The Adapter Protein Shc Interacts with the Interleukin-2 (IL-2) Receptor upon IL-2 Stimulation*

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Binding of interleukin-2 (IL-2) to the IL-2 receptor (IL-2R) stimulates Src family kinases, tyrosine phosphorylation of several proteins, conversion of Ras to its active GTP-bound form, and eventually c-fos, c-jun, and c-myc induction. The IL-2Rβ chain plays a crucial role in IL-2R signaling. Within the cytoplasmic domain of the β chain, a region essential for mitogenesis and another involved in binding the Src family kinase Lck have been defined. The β chain itself is tyrosine-phosphorylated upon IL-2 stimulation. Since the adapter protein Shc acts upstream of Ras and is involved in T cell receptor-mediated Ras activation, we examined the role of Shc in IL-2 signaling. Shc was found to be tyrosine-phosphorylated upon IL-2R stimulation in CTLL-20 cells. After its phosphorylation, Shc interacted with another adapter protein, Grb2, and, via Grb2, with the Ras GTP/GDP exchange factor mSOS. After IL-2 stimulation, Shc also associated with the IL-2Rβ chain. Thus, during IL-2 signaling, the interaction of Shc with the IL-2Rβ chain and its simultaneous association with Grb2 and mSOS may couple IL-2R stimulation to Ras signaling.

The growth stimulatory properties of interleukin 2 (IL-2) are mediated via the multi-subunit IL-2 receptor (IL-2R). The IL-2R is composed of a 55-kDa α chain, a 70–75-kDa β chain, and a 64-kDa γ chain (reviewed in Refs. 1 and 2). Gene transfer studies using cDNAs encoding the different chains of the IL-2R revealed that, while expression of different combinations of the IL-2R chains can bind IL-2 with varying affinities, only the βγ and αβγ combinations confer the highest affinities for IL-2 (Kd of 10−9 and 10−11, respectively) and are capable of delivering an IL-2 mediated mitogenic signal (1, 2). One of the early events during IL-2R stimulation involves the activation of cytoplasmic protein-tyrosine kinases. Since none of the IL-2 receptor chains appear to contain an intrinsic kinase domain, attention has focused on kinases and other intracellular proteins that may interact with the cytoplasmic domains of the IL-2R chains.

The α chain consists of a short 13-amino acid cytoplasmic tail, while the β and γ chains have cytoplasmic domains of 286 and 86 amino acids, respectively (3–5). While biochemical studies of the recently cloned γ chain have just begun, the crucial role of the cytoplasmic domain of the β chain in IL-2 signaling has been well characterized. Within the tail of the β chain, a “serine-rich” region indispensable for growth stimulation, and an "acidic" region involved in binding the Src family kinase Lck have been defined (6–9). One of the several substrates that is phosphorylated on tyrosine after IL-2 stimulation is the IL-2Rβ chain itself (10, 11). Both the acidic and the serine-rich regions of the IL-2Rβ chain appear to be essential for the activation of Lck, for conversion of Ras to its active GTP-bound state, and for induction of c-fos and c-jun expression (8, 12). However, the mechanisms by which different regions of the β chain mediate mitotic signaling remain unclear.

Activation of Ras, which is downstream of tyrosine kinases, appears to be a crucial early event in mitogenic signaling initiated by a number of receptors (13). Recently, the mechanism of Ras activation via growth factor receptors that are tyrosine kinases (such as the epidermal growth factor (EGF) receptor) and receptors that are not tyrosine kinases (such as the T cell receptor (TCR)) have been elucidated. In the case of the EGF receptor, the autophosphorylated EGF receptor interacts with the adapter protein Grb2 (via its Src homology 2 (SH2) domain) and Grb2 simultaneously associates (via its Src homology 3 (SH3) domains) with the guanine nucleotide exchange factor for Ras, mSOS (14–16). SH2 and SH3 domains are found in a number of intracellular signaling molecules, and they bind respectively to specific phosphotyrosine containing sequences and proline-rich sequences (17, 18). Thus Grb2, acting as an adapter protein, helps to shuttle the nucleotide exchange factor mSOS to the membrane. mSOS can then induce loading of GTP for GDP on membrane-bound Ras.

During T cell receptor signaling, which involves activation of several Src family kinases, another protein Shc links TCR clustering to Ras activation (19). Shc is also an adapter protein with one SH2 domain but no obvious catalytic domain (20). It is phosphorylated on tyrosine by Src family kinases (21), and tyrosine-phosphorylated Shc also interacts with Grb2 (22). During T cell activation, Shc may help to shuttle mSOS to the membrane by directly interacting via its SH2 domain to the phosphorylated TCR-ζ chain, and simultaneously binding via its phosphotyrosine residue to Grb2.

The similarities in TCR and IL-2R signaling, i.e. receptors that do not have intrinsic tyrosine kinase activity but signal through activation of Src family kinases, prompted us to examine the role of Shc in IL2R-mediated signal transduction. In the IL-2R-dependent T cell line CTLL-20, Shc was phosphorylated on tyrosine upon IL-2 stimulation. After its phosphorylation, it also interacted with Grb2 and, in turn, via Grb2 with mSOS. In addition, Shc was co-precipitated with the IL-2Rβ chain after IL-2 stimulation and IL-2Rβ chain was co-precipitated with Shc. Taken together, the interaction of Shc with the IL-2Rβ chain and the association of phosphorylated Shc with Grb2 and mSOS may also couple IL-2R stimulation to Ras signaling.

MATERIALS AND METHODS

Cells—CTLL-20 cells were grown in complete medium (RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine,
penicillin and streptomycin, 2 x 10^{-5}M 2-mercaptoethanol, and 1% (v/v) of supernatants from rat spleen cells stimulated with concanavalin A.

Reagents—Affinity-purified polyclonal and monoclonal anti-Shc antibody, anti-Grb2 monoclonal antibody, and horse-radish peroxidase-linked anti-phosphotyrosine antibody (RC20) were obtained from Transduction Laboratories (Lexington, KY). Polyclonal anti-Grb2 antibody was purchased from Upstate Biotechnology Inc. and anti-m-sos-1 antibody was obtained from AMRAD Corp. (Victoria, Australia). Antibody to the murine IL-2Rβ chain was purchased from Biosource International (Camarillo, CA). The fusion protein encoding the SH2 domain of Grb2 was a gift from Dr. Tony Pawson (Toronto, Canada).

IL-2-Stimulation—CTLL-20 cells were starved for 4 h without IL-2 in complete medium. Cells (10^{-15} x 10^{10} per sample) were stimulated with recombinant human IL-2 (a gift from Hoffman-La Roche, Switzerland) at 50 ng/ml. The cells were lysed in a buffer containing 1% Nonidet P-40, 50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM Na_{3}VO_{4}, 10 mM NaF, 10 mM sodium pyrophosphate, 10 μg/ml each of aprotinin and leupeptin, and 2 mM phenylmethylsulfonyl fluoride.

Immunoprecipitations and Immunoblotting—Anti-Shc or anti-IL-2Rβ or anti-Grb2 antibody was added to the lysates along with protein A-Sepharose beads and incubated for 2 h at 4 °C. The beads were washed extensively, and the proteins bound to the beads were resolved by SDSPAGE, transferred to nitrocellulose, immunoblotted with the indicated antibody, and developed by the enhanced chemiluminescence (ECL) system (Amersham Corp.). For precipitations with SH2 domains, GST alone or GST-Grb2SH2 fusion proteins (2-4 μg) bound to glutathione-agarose beads were incubated with lysates for 2 h at 4 °C. The beads were washed four times in IP wash buffer, and the proteins bound to the beads were analyzed as described above.

RESULTS AND DISCUSSION

To identify a potential mechanism for coupling the IL-2R to Ras activation, we first examined whether Shc would be tyrosine-phosphorylated upon activation through the IL-2R. After incubation of IL-2-starved CTLL-20 cells with IL-2 for various times, Shc was immunoprecipitated using antibodies to Shc (anti-Shc) and its phosphorylation was assessed by anti-phosphotyrosine immunoblotting (Fig. 1). Tyrosine phosphorylation of both the 48- and 52-kDa isoforms of Shc was detected within 1 min after stimulation, indicating that Shc tyrosine phosphorylation was an early event. Shc phosphorylation peaked by about 5 min and started to disappear by 30 min. Anti-Shc immunoblotting of the same blot showed that equal levels of Shc were present in all lanes.

In v-Src-transformed fibroblasts as well as after TCR stimulation, phosphorylated Shc interacts with the adapter protein Grb2 (22). When the precipitates of Shc were examined for Grb2 by immunoblotting, as early as 1 min, Grb2 was found to be in a complex with Shc (Fig. 1). Consistent with the level of Shc phosphorylation, the amounts of Grb2 co-precipitated with Shc peaked by 5 min. As in other systems, the interaction of Grb2 with Shc occurred via the SH2 domain of Grb2 since immobilized fusion proteins of glutathione S-transferase (GST) and the SH2 domain of Grb2 (Grb2SH2) specifically precipitated Shc only from IL-2-activated cell lysates (data not shown).

Since Grb2 has been shown to associate via its SH3 domains with the guanine nucleotide exchange factor mSOS (18-22), we examined whether Shc immunoprecipitates also contained mSOS (Fig. 1). Immunoblotting of Shc immunoprecipitates revealed the presence of mSOS as early as 1 min after addition of IL-2. The amount of mSOS in the complex was decreased by 15 min. Thus it appears that Shc, Grb2, and mSOS are all part of a complex as early as 1 min after stimulation via the IL-2R.

These data suggested that the phosphorylation of Shc may be an intermediate event in coupling IL-2 stimulation to Ras activation. Since Shc is a cytoplasmic protein and the activation of Ras occurs on the membrane, we determined whether Shc may interact with one of the membrane-associated tyrosine-phosphorylated proteins. Consistent with this idea, in Shc immunoprecipitates from IL-2-activated cells, we also observed a diffuse tyrosine-phosphorylated band of ~80 kDa (Fig. 2A). Since the IL-2Rβ chain has been shown to be tyrosine-phosphorylated and migrates with a molecular mass of ~80 kDa (3-7), we tested whether this band was the IL-2Rβ chain. Parallel immunoprecipitations of Shc and the IL-2Rβ chain were performed after IL-2 stimulation, and the precipitated proteins were analyzed by anti-phosphotyrosine immunoblotting (Fig. 2B). From lysates of IL-2-stimulated cells, but not from unstimulated cells, a phosphorylated band migrating at 75-80
**Interaction of Shc with the IL-2 Receptor**

**Fig. 2. Association of Shc with IL-2Rβ chain after IL-2 stimulation.** A, lysates from IL-2-stimulated or unstimulated CTLL-20 cells were immunoprecipitated with anti-Shc antibody, resolved by 8% SDS-PAGE, and analyzed by anti-phosphotyrosine immunoblotting. B, lysates from IL-2-stimulated or unstimulated CTLL-20 cells were immunoprecipitated with anti-Shc or anti-murine IL-2Rβ chain antibody and analyzed by anti-phosphotyrosine immunoblotting. The 48- and 52-kDa isoforms of Shc, as well as the IL-2Rβ chain, are indicated by arrows. The ~60-kDa band seen in IL-2Rβ chain immunoprecipitates from unstimulated lysates is a nonspecific band. Molecular size standards are indicated on the left. C, lysates from IL-2-stimulated and unactivated cells were immunoprecipitated with anti-IL-2Rβ chain antibody or an isotype-matched control antibody (anti-L3T4), resolved by 8% SDS-PAGE, and analyzed by anti-Shc immunoblotting. The Ig heavy chain band at 75-80 kDa observed by several groups and the

**Fig. 3. Anti-phosphotyrosine blotting of Grb2SH2 and anti-Grb2 immunoprecipitates.** A, lysates from IL-2-activated and unactivated cells were incubated with GST alone or GST-Grb2SH2 fusion proteins bound to glutathione-agarose beads. After washing, the proteins bound to the beads were resolved by 8% SDS-PAGE and immunoblotted with anti-phosphotyrosine. The bands designated as Shc were identified by stripping and re-probing the blot with anti-Shc antibody (data not shown). The band at 75–80 kDa is also indicated. B, lysates from unactivated or activated cells (after preclearing with anti-Shc) were immunoprecipitated with anti-Grb2 and analyzed by anti-phosphotyrosine immunoblotting (top panel). Same level of Grb2 was immunoprecipitated in all lanes as determined by anti-Grb2 immunoblotting (bottom panel). kDa was precipitated by an anti-IL-2Rβ chain antibody, as well as by the anti-Shc antibody (although with less intensity). This suggested that the 75–80-kDa phosphoprotein seen in Shc immunoprecipitates was the IL-2Rβ chain. In addition, a phosphoprotein migrating similar to the 52-kDa isoform of Shc was also seen in IL-2Rβ chain immunoprecipitates. Anti-Shc immunoblotting of the IL-2Rβ chain immunoprecipitates revealed that the 52-kDa band was indeed Shc, and it was found associated with the IL-2R only after IL-2 stimulation (Fig. 2C). An isotype-matched control antibody did not precipitate Shc from IL-2-activated cell lysates. We could not directly identify the 75–80-kDa band, since the available anti-IL-2Rβ chain antibodies were unable to immunoblot the IL-2Rβ chain. Based on the characteristic diffuse migration of the phosphorylated IL-2Rβ chain of 75–80 kDa observed by several groups and the
presence of the same band in both IL-2Rβ chain and Shc immunoprecipitates, it appears likely that this band is the IL-2Rβ chain.

To further define Shc-Grb2 interaction during IL-2 stimulation, lysates from IL-2-stimulated and unactivated cells were incubated with GST or GST-Grb2SH2 fusion proteins bound to glutathione-agarose beads and the proteins bound to the beads were revealed by immunoblotting with anti-phosphotyrosine (Fig. 3A). In addition to the 48- and 52-kDa isoforms of phosphorylated Shc (identified by anti-Shc immunoblotting; data not shown), the Grb2SH2 domain also precipitated a diffuse phosphorylated band at 80 kDa identical to that seen for IL-2Rβ chain. The Grb2SH2 domain could have bound to the IL-2Rβ chain either directly or indirectly through Shc. To address this, lysates from unactivated cells or activated cells (with or without initial preclariﬁng with anti-Shc antibody) were immunoprecipitated with anti-Grb2 antibody. Anti-phosphotyrosine immunoblotting revealed that like with Grb2SH2 domain, native Grb2 co-precipitated the 52-kDa Shc band and a 75–80-kDa band after IL-2 stimulation. However, after preclariﬁng with anti-Shc, the 52-kDa Shc band was lost and the majority of the 75–80-kDa band was also lost from anti-Grb2 immunoprecipitates, indicating that most of the IL-2Rβ chain precipitated by Grb2 was through Shc (Fig. 3B, top panel). Anti-Grb2 immunoblotting revealed that the same levels of Grb2 was immunoprecipitated under all conditions (Fig. 3B, bottom panel). These data suggest that Shc directly interacts with the IL-2Rβ chain. However, we cannot exclude the possibility that small amounts of Grb2, independent of Shc, may also interact with the IL-2Rβ chain.

Taken together, it appears that upon IL-2 stimulation and phosphorylation of the IL-2Rβ chain, Shc may associate with the phosphorylated IL-2 receptor, possibly through its SH2 domain, native Grb2 co-precipitated the 52-kDa Shc band and a 75–80-kDa band after IL-2 stimulation. However, after preclariﬁng with anti-Shc, the 52-kDa Shc band was lost and the majority of the 75–80-kDa band was also lost from anti-Grb2 immunoprecipitates, indicating that most of the IL-2Rβ chain precipitated by Grb2 was through Shc (Fig. 3B, top panel). Anti-Grb2 immunoblotting revealed that the same levels of Grb2 was immunoprecipitated under all conditions (Fig. 3B, bottom panel). These data suggest that Shc directly interacts with the IL-2Rβ chain. However, we cannot exclude the possibility that small amounts of Grb2, independent of Shc, may also interact with the IL-2Rβ chain.

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