognizes the carboxyl-terminal domain of LTK, was produced as described previously (4). The monoclonal antibody directed against the extracellular domain of EGF-receptor (Ab-1) and anti-Grb2/Ash monoclonal antibody (3F2) were purchased from Oncogene Science Inc. and MBL Inc., respectively. The rabbit anti-Shc antibody and the anti-phosphotyrosine monoclonal antibody 4G10 were purchased from Upstate Biotechnology Inc. Anti-IRS-1 antibody B51 was provided by T. Kadowaki and J36 was a gift from M. Nishiyama. Anti-Syp antibody was a gift from T. Pawson.

Human embryonic kidney fibroblast 293 cells (Japanese Cancer Research Resources Bank; CRL1573) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (FCS). Ba/F3 cells were cultured in RPMI 1640 containing 10% FCS and 0.25 unit/ml aprotinin.

Construction of cDNAs for EGF-LTK Chimeric Receptors and LTK Mutant—The EGF-LTK chimeric receptor (EL) cDNA was constructed by ligating the extracellular domain of human EGFRI with the transmembrane and the cytoplasmic domains of LTK. Tyrosine-phenylalanine LTK mutants Y485F, Y721F, Y753F, Y779F, Y862F, and Y485F/Y862F were generated as described elsewhere (6). To construct mutant chimeric receptors, the HindIII-EcoRI fragments of the mutant LTK cDNAs which encode the cytoplasmic domain were substituted for that of wild-type EL receptor cDNA.

Transfection—Transfection into 293 cells was carried out according to the protocol of Chen and Okayama (24). Eighteen hours after transfection, cells were washed once with Dulbecco’s modified Eagle’s medium and cultured in fresh medium containing 5% FCS for 24 h.

Retrovirus vector was used to transfect cDNAs for chimeric receptors into Ba/F3 cells. Replication-deficient retroviral stocks were prepared by transient hyperexpression in COS7 cells. These constructs were transfected together with v-packaging plasmid by the DEAE-dextran method (25). Viral infections were performed by exposing cells to virus stocks with 8 μg of Polybrene/ml at 37 °C for 12 h, and G418-resistant populations were selected in the presence of 1000 μg of G418/ml for at least 3 weeks following 2 days of infection.

Immunoprecipitation, Immunoblotting, and in Vitro Kinase Assay—Prior to stimulation, cells were starved in RPMI 1640 containing 0.5% FCS for 10 h. Cells were then stimulated with 200 ng/ml EGF for 5 min at 37 °C, washed twice with ice-cold phosphate-buffered saline, and lysed with Triton X-100 (0.5% v/v), Tris-HCl pH 7.4, 2 mM phenylmethylsulfonyl fluoride, 10 units/ml aprotinin, 1 mM sodium orthovanadate, 1 mM EDTA). Cell lysates were centrifuged and the supernatant was collected.

RESULTS

The EGFR-LTK Chimeric Receptor Phosphorylates IRS-1 in a Ligand-dependent Manner, and Tyrosine 485 of LTK Is Responsible for Phosphorylation of IRS-1—Recently, it was reported that the PTB domain of IRS-1 binds to the NPXY motif encompassing tyrosine 960 of the insulin receptor (7). Because LTK belongs to the insulin receptor family, we hypothesized that LTK also utilizes IRS-1 as a substrate. To test this hypothesis, we constructed chimeric receptors composed of the extracellular domain of EGFR and the transmembrane and cytoplasmic domains of LTK, whose ligand is not identified yet. Since the expression level of IRS-1 in EL3-3, a stable line of 293 cells transfected with the chimeric receptor (6), was low (data not shown), we transiently expressed IRS-1 in EL3-3 cells. Cell lysates in the presence or in the absence of EGF were immunoprecipitated with anti-IRS-1 antibody, followed by immunoblotting with anti-phosphotyrosine antibody. IRS-1 in EL3-3 cells stimulated with EGF was found to be phosphorylated on tyrosine, whereas IRS-1 in mock cells was not (Fig. 1A), indicating that IRS-1 is a substrate of an LTK-mediated signal.

The evidence that the PTB domain of IRS-1 binds to the NPXY motif located at the juxtamembrane domain of insulin receptor led us to hypothesize that the NPXY motif located at the juxtamembrane domain of LTK is responsible for phosphorylation of IRS-1. To examine this, we transiently expressed IRS-1 and LTK in 293 cells. In this system, LTK was autophosphorylated and, at the same time, induced phosphorylation of IRS-1 (Fig. 1B). We then introduced several LTK mutant cDNAs instead of the wild-type LTK cDNA with IRS-1 cDNA. These mutants include receptors containing point mutations at tyrosines 485, 721, 753, 779, and 862 or at both tyrosines 485 and 862 to phenylalanine (Y485F, Y721F, Y753F, Y779F, Y862F, and Y485F/Y862F) or at lysine 544 to methionine (K544M). The K544M LTK mutant is known to be a kinase-inactive form of LTK (27). The expression of these LTK mutants were confirmed by the immunoblot with anti-LTK monoclonal antibody, ID3-1. As expected, all these mutants were autophosphorylated on tyrosine residues except for the K544M mutant. In this experiment, Y485F, Y485F/Y862F, and K544M LTK mutants could not phosphorylate IRS-1, although the expression levels of IRS-1 were similar to one another (Fig. 1B). These results indicate that phosphorylation of IRS-1 is dependent on the LTK kinase activity and that tyrosine 485 located at the juxtamembrane domain of LTK is responsible for the phosphorylation of IRS-1.

The binding motifs for the PTB domains of IRS-1 and Shc are aligned in Fig. 1C. Although both of the PTB domains recognize the NPXY sequence, there should be specificity for them. For example, the PTB domain of Shc specifically binds to the NPXY site of TrkA (Tyr-490), while the PTB domain of IRS-1 specifically recognizes the NPXY sequence of the α chain of IL4 receptor (Tyr-497) (7). From our data, we propose that, except for the NPXY sequence, leucine or isoleucine at −8 position seems critical for the specific binding to the PTB domain of IRS-1. Moreover, as Batzer et al. proposed (28), leucine or...
tyrosines were mutated to phenylalanine; EL3-3 cells transfected with IRS-1 cDNAs were stimulated with or without EGF, lysed, and immunoprecipitated with anti-IRS-1 antibody (J36). The immunoprecipitates were subjected to the immunoblotting with anti-Syp antibody (upper panel) or anti-Grb2 antibody (3F2) (lower panel).

IRS-1 Phosphorylated by the Chimeric Receptor Associates with Grb2 and Syp in Vivo in a Ligand-dependent Manner—To test whether IRS-1 in EL3-3 cells treated with EGF can transmit signals, we examined association between IRS-1 and several signaling molecules that have been shown to associate with IRS-1 in the insulin receptor signaling system. The immunoprecipitates with anti-IRS-1 antibody were subjected to the immunoblotting with appropriate antibodies. In this experiment, Grb2 and Syp were found to associate with IRS-1 in vivo in a ligand-dependent manner (Fig. 2). These data suggest that IRS-1 phosphorylated by LTK utilizes the same signaling molecules as were used in the insulin receptor system.

The signaling pathway downstream of LTK is summarized in Fig. 3. Two independent signaling pathways through LTK activate Ras. Shc binds to tyrosine 862 of LTK and connects LTK and the Grb2-Sos complex in a growth factor-dependent manner (6). Tyrosine 485 of LTK is essential for phosphorylation of IRS-1. IRS-1, then, associates with Syp and Grb2 in a ligand-dependent manner. In this scheme, both Shc and IRS-1 can contribute to activation of the Ras pathway, because they associate with Grb2, which is an adaptor molecule linking receptor tyrosine kinases with the Ras signaling pathway. This scheme has some similarity to that of insulin receptor in that it utilizes IRS-1 and Shc as major substrates, whereas the difference is that binding sites in LTK for IRS-1 and Shc are separated.

Construction and Expression of Mutant Chimeric Receptors Containing a Mutation at Each NPYXY Site and Ligand-induced Autophosphorylation of These Receptors and Transphosphorylation of Shc—To investigate the roles of tyrosines 485 and 862 in the LTK signaling pathway, we constructed mutant EGFR-LTK chimeric receptors in which each tyrosine or both are substituted for phenylalanine. These mutant receptors were designated as EL-Y485F, EL-Y862F, and EL-Y485F/Y862F (Fig. 4A). Stable transfectants of Ba/F3, a mouse interleukin 3 (mIL3)-dependent cell line derived from a mouse pro-B cell that does not express endogenous EGFR, were produced by retrovirus vectors carrying the wild-type and mutant cDNAs (EL, EL-Y485F, EL-Y862F, and EL-Y485F/Y862F) (Fig. 4A). Stable transfectants of Ba/F3, a mouse interleukin 3 (mIL3)-dependent cell line derived from a mouse pro-B cell that does not express endogenous EGFR, were produced by retrovirus vectors carrying the wild-type and mutant cDNAs (EL, EL-Y485F, EL-Y862F, and EL-Y485F/Y862F). We confirmed the expression of the 140-kDa chimeric receptor by a combination of immunoprecipitation with anti-EGFR antibody and immunoblotting with 1D3-1. The expression levels of these chimeric receptors were approximately similar among a series of the mutants (Fig. 4B). To investigate the ability of EGF to induce autophosphorylation of these receptors in Ba/F3 cells, the stable transfectants were starved for 10 h in medium containing 0.5% FCS, and stimulated with 200 ng/ml EGF for 5 min at 37 °C. Cells were then lysed and immunoprecipitated with anti-EGFR antibody and subjected to the immunoblotting with anti-phosphotyrosine monoclonal antibody 4G10. As shown in Fig. 5A, 140-kDa tyrosine-phosphorylated proteins were detected in cells expressing chimeric molecules when...
stimulated with EGF, whereas tyrosine-phosphorylated proteins were detected in mock cells treated with EGF.

To confirm the expressed receptors possess the mutation at the Shc binding site, we examined the ability of these receptors to phosphorylate Shc. As expected from the receptor constructions, EL-Y485F and EL-Y862F/Y862F receptors could not phosphorylate Shc on tyrosine residues (Fig. 5B). The phosphorylation of Shc by EL-Y485F, EL-Y862F, EL-Y485F/Y862F, and mock cells were stimulated with or without EGF, lysed, and immunoprecipitated with anti-Shc antibody. The immunoprecipitates were subjected to immunoblotting with 4G10. Furthermore, the kinase activity of MAP kinase in these cell lines was evaluated by an in vitro kinase assay using immunoprecipitates with anti-MAP kinase antibody and MBP as a substrate. We confirmed that the result was consistent with the level of tyrosine phosphorylation of MAP kinase (Fig. 6C). These data indicate that the EGFR-LTK chimera receptor can activate the Ras pathway and generate mitogenic signals in Ba/F3 cells, whereas the mutant receptor in which two NPXY motifs were disrupted cannot. In summary, these data suggested that, in LTK signaling, IRS-1 and Shc contribute equally to the mitogenic signals and the activation of the Ras pathway.

Tyr-485 Is Critical for the Anti-apoptotic Activity of the Chimeric Receptor in Ba/F3 Cells—Since IRS-1 and Shc are not tyrosine-phosphorylated in these stable transfectants cultured in the medium containing EGF but not mIL3, we can directly compare the roles of Shc and IRS-1 in the cell growth signals by analyzing the biological feature of EL-Y485F and EL-Y862F cells. We checked the growth and morphology of the stable transfectants in the medium containing EGF but not mIL3. In this experi-
ment, mock cells immediately died within 4 days because Ba/F3 cells cannot survive without mIL3. In contrast, EL cells continuously proliferated in the presence of EGF even if mIL3 was depleted. EL-Y862F cells proliferated slower than EL cells for 96 h and gradually stopped growth. EL-Y485F cells proliferated for 24 h and then rapidly lost their viability and died. EL-Y485F/Y862F cells showed a similar growth tendency to that of EL-Y485F cells (Fig. 7A). When dying, the cell body and nuclei of EL-Y485F and EL-Y485F/Y862F cells were fragmented and they formed apoptotic bodies (Fig. 7B). The low molecular weight DNAs were extracted from these cell lines cultured in the presence of EGF for 3 days and electrophoresed in an agarose gel. Notably, the ladder pattern was observed for DNAs extracted from mock cells, EL-Y485F cells, and EL-Y485F/Y862F cells but not for those of EL cells and EL-Y862F cells (Fig. 7C). Although the MAP kinase activity of EL-Y485F cells were as same as that of EL-Y862F cells and the mitogenic activities of both cell lines were approximately equal, the sur-
vival of EL-Y862F cells were significantly prolonged compared with that of EL-Y485F cells in the presence of EGF. These results indicate that tyrosine 485 of LTK is critical for the anti-apoptotic activity of LTK and suggest that this survival signal is transmitted through IRS-1, because tyrosine 485 of LTK is the binding site for IRS-1. Furthermore, since the MAP kinase activities of EL-Y485F and EL-Y862F were comparable but the survival properties of them were completely different from each other, we can conclude that the survival signal pathway of LTK transmitted via tyrosine 485 is distinct from the Ras pathway.

The Anti-apoptotic Activity of the Chimeric Receptor Is Resistant to Rapamycin—Since tyrosine 485 of LTK is critical for IRS-1 phosphorylation, we hypothesized that the survival signal transmitted via tyrosine 485 of LTK is generated by IRS-1. However, anti-apoptotic effects of IRS-1 have not been reported so far; therefore, we tried to determine which pathway is responsible for transmitting cell survival signals. Except for the Ras pathway, it is reported that IRS-1 transmits signals activating p70S6 kinase in the insulin receptor signaling (29). Therefore, we supposed that the p70S6 kinase pathway may be involved in the survival signal of IRS-1. To test this hypothesis, we cultured EL cells and EL-Y862F cells in the medium containing rapamycin, which is an immunosuppressant known to inhibit the p70S6 kinase activity (30). Therefore, we supposed that the p70S6 kinase pathway may be involved in the survival signal of IRS-1. To test this hypothesis, we cultured EL cells and EL-Y862F cells in the medium containing rapamycin, which is an immunosuppressant known to inhibit the p70S6 kinase activity (30). Therefore, we supposed that the p70S6 kinase pathway may be involved in the survival signal of IRS-1. To test this hypothesis, we cultured EL cells and EL-Y862F cells in the medium containing rapamycin, which is an immunosuppressant known to inhibit the p70S6 kinase activity (30).

**DISCUSSION**

Growth factor receptors utilize various sets of signaling molecules downstream of them, and those signaling molecules they utilize may determine the specificity of the biological activities of the growth factors. In this study, we showed that LTK utilizes two signaling molecules, Shc and IRS-1. Recent studies have shown that both of them possess the PTB domain at the aminoterminal region and the binding sites for these domains are the NP\_X\_Y motif (7). The LTK receptor has two NP\_X\_Y motifs, tyrosine 485 at the juxtamembrane domain and tyrosine 862 at the carboxyl-terminal domain. In consistent with these findings, our data indicate that the major binding sites for Shc is tyrosine 862 (6) and tyrosine 485 is critical for IRS-1 phosphorylation. We also checked if IRS-1 directly binds to LTK in vivo, but failed to demonstrate the association between them (data not shown). This is not surprising because the interaction between insulin receptor and IRS-1 is also difficult to demonstrate by coimmunoprecipitation (31, 32), suggesting that the binding of the PTB domain of IRS-1 with the NP\_X\_Y motif may be weak or transient. The interaction between insulin receptor and IRS-1 was recently shown by the two-hybrid system (33).

Although recent studies have revealed that IRS-1 and Shc play essential roles in the signal transduction pathway of insulin receptor, their distinctive roles are not well defined. The major reason for this is that the PTB domains of IRS-1 and Shc recognize the same NP\_X\_Y motif located at the juxtamembrane domain of insulin receptor \(\beta\) subunit (7) and that the mutation at tyrosine 960 in the NP\_X\_Y motif disrupts phosphorylation of both IRS-1 and Shc (23). Some reports exert that Shc is the predominant molecule to activate the Ras pathway in insulin.
In this study, we have shown that existence of growth and survival signals transmitted through two distinct NPXY motifs of LTK. Although both NPXY motifs can transmit growth signals, only tyrosine 485 is critical for anti-apoptotic effects of LTK. Our analyses showed that this survival signal is suggested to generated through IRS-1 and to be distinct from the Ras pathway and the p70S6 kinase pathway. Taken together, all these results suggest the existence of another unidentified signaling pathway downstream of IRS-1, which is relevant to the anti-apoptotic activity. To determine the mechanism by which IRS-1 generates survival signals, further investigation will be required.

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