The interleukin (IL)-2 receptor system has previously been shown to signal through the association and tyrosine phosphorylation of Shc. This study demonstrates that the IL-2 receptor β (IL-2Rβ) chain is the critical receptor component required to mediate this effect. The use of IL-2Rβ chain deletion mutants transfected into a Ba/F3 murine cell model describes a requirement for the IL-2Rβ "acid-rich" domain between amino acids 315 and 384 for Shc tyrosine phosphorylation and receptor association. COS cell co-transfection studies of IL-2Rβ chain constructs containing point mutations of tyrosine to phenylalanine along with the tyrosine kinase Jak-1 and a hemagglutinin-tagged Shc revealed that the motif surrounding phosphorylated tyrosine 338 within the acid-rich domain of the IL-2Rβ is a binding site for Shc. Deletion of this domain has previously been shown to abrogate the ability of IL-2 to activate Ras but does not affect IL-2-dependent mitogenesis in the presence of serum. Proliferation assays of Ba/F3 cells containing IL-2Rβ chain deletion mutants in serum-free medium with or without insulin shows that deletion of the acid-rich domain does not affect IL-2-driven mitogenesis regardless of the culture conditions. This study thus defines the critical domain within the IL-2Rβ chain required to mediate Shc binding and Shc tyrosine phosphorylation and further shows that Shc binding and phosphorylation are not required for IL-2-dependent mitogenesis. Neither serum nor insulin is required to supplement the loss of induction of the Shc adapter or Ras pathways, which therefore suggests a novel mechanism for mitogenic signal transduction mediated by this hematopoietin receptor.

Interleukin-2 (IL-2) is a multifunctional cytokine that has been shown to affect the physiology of cells of immune and nonimmune tissue (reviewed in Ref. 1) by direct interaction with a high or intermediate affinity IL-2 receptor (IL-2R). The high affinity IL-2R (K_d = 10^{-11}) is composed of three receptor subunits (reviewed in Ref. 2); α (55 kDa), β (75 kDa), and the common γ (γ_c) chain (64 kDa), which is shared by several hematopoietic cytokine receptors (3). The intermediate affinity receptor (K_d = 10^{-9}) is a β-γ_c complex only (2). Signal transduction by IL-2 minimally requires the intermediate affinity β-γ_c receptor complex and does not require the α chain (2). Thus the apparent function of the α chain is to affect IL-2 affinity only and not the mechanism of IL-2 signal transduction.

Signal transduction by IL-2 is initiated by the activation of several tyrosine kinases associated with specific receptor molecules of the IL-2R. Among these are members of the Src family of tyrosine kinases, which have been shown to functionally couple to the IL-2Rβ chain (4-6); the Janus family tyrosine kinase Jak-1, which associates with the IL-2Rβ chain (7, 8); and Jak-3 (9-11), which associates with the γ_c chain (7, 8). The first detectable event following treatment of T cells with IL-2 is the formation of an activated α-β-γ_c IL-2R complex containing tyrosine-phosphorylated and activated Jak-1 and Jak-3 (7, 8, 10). This is soon followed by the activation of Src family tyrosine kinases (4-6) and the tyrosine phosphorylation of multiple substrates.

Among the proteins that are tyrosine phosphorylated in response to IL-2 is the recently identified protein Shc (12). Shc is an SH2 domain containing protein found as two dominant, widely expressed, and tyrosine-phosphorylated forms of 52 and 46 kDa as well as a 66-kDa protein with more restricted expression (13). Shc functions to link receptor tyrosine kinase activation and tyrosine phosphorylation to the downstream activation of Ras and Ras-like pathways (reviewed in Ref. 14) and accomplishes this by recognizing and binding to a phosphotyrosine-containing motif within the receptor or a receptor-associated protein. Binding to the receptor complex brings Shc into proximity for phosphorylation by an activated tyrosine kinase and establishes a secondary "docking" site for the protein Grb2 (15). The subsequent binding of Grb2 to the receptor complex brings guanine nucleotide releasing factor activity, such as Sos (16) or Vav (17), to the membrane where it can catalyze the release of GDP from inactive Ras-GDP, allowing
The formation of an activated Ras-GTP complex. Thus it is clear
that the activation of Ras via the Shc/Grb2 adapter pathway is
dependent on the ability of Shc to recognize and bind to a
 tyrosine-phosphorylated receptor complex.

Tyrosine phosphorylation of p52Shc and activation of the
Shc/Grb2 adapter pathway can be induced by a number of
mitogenic cytokines and growth factors (13, 18-22) and in-
volves the ligand-dependent association of Shc with the recep-
tor complex (23, 24). As in other systems, Shc has been found
associated with the IL-2R complex (25) and the induction of
this pathway by IL-2 has been reported to correlate with the
ability of IL-2 to elicit a mitogenic response (26). Because of this
and the multi-protein nature of the IL-2R complex, we were
interested in determining the precise mechanism by which IL-2
stimulates Shc/receptor binding and tyrosine phosphorylation.

MATERIALS AND METHODS

Tissue Culture—Human T cells were obtained from normal donors
and isolated by counter flow centrifugal elutriation (27). Isolated T cells
were activated and cultured for 3 days in RPMI media (Mediatech)
supplemented with 10% fetal calf serum (Intergen), 1 μg/ml phyto-
hemagglutinin, glutamine, and antibiotics (28). Cells were recovered
and G418-enriched (28) by washing several times in low pH RPMI media
and culturing for 24 h in RPMI media supplemented with 1% fetal calf
serum, glutamine, and antibiotics. Cells were treated with 100 ng/ml
recombinant human IL-2 or IL-4 (Peprotech) for up to 30 min as de-
dcribed (29), recovered by centrifugation, and prepared for lysis
and immune precipitation.

Ba/F3 cells transfected with various IL-2Rβ chain deletion mutants
(FL, AD, BD, SD, and BS) were maintained at 37°C in a humidified
CO₂ incubator in RPMI1640 medium supplemented with 10% fetal calf
serum, 5% WEHI-3B culture supernatant (30), glutamine, antibiotics,
and 250 μg/ml hygromycin-B (Calbiochem). IL-2 treatments were per-
formed on these cells using 100 ng/ml recombinant IL-2 at a cell density of
50,000 cells/ml in RPMI1640 medium supplemented with 5% fetal
 calf serum. Cells were incubated at 37°C for 15 min, recovered by
centrifugation, and prepared for lysis and immune precipitation.

Transfections—Constructs containing IL-2Rβ chain deletion muta-
tions were prepared as described (31), transfected into Ba/F3 cells, and
cultured as described (31). Plasmids constructs containing wild type or
mutant IL-2Rβ chain molecules with substitutions of phenylalanine for
 tyrosine were generated as described (32). The jak-1 expression plas-
mid was prepared by subcloning the 4.2 kbp murine jak-1 cdna (pro-
vided by O. Silvennoinen and J. Ihle) into the Sal/I and Sal/II sites of
pCMV4α (provided by M. Feinberg). A hemagglutinin (HA) epitope-
tagged derivative of human Shc (containing two tandem copies of the
sequence YPYDVPDYD at the 5' end) was prepared by polymerase chain
reaction (PCR) using a 1.6-kilobase pair fragment of genomic DNA
subcloned into the HindIII/KpnI sites of the expression plasmid pCMV4αNeo (provided by M. Feinberg). Plasmids were transfected into COS-7 cells (ATCC) using Lipofectamine (Life Technologies, Inc.) as described (33).

Cell Lysis and Immune Precipitation—Cell pellets were lysed by
resuspension in 1 ml of lysis buffer (10 mM Tris-Cl, pH 7.6, 5 mM EDTA,
50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1
mM sodium ortho-vanadate, 1% Triton X-100, 1 mM phenylmethylsul-
fonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, and 2 μg/ml
leupeptin)/100 x 10⁶ cells and incubated at 4°C for 1 h. Lysates were
denatured by centrifugation at 12,000 × g and subjected to immune
precipitation.

Anti-human jak-3 and anti-Shc immune precipitation of human T
 cells lysates were performed on a 100 x 10⁶ cell lysate equivalent using
2 μl of anti-jak-3 antibody (9) or 1 μl of anti-Shc antibody (Upstate
Biotechnology Inc.). Anti-Shc immune precipitation of Ba/F3 cells was
performed using a 300 x 10⁶ cell lysate equivalent and 4 μl of anti-Shc
antibody/sample. All immune precipitations were performed for 4 h at
overnight. Lysates were then lysed by boiling for 10 min in the presence of
2 μl of protein A-Sepharose (Sigma). Immune precipitations were washed
6 times in lysis buffer, and proteins were eluted by boiling in 50 μl of 2 x SDS-
polyacrylamide gel electrophoresis sample buffer. Proteins were re-
solved by SDS-polyacrylamide gel electrophoresis, transferred to
Immobilon polyvinylidene difluoride membranes (Millipore), and probed
using anti-phosphotyrosine immunoblotting.

IL-2Rβ immune precipitation was performed using anti-IL-2Rβ
monoclonal antibody 561 (provided by R. Robb) directly conjugated to
protein A-Sepharose (PAS). Preparation of 561-PAS was initiated by
incubating 1 mg of 561 with 1 ml of packed PAS beads in 3 ml of lysis
buffer overnight at 4°C. The beads were washed extensively with lysis
buffer, 2 times with 0.1 M borate, pH 9.0 (BB) and resuspended in 10 mls
of BB. Dimethyl pimelimidate (Sigma) was added to 20 μw, and beads
were incubated at room temperature for 1 h. 561-PAS was washed once
with BB, resuspended in 40 μw ethanandine pH 8.0, and incubated for
1 h at room temperature. Beads were washed several times with alter-
ning cycles of BB and 0.1 μw glycine, pH 3.0. 561-PAS was resuspended
at a final concentration of 1 mg/ml 561/packed beads in phosphate-
buffered saline, supplemented with 0.1% sodium azide, and stored at
4°C.

For IL-2Rβ chain immune precipitations, treated Ba/F3 cells con-
taining different IL-2R chains or transfected COS-7 cells were lysed,
collected, and recovered using 30 μl of 561-PAS (30 μg of antibody equivalent). Immune precipitations were incubated for 4 h at 4°C and washed six times with lysis buffer. Proteins were eluted by boiling in 50 μl of nonreducing 2 × SDS-polyacrylamide gel
electrophoresis sample buffer (no β-mercaptoethanol). Samples were
centrifuged, and the supernatant was removed and adjusted to 5% β-
mercaptoethanol. Proteins were resolved by SDS-polyacrylamide gel
electrophoresis, transferred to Immobilon polyvinylidene difluoride
membranes (Millipore), and probed with Shc or Shc-HA by anti-Shc or
anti-HA immunoblotting.

Immune Blotting—All immune blotting was performed as described
(34) using the following antibodies. For the detection of changes in
tyrosine phosphorylation, immune precipitated samples were lysed,
diluted as described, and immune precipitated using 30 μl of 561-PAS
(30 μg of antibody equivalent). Immune precipitations were incubated for
4 h at 4°C and washed six times with lysis buffer. Proteins were eluted by
boiling in 50 μl of nonreducing 2 × SDS-polyacrylamide gel
electrophoresis sample buffer (no β-mercaptoethanol). Samples were
centrifuged, and the supernatant was removed and adjusted to 5% β-
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membranes (Millipore), and probed with Shc or Shc-HA by anti-Shc or
anti-HA immunoblotting.

RESULTS

The IL-2Rβ Chain Mediates IL-2-dependent Shc Phosphoryla-
tion—Several hematopoietic cytokines induce p52Shc tyro-
size phosphorylation (13, 19, 21, 22), which correlates with their
ability to activate Ras and induce mitogenesis (26). IL-2 has been
demonstrated to induce Shc association with the IL-2 recep-
tor complex (25), stimulate the tyrosine phosphorylation of Shc (12),
and activate Ras in human T cells (36). For these reasons we were
interested in determining the mechanism by which IL-2 induces Shc phosphorylation leading to activation of the
Shc adapter pathway.

The IL-2 receptor complex contains four dominant tyrosine-
phosphorylated proteins: the IL-2Rβ chain, the common γ chain,
and the receptor-associated tyrosine kinases jak-1 and
jak-3. To determine which of these phosphotyrosine containing
proteins was most important in mediating Shc phosphorylation
we used the IL-4 receptor system, which also contains the
common γ chain and activates jak-1 and jak-3 (7), and asked
whether IL-4 was capable of inducing Shc phosphorylation in
a manner similar to that observed with IL-2.

Activated human T cells were treated with IL-2 or IL-4 and
assayed for receptor tyrosine kinase activation, as represented
by increased tyrosine phosphorylation of jak-3, and factor-de-
pendent tyrosine phosphorylation of Shc. This analysis clearly
demonstrates that IL-4 does not induce Shc tyrosine phos-
phorylation in spite of inducing receptor tyrosine kinase activity
and phosphorylation of jak-3 in human T cells (Fig. 1). This
observation is supported by additional results that describe an
inability of IL-4 to induce Shc tyrosine phosphorylation in other cell types (37). This therefore implies a direct role for the IL-2Rβ chain in controlling IL-2-dependent Shc phosphorylation.

The "Acid-rich" Domain of the IL-2Rβ Chain Is Required for Shc Tyrosine Phosphorylation and Shc Association with the IL-2R—Previously generated and characterized IL-2Rβ molecules containing various deletion mutations within the cytoplasmic domain (31) (Fig. 2) were transfected into murine IL-3-dependent Ba/F3 cells and analyzed for their ability to support IL-2-induced receptor tyrosine kinase activation and substrate phosphorylation leading to mitogenesis (summarized in Table I). As previously reported, only wild type (FL), acid-rich domain deletion mutants (AD), and truncation deletion mutants from amino acid 384 to the carboxyl terminus (BD) were capable of supporting IL-2-induced tyrosine phosphorylation and mitogenesis (Table I and Ref. 31). We next asked whether there was a difference in the ability of these mutants to transduce IL-2-dependent signal leading to Shc phosphorylation. Receptor-containing Ba/F3 cells were treated with IL-2 and assayed for IL-2-induced Shc phosphorylation by anti-Shc immuno-precipitation followed by anti-phosphotyrosine immunoblotting. As shown in Fig. 3, the wild type receptor (FL) and the BD mutant, containing an intact acid-rich domain, were capable of inducing IL-2-dependent tyrosine phosphorylation of Shc. Deletion of this domain in the AD receptor resulted in an inability to transduce signal leading to Shc phosphorylation (Fig. 3). As a control, IL-2-induced tyrosine phosphorylation of murine Jak-3 and downstream substrates was verified in all three mutant receptor-containing cells (Ref. 31 and data not shown). SD- and BS-containing cells were incapable of inducing tyrosine phosphorylation of Shc or Jak-3 (data not shown), supporting previous observations (31). This analysis suggests that a domain within the acid-rich region of the IL-2Rβ is required to mediate IL-2-dependent Shc phosphorylation and may represent a binding site for Shc.

FL-, AD-, and BD-containing cells were treated with IL-2, the IL-2Rβ chain was immune precipitated, and an immuno-blot was performed using an anti-Shc antibody. Results from this experiment show an IL-2-dependent association of Shc with FL and BD receptors but not in cells containing the AD mutant (Fig. 4). This clearly shows that IL-2-dependent tyrosine phosphorylation of Shc involves direct binding of Shc to a motif within the acid-rich domain of the receptor and implies a requirement for IL-2-dependent tyrosine phosphorylation of this region as a prerequisite for Shc association.

The Motif Surrounding Phosphorylated Tyrosine 338 within the IL-2Rβ Chain Cytoplasmic Domain Provides the Binding Site for Shc—There are six tyrosines within the IL-2Rβ chain cytoplasmic domain that upon phosphorylation may serve as binding sites for SH2 domain-containing proteins such as Shc (Fig. 5). Four of these are found within the acid-rich domain. To further clarify which of these phosphotyrosine-containing motifs is involved in mediating Shc binding, COS cells were co-transfected with a hemagglutinin-tagged Shc (Shc-HA) construct, wild type IL-2Rβ, and IL-2Rβ constructs containing point mutations of tyrosine to phenylalanine, and a construct encoding the tyrosine kinase Jak-1; co-transfection of these constructs results in tyrosine phosphorylation of each of the IL-2Rβ cytoplasmic tyrosine residues in this assay (data not shown). As shown in Fig. 5, the wild type receptor (FL) and the BD mutant, containing an intact acid-rich domain, were capable of inducing IL-2-dependent Shc phosphorylation and mitogenesis (summarized in Table I). As previously reported, only wild type (FL), acid-rich domain deletion mutants (AD), and truncation deletion mutants from amino acid 384 to the carboxyl terminus (BD) were capable of supporting IL-2-induced tyrosine phosphorylation and mitogenesis (Table I and Ref. 31). We next asked whether there was a difference in the ability of these mutants to transduce IL-2-dependent signal leading to Shc phosphorylation. Receptor-containing Ba/F3 cells were treated with IL-2 and assayed for IL-2-induced Shc phosphorylation by anti-Shc immuno-precipitation followed by anti-phosphotyrosine immunoblotting. As shown in Fig. 3, the wild type receptor (FL) and the BD mutant, containing an intact acid-rich domain, were capable of inducing IL-2-dependent tyrosine phosphorylation of Shc. Deletion of this domain in the AD receptor resulted in an inability to transduce signal leading to Shc phosphorylation (Fig. 3). As a control, IL-2-induced tyrosine phosphorylation of murine Jak-3 and downstream substrates was verified in all three mutant receptor-containing cells (Ref. 31 and data not shown). SD- and BS-containing cells were incapable of inducing tyrosine phosphorylation of Shc or Jak-3 (data not shown), supporting previous observations (31). This analysis suggests that a domain within the acid-rich region of the IL-2Rβ is required to mediate IL-2-dependent Shc phosphorylation and may represent a binding site for Shc.

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Serum or Insulin Supplements

The acid-rich domain of the IL-2R chain requires the IL-2Rβ acid-rich domain for IL-2 signal transduction leading toward mitogenesis of Ba/F3 cells containing only tyrosine 338 (YF:338), tyrosines 355, 358, and 361 as a group (YF:355Y), tyrosine 392 (YF:5Y), or tyrosine 510 (YF:6Y). Cells were cultured, lysed, and immune precipitated with antibody 561 and probed for Shc-HA association using anti-HA immunoblot (upper panel). Tosterivate expression in transfected cells, lysates were subjected to anti-HA immunoblot (middle panel) or anti-β chain immunoblot using antibody 561 (lower panel).

Shc-HA + JAK1

Fig. 6. IL-2Rβ point mutations of tyrosine to phenylalanine establish the motif surrounding phosphorylated tyrosine 338 as the IL-2Rβ Shc binding site. COS-7 cells were transfected with constructs containing Shc-HA, Jak-1, and/or wild type IL-2Rβ chain (pWT) or tyrosine to phenylalanine mutants of the IL-2Rβ chain containing only tyrosine 338 (pYF:338Y), tyrosines 355, 358, and 361 as a group (pYF:355Y), or tyrosine 392 (pYF:5Y), or tyrosine 510 (pYF:6Y). Cells were cultured, lysed, and immune precipitated with antibody 561 and probed for Shc-HA association using anti-HA immunoblot (upper panel). Tosterivate expression in transfected cells, lysates were subjected to anti-HA immunoblot (middle panel) or anti-β chain immunoblot using antibody 561 (lower panel).
**DISCUSSION**

Using mutational analysis of the IL-2R system, we have shown that IL-2 induction of Shc tyrosine phosphorylation is dependent on Shc association with the IL-2Rβ. We further show that this association is dependent on the presence of phosphorylated tyrosine 1 at amino acid 338 within the acidic-rich region of the IL-2Rβ cytoplasmic domain. This tyrosine is found within a motif containing the sequence TNQpYFFFF. Structural studies of motifs required for Shc binding to various receptor complexes (40–42) have revealed a consensus binding site for the Shc phosphotyrosine binding domain (40), also referred to as the SAIN (Shc and IRS-1 NPYX binding) domain (42) of NPXYpY. Considering the structural similarities between proline and glutamine, the motif within the IL-2Rβ chain of NOGY appears quite similar to the predicted SAIN domain recognition consensus of NPXY. Using the predicted IL-2 receptor binding domain for Shc, NOXY, a search was initiated for proteins containing this motif (43). Generally, this search identified proteins that have been shown to have regulatory functions in various signal transduction pathways including Ras-Gap (motif from amino acid 178PTNOWYH-), which is involved in the regulation of Ras and Ras-like pathways, and several receptor molecules such as the fibroblast growth factor receptor (motif from amino acid 761TSNOQEL-). Experiments aimed at investigating the potential role that Shc association with these and other proteins may play in regulating signal transduction are presently underway.

It has been shown (31, 38) that deletion of the acid-rich region of the IL-2Rβ chain abrogates the ability of IL-2 to induce Ras activation as well as increased transcription of fos and jun but did not affect IL-2-dependent mitogenesis in the murine IL-3-dependent cell Ba/F3. This analysis did not preclude the possibility of IL-2-dependent activation of Ras-like but Ras-distinct pathways, which would support IL-2-driven cell growth. By focussing on the analysis of IL-2 activation of upstream Ras pathway regulators (Shc adapter pathway), it becomes much easier to define the potential role that these pathways play in IL-2 signal transduction. Results from this study clearly show that Shc phosphorylation that acts to initiate this pathway is not required for mitogenesis. This analysis does not preclude the possibility that Grb2 may interact directly with this receptor complex and function to activate other guanine nucleotide releasing factor activities in the AD mutant, which may lead to mitogenesis. If this were the case, however, one would expect AD receptors to also activate Ras. This clearly does not occur in this mutant (38). Additionally, in the insulin receptor system, which has been shown to associate independently with both Shc and Grb2, Shc binding and tyrosine phosphorylation is the primary mechanism utilized in vivo to bring Grb2 and guanine nucleotide releasing factor activity to the membrane resulting in Ras activation (44). These data thus suggest that Shc binding to the IL-2Rβ chain, which results in its tyrosine phosphorylation and initiation of the Shc/Grb2 adapter pathway, is the mechanism used by IL-2 to activate Ras and Ras effector pathways.

These data also strongly suggest that IL-2 receptor signal transduction functions to activate specific mitogenic signals without a strict requirement for Ras in IL-2 receptor-containing Ba/F3 cells. This does not preclude the possibility, however, that in the context of a different cellular background IL-2 may have different requirements for Ras and Ras signaling pathways.

It is well described that Ras activation and regulation of downstream pathways is critical in controlling proliferation (14). It seems plausible therefore that mitogenic signal transduction by IL-2 in Ba/F3 cells may activate one or more of these downstream pathways by functionally bypassing Ras activation. A key mitogenic pathway activated by upstream activation of Ras is the Raf/Map kinase cascade (45, 46). This pathway has been described as a “mitogenic bottleneck” due to the wide variety of mitogenic signals that funnel into Raf activation (46) and because antisense elimination of Raf blocks cytokine-triggered mitogenesis (47). IL-2 has been shown to activate Raf, and Raf has been found to be associated with the IL-2 receptor complex by ourselves (data not shown) and others (48, 49). Furthermore, IL-2-dependent activation of Raf involves a tyrosine kinase-dependent mechanism (48). As has been shown in other systems, this is presumed to involve the tyrosine phosphorylation of Shc, the subsequent activation of Ras, and the downstream activation of Raf and Map kinases (14, 46). However, Raf can also be activated by direct tyrosine phosphorylation (50). The possibility exists, therefore, that IL-2 signaling may involve the direct tyrosine phosphorylation of Raf as an activating mechanism. This would theoretically abrogate the requirement for Ras activation in an IL-2-driven mitogenic response. To further elucidate this, the role that Raf activation plays in IL-2 signal transduction in IL-2 receptor mutants is being investigated.

With the data presented here, a potentially novel mechanism by which IL-2, and perhaps other IL-2Rβ chain containing receptors, controls cell growth is being established. In contrast to the results presented from studies within the granulocyte macrophage colony-stimulating factor receptor system (39), which clearly establishes an absolute requirement for some level of Ras activation in order for this receptor system to support cytokine-induced mitogenesis, the model of IL-2 signal transduction that has been generated in Ba/F3 cells establishes that mitogenesis can be fully supported by IL-2 receptors that lack the ability to activate this or similar pathways through...
IL-2 Signaling and Shc Phosphorylation

utilization of the Shc adapter pathway. IL-2 signal transduction thus forms a novel paradigm for mitogenic signaling by hematopoietic cytokines.

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