The ST2/T1 receptor, a homologue of the interleukin 1 receptor (IL-1R), was expressed in COS and Drosophila S2 cells as a human IgG-Fc fusion protein. While a type I IL-1R Fc fusion protein bound human IL-1 in vitro, the ST2 Fc fusion protein did not. Furthermore, IL-1 stimulated a synthetic interleukin-8 promoter reporter gene that was cotransfected into Jurkat cells with a full-length IL-1R type I (IL-1R1) or a chimeric receptor composed of the IL-1R1 extracellular domain and ST2 intracellular domain. In contrast, IL-1 did not stimulate the interleukin-8 promoter when cotransfected with a full-length ST2 or an ST2 extracellular/IL-1R intracellular domain fusion protein. Both IL-1R1 and the IL-1R/ST2 chimeric receptor also activated a receptor-associated kinase and CSBP/p38 MAP kinase. Using ST2 Fc receptor, we have identified, through receptor precipitation, receptor-dot blot and surface plasmon resonance, a putative ligand of ST2 secreted from Balb/c 3T3 and human umbilical vein endothelial cells. The putative ligand was also able to stimulate CSBP/p38 MAP kinase through the ST2 receptor. These results suggest that the ST2 is not an IL-1 receptor but rather has its own cognate ligand.

ST2/T1 was identified as a late response gene induced by either serum or overexpression of v-mos or Ha-ras oncogenes in Balb/c 3T3 or NIH 3T3 cells (1, 2). The ST2/T1 (designated ST2 hereafter) gene encodes a 38.5-kDa peptide that is secreted from 3T3 cells as a heavily glycosylated protein of 50–60 kDa. Subsequently, an alternatively spliced form of murine ST2 and rat Fit-1 were cloned that encoded a single transmembrane-spanning protein retaining the extracellular domain found in the soluble ST2 receptor (4, 5).

ST2 belongs to the immunoglobulin superfamily and bears significant amino acid identity (~25%) to the extracellular portion of both type I and type II interleukin 1 receptors (IL-1R) (2). Some of the intracellular residues that are required for signal transduction through the IL-1R and are conserved in the Drosophila Toll protein are also found in the membrane form of ST2 (6, 7). Furthermore, the gene for ST2 was mapped to mouse chromosome 1 closely linked to the il-1r locus containing both type I and type II receptor genes in support of their common ancestry (8).

Both soluble and membrane bound ST2 receptors are predominantly expressed in hematopoietic tissues in vivo and in established hematopoietic, epithelial, and fibroblast cell lines in vitro (5, 9). This expression pattern partially overlaps with that of the type I and type II IL-1Rs (10, 11). Soluble IL-1Rs have also been identified from various sources (12–14) including vaccinia virus. The vaccinia virus coded protein, B15R, binds to IL-1β and has been shown to be involved in viral pathogenesis by attenuating host response elicited due to IL-1 production (15, 16). Thus, soluble IL-1Rs may modulate IL-1-mediated responses by sequestering it and inhibiting its proinflammatory responses (17). The ST2 receptor may play a similar role for its ligand.

We wished to determine if ST2 is a receptor for IL-1 or some other ligand in order to further understand its function. In the present work we have expressed a soluble and membrane form of ST2 and show that it is not a receptor for IL-1. Instead, we show for the first time that the ST2 receptor binds a previously uncharacterized ligand and signals in a manner similar but not identical to IL-1.

**MATERIALS AND METHODS**

CdcL Lines, Culture Conditions, and Metabolic Labeling—Cos-1 and human umbilical vein endothelial cells (HUVEC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies Inc.). Drosophila Schneider 2 (S2) cells were grown in M3 medium supplemented with 10% heat-inactivated fetal bovine serum. J urkat cells were maintained in RPMI 1640, 10% heat-inactivated fetal bovine serum. Balb c 3T3 cells were maintained in DMEM, 10% heat-inactivated calf serum. For metabolic labeling, serum-starved (quiescent) cells were incubated for 4 h in methionine and cysteine-free medium containing 100–150 μCi/ml of trans-[35S]-label ([35S]methionine/cysteine), specific activity 1000 Ci/mm (ICN Biomedicals, Costa Mesa, CA). For exponentially growing cells, this medium was supplemented with 5% dialyzed fetal bovine serum. Cells were made quiescent by serum starvation, and serum-free medium was added and collected after 48 h. This medium was concentrated using a 10 K cut-off Centricon spin column (Amicon, Danvers, MA).

Cloning of Murine and Human ST2 cDNA—Human ST2 cDNA was generated by reverse transcriptase-mediated polymerase chain reaction (18) from mRNA isolated from Balb/c 3T3 cells based on the published sequence (2). The forward primer, 5'-GAATTCCGTTACCCGATATCTT-GCTCTTGATTGATAAAC-3', corresponds to nucleotides 23 to 4 (the +1 base being the first base of initiator methionine codon) and contains EcoRI and BstEII restriction sites. The reverse primer, 5'-CGCGGCTTCTCCCTCAGAAACACTCTCTATTGGAT-3', corresponding to codons 321–328, contained an in frame recognition sequence for factor Xa (EGR) and an in frame KpnI site. PCR products were cloned that encoded a single transmembrane-spanning protein retaining the extracellular domain found in the soluble ST2 receptor (4, 5).

The abbreviations used are: IL-1R, interleukin 1 receptor; IL-1 receptor; HUVEC, human umbilical vein endothelial cells(s); sIL-1R, soluble interleukin 1 receptor; MST2R, murine ST2 receptor; HST2R, human ST2 receptor; shST2R, soluble HST2R; ST2Fc, ST2 receptor Fc fusion; HSTFc, human ST2Fc; IL-1R Fc fusion; IL-5R Fc fusion; PCR, polymerase chain reaction; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; CAT, chloramphenicol acetyltransferase; MAP kinase, mitogen-activated protein kinase; EPORFc, human erythropoietin receptor Fc; MBP, myelin basic protein; CSBP, CS AID (14) (Cytokine suppressive antiinflammatory drug) Binding Protein.
were first cloned into the pCIIR vector (Invitrogen, San Diego, CA), to confirm the sequence, and insert was then excised with BstEII and KpnI and cloned into the MtaI vector for stable expression in Drosophila cells (driven by the inducible metallothionine promoter) and into the COSF vector for transient expression in COS cells (driven by the constitutive cytomegalovirus promoter). Both of these vectors contain a polylinker corresponding to the human IgG1 hinge region and an XbaI restriction site at the start of the hinge segment (19). Mouse ST2Fc and human IL-1R fusion proteins were first cloned into the PCRII vector (Invitrogen, San Diego, CA), to facilitate reverse transcriptase-mediated PCR using the forward primer 5'-AAGTTCCAGCAATGACATGGATTG-3' and reverse primer, 5'-GTCTCTAGATCCAAAGCTTCAGATGGCTTTTGCTCAAAGTGTTTCAGGTCTAAGCATGCCTTG-3' (codons 330–337); and human IL-1Rc, 5'-GGATTCCGTGATCATACTATAAGTTGTTACCTGCAGC-3' (codons 559–567), followed by an XbaI restriction site. After confirmation of sequence, the PCR product was cloned in place of the XbaI end of the soluble ST2 and FC sequence in the COSF vector between the XcmI and XbaI sites, yielding the 567-amino acid full-length COSMST2R. The MST2/L1-IR chimera was constructed by amplifying the intracellular portion of IL-1R from amino acids 330–367 with the following primers: 5'-CCAATGACCATCAATTTACAAGCTTTGCTCAAAGTGTTTCAGGTCTAAGCATGCCTTG-3' (codons 330–337) with in frame BclI restriction site and 5'-TTTTCTAGATCCAAAGCTTCAGATGGCTTTTGCTTTCGAGAGGCACGTGAGCCTCTCTTTGCAGTTT-3' (codons 559–567) followed by the XbaI site, stop codon, and a XbaI site. The PCR product was used to replace the intracellular portion of the ST2 receptor from BclI to XbaI. The final product MST2/L1-IR contained amino acids 1–330 and the ST2 receptor fused to amino acids 330–567 of the IL-1R.

The full-length IL-1R construct and the truncated version, IL-1Ra360, which lacks all but five amino acids after the transmembrane domain, was a kind gift from Dr. R. Einstein. For the IL-1R/MST2 chimera, the intracellular portion of ST2 receptor was amplified using the forward primer 5'-TGCTCTAGATCGAAGCACATGATT-3' and reverse primer 5'-CTTTCTAGATCACAAGTCCTCTTCAGAAATGAGCTTTTGCTCAAAGTGTTTCAGGTCTAAGCATGCCTTG-3' (codons 378–385 with a HindIII site) and the reverse primer 5'-CAGGTGACCTCAACAAGCTTCATTCAAGAATGAGCTTTTGCTTTCCGAGAGGCACGTGAGCCTCTCTTTGCAGTTT-3' (codons 557–567 with myc epitope, stop codon, and a BstEII site).

The intracellular portion of IL-1R from the HindIII site (amino acid 378) was replaced with the PCR-amplified intracellular portion of MST2 (from amino acid 378 to 567). The expression vector for these receptors contained a cytomegalovirus promoter and a bovine growth hormone polyadenylation sequence. The authenticity of each construct was confirmed by transient expression of the corresponding proteins in COS cells as analyzed by immunoprecipitation from 35S metabolically labeled cells.

The IL-8 promoter from 185 to +44 (21, 22) was made by PCR-mediated gene synthesis containing a 5' HindIII and a 3' XbaI site. The PCR product was first cloned into pCIIR to confirm the sequence. The insert was then removed by digestion with HindIII and XbaI and subcloned into corresponding sites in the PCAT vector (Promega, Madison, WI), which contains a bacterial chloramphenicol acetyl transferase (CAT) gene cassette, to generate the reporter plasmid IL-BP/CAT.

Expression of Fc Fusion Protein and Its Purification—For transient expression, all Fc fusion constructs were transfected into COS cells by the DEAE-dextran method (18). 24 h post-transfection, serum-free media was removed, cells and collected, and supernatants from COS cells were harvested, adjusted to pH 7.5, and passed over a protein A-Sepharose CL-4B (Pharmacia Biotech Inc.) column. The column was washed with 20 column volumes of 0.1 M Tris-HCl, pH 7.5, and the Fc fusion protein eluted with 0.1 M glycine buffer, pH 2.8. The peak eluate fractions were neutralized immediately, pooled, and dialyzed against phosphate-buffered saline (PBS) and stored frozen at –70°C. The yield was 2–5 μg/ml. These recombinant proteins were detected by immunoblotting with anti-Fc, anti-ST2, or anti-IL-1R antibodies and horseradish peroxidase-conjugated secondary antibody followed by enhanced chemiluminescence detection (ECL, Amersham Corp.). Soluble ST2 was obtained by digesting ST2Fc fusion protein with factor Xa (New England Biolabs, Beverly, MA) according with an in vitro digestion protocol and passing the digest over a protein A-Sepharose column. The identity of each protein was also confirmed by N-terminal sequencing.

IL-1 Binding Assays and Receptor Precipitation or Immunoprecipitation—For binding assays, Fc fusion proteins were first allowed to bind protein A-Sepharose for 15 min at room temperature. 5,000–250,000 cpm of [3H]IL-1Ra360 (0.001 μM) was incubated at room temperature for 3 h. For competition assays a 1000-fold molar excess of unlabeled IL-1 and a 200-fold molar excess of soluble receptors were used. The reaction mixture was then centrifuged in a microfuge at high speed, and the pellets were washed 3 times with 500 μl of ice-cold binding buffer. The pellets were counted in a γ counter (Bedboard Instruments) and resuspended in SDS sample buffer for SDS-PAGE analysis. All experiments were done in triplicate, and results from one representative experiment are shown. Less than 10% of the total iodinated ligand bound at the highest concentration tested and that exceeded 5% in all binding studies.

For receptor precipitation, 1–2.5 μg of various purified Fc fusion proteins were mixed with 35S-labeled 3T3 conditioned medium and 20 μl of protein A-agarose (Life Technologies, Inc.) and incubated overnight at 4°C. Protein-A-agarose pellets were collected by centrifugation and washed 3 times with PBSTDS buffer (PBS containing 1% Triton X-100, 0.1% SDS, and 0.01% sodium deoxycholate). Pellets were solubilized in sample buffer and resolved through SDS-PAGE, fixed, dried, and visualized by autoradiography. In some experiments the pH of 3T3 conditioned medium was lowered to 3.0 by 0.1 M HCl and then immediately neutralized before the receptor precipitation assay. For immunoprecipitation, polyclonal rabbit antiserum (preimmune or immune) against ST2 and IL-1R were used. For myc tagged fusion proteins, soluble ST2 fusion proteins were mixed with 35S-labeled 3T3 conditioned medium and 20 μl of protein A-agarose. For competition studies, soluble ST2 fusion proteins were mixed with 35S-labeled 3T3 conditioned medium and 1 μl of unlabeled IL-1Ra360, 1 μg/ml leupeptin, and 5 units/ml aprotinin for 20 min on ice followed by centrifugation at 15,000 × g for 20 min to remove nuclei and cell debris. For cross-linking experiments, the homobifunctional cross-linker disuccinimidyl suberate (Pierce) was added to form cross-linker-receptor complexes in binding buffer at a final concentration of 1 μM for 30 min, followed by the addition of Tris-HCI, pH 7.4 to 10 μM. Sample buffer was then added, and cross-linked protein was resolved by SDS-PAGE. Endogenous soluble ST2 protein was partially purified by immunoprecipitation from concentrated 3T3 cell conditioned medium using anti-ST2 polyclonal antibody. ST2 was eluted from the agarose-bound antibody-bearing beads using 100 μg/ml of pCMV-β-galactosidase reporter DNA as transgenes using Lipofectamine reagent (Life Technologies, Inc.) according to the manufacturer’s recommendations. After recovery of cells in serum containing media for 24 h, cells were split into two flasks. One flask was left untreated, and the other flask was treated with 10 ng of IL-1α or IL-1β for 16 h. Cells were washed with PBS, and extracts were prepared by three freeze-thaw cycles. The extracts were normalized for β-galactosidase expression, which was used as an internal control for transfection efficiency. The CAT activity was determined in duplicate as described (24). The radioactivity was quantitated in a Betascope 603 blot analyzer (Betagen, Mountain View, CA).

For the CSBP/p38 kinase assay, 2 × 107 cells were transfected with different receptor constructs, and 48 h later one-half of cells were stimulated with IL-1α or β and neutralized with IL-1α for 30 min at 30°C as in Raingeaud et al. (25) using anti-CSBP polyclonal antibodies (26). The kinase reaction was stopped by the addition of SDS-PAGE buffer, boiled for 2 min, and resolved by SDS-PAGE. The half-life of the gel containing the myd-in basic protein (MBP) was prepared for autoradiography, and the half-life of the gel containing CSBP was prepared for immunoblotting. The radioactivity in each band was quantitated in a Betascope. For the receptor-associated kinase assay, cells were lysed and immunoprecipitated with a monoclonal anti-IL-1R antibody (Genzyme, Cambridge, MA), and the
kinase assay was performed as in Croston et al. (27) with the exception that MBP (5 μg) was also added as an exogenous substrate in the kinase assay. These experiments were performed with cells stimulated with both IL-1α and β, and comparable results were obtained. Therefore, the data only for IL-1α are presented.

Size Exclusion Chromatography of 3T3 Conditioned Medium and Dot Blot Assay—5 ml of serum-free conditioned medium from 3T3 cells was concentrated 10-fold by ultrafiltration (Amicon YM-10 membrane) and applied to a 25-ml (10 × 300-mm) Superdex-75 column (Pharmacia) equilibrated with 50 mM sodium acetate (pH 4.5) and 100 mM NaCl. After the void volume, 1-ml fractions were collected. Molecular weights for each fraction were calculated by linear regression based on standard proteins (Bio-Rad) for gel filtration. 100 μl of each fraction were concentrated 10-fold by ultrafiltration (Amicon YM-10 membrane) and visualized by ECL. The position of the molecular weight markers is indicated on the left.

Binding of ST2 and IL-1R Fc Fusion Proteins to IL-1—We used soluble human ST2Fc and IL-1RFc proteins in a receptor precipitation assay to study the binding of human IL-1. The IL-1RFc showed saturable binding of 125I-IL-1α (Fig. 2), whereas human ST2Fc failed to show any significant binding of 125I-IL-1α over that of control IgG. We also performed a competition assay to determine if soluble ST2 competed in the binding of IL-1 to IL-1RFc. Human IL-1RFc bound an average of 7000 cpm (0.06%) of 125I-IL-1α; this binding was competed by excess cold IL-1α, IL-1β, IL-1 receptor antagonist, or soluble IL-1 receptor (sIL-1R) but not by soluble ST2 (hST2R) (Fig. 3, lanes 1-6). In contrast, HST2Fc fusion protein did not precipitate any significant amount of labeled IL-1α (Fig. 3, lanes 7-12). Purified human IgG, IL-1RFc, or protein A-agarose beads also did not precipitate IL-1α above background. HST2R did not compete with the binding of IL-1α to IL-1RFc even when added in a 1000-fold molar excess. In contrast, more than 50% inhibition of binding was observed with a 200-fold molar excess of sIL-1R (lane 5). Also, no binding was observed when sIL-1R was included with HST2Fc receptor in the precipitation assay (Fig. 3, lane 11), suggesting that ST2 is not a second subunit of the IL-1R, which might have led to an increased binding. These data show that the HST2Fc fusion protein does not bind IL-1. Similar results were obtained when iodinated IL-1β or IL-1 receptor antagonist were used as ligands (data not shown). We were also unable to show any binding of IL-1 to ST2 by adding metal ions in this assay. Metal ions, especially zinc, have been shown to increase the binding affinity of growth hormone to prolactin receptor (33).

RESULTS

Expression and Purification of ST2Fc Protein—In order to study the role of ST2 protein, we chose to express it as an IgG-Fc fusion. Several fusion proteins including Fc fusions have been successfully used for receptor-ligand binding experiments (30, 31). Both human and murine ST2 and human type I IL-1R Fc fusion proteins were expressed in COS and Drosophila cells and purified by affinity chromatography on a protein A-Sepharose CL-4B column. Fig. 1 shows the immunoblot of recombinant ST2Fc and IL-1RFc proteins with an anti-Fc antibody. Proteins expressed in COS cells (lanes 1-3) are >100 kDa, whereas proteins expressed in Drosophila cells (lanes 4-6) are slightly smaller, ~100 kDa. Since the predicted molecular weight for these proteins is ~70 kDa, the apparent increase is likely due to glycosylation, in agreement with previous reports (3, 32) with differences between Drosophila versus COS expressed proteins reflecting the differences in glycosylation complexity between mammals and insects. Unless otherwise indicated, all experiments were done with both Drosophila- and COS-expressed proteins, and results were comparable.
sion may not behave as authentic ST2 protein, we used immu-

mumoreactivity that recombinant ST2 protein expressed as Fc fu-

to or with 2 ng each of various unlabeled competitors as indicated. 

After incubation the beads were collected by centrifugation, washed 3

times to remove unbound ligand, counted in a γ counter (A), and then 

resuspended in sample buffer and resolved by SDS-PAGE (B).

Fig. 3. Receptor-mediated precipitation of 125I-IL-1α by hu-

man IL-1RFc and HST2Fc fusions. A, 100 ng of Fc fusion proteins 

immobilized on 20 μl of protein A-Sepharose were incubated for 3 h with 

2 ng of 125I-IL-1α (specific activity, 60,000 cpm/ng) without any compet-

itor or with 2 μg each of various unlabeled competitors as indicated. 

Fig. 4. Cross-linking of iodinated IL-1α and IL-1β to ST2 and 

IL-3R. ST2 immunopurified from 3T3 condition medium (3T3-ST2, 

lanes 1 and 2 and lanes 5 and 6) or 10 ng of purified sIL-1R (lanes 3 and 

4 and lanes 7 and 8) or 100 ng of HST2Fc (lanes 9–12) or 10 ng of human 

IL-1RFc (lanes 13 and 14) were incubated with 2 ng of iodinated IL-1s 

(specific activity, 60,000 cpm/ng) as indicated. After 3 h at room tem-

perature, the homobifunctional cross-linker disuccinimidyl suberate 

was added to a 1 ml final concentration and incubated for an additional 

30 min. Cross-linked products were analyzed by SDS-PAGE and auto-

radiography. Even numbered lanes show cross-linking in the presence of 

1000-fold molar excess of unlabeled ligands.

either IL-1α or IL-1β (lanes 9 and 11), whereas siL-1R or 

IL-1RFc was cross-linked to both (lanes 3, 7, and 13). The 

cross-linked product of siL-1R with IL-1α (lane 7) was slightly 
larger than that of sL-1R with IL-1β (lane 3). The reason for 

this difference is not clear at this time. However, these cross-

linked bands were specific since they could be competed by the 

appropriate excess cold IL-1 (lanes 4, 8, and 14). To exclude the 

possibility that recombinant ST2 protein expressed as Fc fu-

sion may not behave as authentic ST2 protein, we used immu-

nopurified ST2 protein from 3T3 cell conditioned medium, but 

it also failed to cross-link to either IL-1α or IL-1β (Fig. 4, lanes 

1 and 2 and lanes 5 and 6).

BIACore Assays—As a more sensitive means of detection we 

used a BIACore biosensor instrument to determine if ST2 could 

bind IL-1. Human ST2Fc protein was captured on the biosensor 

chip surface via protein A immobilized to the activated carboxymethyl dextran surface. As shown in Fig. 5, 

no binding was observed with any of the three IL-1 ligands 

(white brick bars). The results were negative at various con-

centrations of IL-1 (10 pM to 10 μM) and over a wide concen-

tration range of captured ST2Fc protein. In contrast, a polye- 


conal immunoblot against ST2 protein and the IL-1R protein 

revealed binding (white brick bars) and the various IL-1s also bound to IL-1RFc cap-

tured in a similar way (black brick bars).

Signal transduction through the ST2 and IL-1R—It is pos-

sible that the soluble ST2 receptor has a very low binding 

affinity for IL-1 but that the membrane-anchored full-length 

ST2 receptor may respond to IL-1 binding. To test this hypo-

thesis, we transiently coexpressed membrane ST2 (MST2R) or 

IL-1R in J urkat cells together with a synthetic IL-8 promoter-

CAT reporter gene. J urkat cells lack IL-1Rs (34), but have 

previously been shown to be responsive to IL-1 once transfected 

with the type I IL-1R cDNA (6). It has also been shown that 

IL-1 induces IL-8 production in many cell types (35), and IL-8 

promoter sequences responsible for this induction have been 

identified (36). We also created fusions of the extracellular 

domain of the ST2 receptor with the intracellular domain of 

IL-1R and vice versa (Fig. 6A, top panel). As shown in Fig. 6A, J 

urkat cells cotransfected with the IL-1R and the IL-8P/CAT 

expression vectors showed a 5-fold induction of CAT activity 

in response to IL-1α. An IL-1R construct truncated at amino 

acid 360 (IL-1RΔ360), with all but five amino acids of the 

intracellular portion deleted, did not respond to IL-1, showing 

that the intracellular domain of IL-1R was required for signal 

transduction. Expression of a chimeric protein containing 

the extracellular portion of human IL-1R and the intracellular 

portion of mouse ST2 receptor (IL-1R/MST2R), also resulted in 

a 7-fold induction of CAT activity in response to IL-1, sug-

gesting that the intracellular domain of ST2 shared signaling 

determinants with IL-1R. In contrast, neither the full-length 

ST2 receptor (MST2R) nor a chimeric receptor containing the 

extracellular portion of MST2 and the intracellular portion of 

IL-1R (MST2R/IL-1R) responded to IL-1, suggesting that it 

does not bind IL-1 even when expressed on the cell surface. 

Similar data were obtained when a HIV1LTR/CAT, another
IL-1-responsive promoter, was used as a reporter gene.2 The observation that the intracellular portion of the ST2 receptor can substitute for the intracellular portion of IL-1R suggests that the signal transduction pathways for the intracellular portion of the two receptors are similar. IL-1 is known to activate a recently discovered stress-activated MAP kinase known as CSBP/p38 (25, 26, 37, 38). We next investigated whether the chimeric receptor also activated this MAP kinase. As shown in Fig. 6, CSBP/p38 was activated in response to IL-1β in cells expressing the IL-1R and the IL-1R/MST2R chimera but not in cells expressing the truncated IL-1RΔ360 receptor. It has also been reported that an ~80-kDa IL-1R-associated protein kinase is required for IL-1-mediated activation of NF-κB (27) and that another protein kinase that phosphorylates MBP co-immunoprecipitates with type I IL-1R in response to IL-1 in T cells (39). We therefore examined if either kinase was activated by the chimeric receptor. While we were unable to detect the ~80-kDa autophosphorylating kinase, we did detect an MBP-phosphorylating protein kinase activity that was induced within 5 min following IL-1β stimulation in cells transfected with the IL-1R and the IL-1R/MST2R chimera but not with the truncated IL-1RΔ360 receptor (Fig. 6C). These data suggest that at least part of the signal transduction pathway between the IL-1 and the ST2 receptors are common. We could not detect activation of these kinases by either MST2R or the MST2R/IL-1R chimera in response to IL-1 (data not shown).

Identification of a Putative Ligand of ST2—The observation that the intracellular portion of the ST2 receptor can substitute for the intracellular portion of IL-1R suggests that the signal transduction pathways for the intracellular portion of the two receptors are similar. IL-1 is known to activate a recently discovered stress-activated MAP kinase known as CSBP/p38 (25, 26, 37, 38). We next investigated whether the chimeric receptor also activated this MAP kinase. As shown in Fig. 6B, CSBP/p38 was activated in response to IL-1β in cells expressing the IL-1R and the IL-1R/MST2R chimera but not in cells expressing the truncated IL-1RΔ360 receptor. It has also been reported that an ~80-kDa IL-1R-associated protein kinase is required for IL-1-mediated activation of NF-κB (27) and that another protein kinase that phosphorylates MBP co-immunoprecipitates with type I IL-1R in response to IL-1 in T cells (39). We therefore examined if either kinase was activated by the chimeric receptor. While we were unable to detect the ~80-kDa autophosphorylating kinase, we did detect an MBP-phosphorylating protein kinase activity that was induced within 5 min following IL-1β stimulation in cells transfected with the IL-1R and the IL-1R/MST2R chimera but not with the truncated IL-1RΔ360 receptor (Fig. 6C). These data suggest that at least part of the signal transduction pathway between the IL-1 and the ST2 receptors are common. We could not detect activation of these kinases by either MST2R or the MST2R/IL-1R chimera in response to IL-1 (data not shown).

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to identify cells that make putative ST2 ligand, we made use of the observation that soluble receptor secretion often accompanies ligand expression. We screened several cell lines for receptor expression by immunoprecipitation. To facilitate the detection of both soluble and membrane-anchored forms of the ST2 receptor, we generated polyclonal antibodies in rabbits using purified soluble ST2 protein expressed in Drosophila cells. These antibodies were used to immunoprecipitate both soluble and full-length ST2 proteins from metabolically labeled cells. As shown in Fig. 7A, a soluble ST2 protein of ≈50–60 kDa (arrow) is precipitated from exponentially growing 3T3 medium (lane 2). The anti-ST2 antibodies precipitated two proteins of ≈40–50 kDa (open arrowhead, lane 4) and ≈70–90 kDa (filled arrowhead, lane 4) from a 3T3 cell extract. The ≈40–50-kDa protein is probably the soluble receptor in the process of being secreted or the unglycosylated full-length receptor, whereas the 70–90-kDa protein is likely to be the cell surface form. These proteins are reported to be highly glycosylated, which is consistent with their higher than predicted molecular weight (1, 3, 32). Immune serum preabsorbed with recombinant soluble ST2 protein or preimmune serum did not precipitate these proteins (Fig. 7A, lanes 1 and 3, and data not shown). Similarly, immune serum but not preimmune serum also precipitated ≈50–60-kDa soluble ST2 protein (Fig. 7B, lane 2, filled arrowhead) from HUVEC.

We used mouse and human ST2Fc fusion proteins to identify ST2 binding proteins in metabolically labeled media from HUVEC and 3T3 cells made quiescent by serum starvation. As shown in Fig. 7B, an ≈18-kDa protein (arrow) and an ≈32-kDa protein (open arrowhead) were precipitated from HUVEC medium by HST2Fc (lane 3) but not by control IgG (lane 4). There are additional proteins also precipitated by HST2Fc. However, only the ≈18- and the ≈32-kDa proteins were precipitated by the mouse ST2Fc (MST2Fc) from quiescent 3T3 cell medium (Fig. 7C, lane 2, arrow and open arrowhead) but not by protein A-agarose beads alone (lane 1) or by control IgG (lane 3). Preincubation of labeled conditioned medium with soluble ST2 protein inhibited the precipitation of both the ≈18- and the ≈32-kDa proteins by ST2Fc (data not shown). These two proteins were not precipitated by ST2Fc from either HUVEC or 3T3 cell lysates (data not shown).

The experiment was repeated with metabolically labeled conditioned media from exponentially growing 3T3 cells in the presence of serum. Both human and murine ST2Fc fusion proteins precipitated an ≈18-kDa protein (Fig. 8, lanes 1 and 2, arrow). However, the intensity of this band was very faint. Since exponentially growing cells secrete a large amount of soluble ST2 protein, whereas quiescent cells do not (32), we suspected that most of the ligand may be bound to the secreted endogenous ST2. To release this potential pool of ligand, labeled conditioned medium from these cells was briefly treated with acid and neutralized before the addition of various Fc fusion proteins. As shown in Fig. 8, lanes 6 and 7, the intensity of the ≈18-kDa band increased dramatically following this brief acid treatment. All control Fc fusion proteins were negative in this assay (Fig. 8, lanes 3–5 and lanes 8–10). Acid treatment also led to an increase in the signal of other proteins in the high molecular weight range which was not reproducible and varied among different experiments. These high molecular weight proteins probably result from aggregation of ST2Fc fusion protein alone or with other labeled proteins in the conditioned medium, perhaps due to denaturation of serum proteins and/or ST2 following acid treatment. Alternatively some of these proteins may represent other accessory proteins coprecipitated with the ST2 ligand-receptor complex.
To confirm the size of ST2 ligand, we passed the concentrated serum-free 3T3 conditioned medium over a Superdex 75 gel filtration column and assayed the resulting fractions by a dot blot assay using ST2Fc. As shown in Fig. 9, fractions corresponding to 47 and 15 kDa were positive in this assay, with maximum signal obtained with fraction corresponding to 20 kDa. These data are consistent with our earlier results from receptor-precipitation studies.

As further evidence for the existence of the ST2 binding proteins, we used BIAcore analysis. A similar assay has been successfully used to identify the ligand for the ECK receptor protein-tyrosine kinase (28). As shown in Fig. 10, both unconcentrated (3T3 1-fold) and a 10-fold concentrated (3T3 10-fold) 3T3 cell conditioned medium showed significant binding (white brick bars) to ST2Fc protein captured through immobilized protein A. Soluble ST2 competed for this binding, thus showing its specificity (+MST2). Similarly, a 10-fold concentrated conditioned medium from HUVEC (HUVEC 10-fold) also showed specific binding. The 20–50-kDa fraction, obtained from concentrated 3T3 conditioned medium after passage through a Superdex 75 gel filtration column (see Fig. 9) was also positive in this assay (data not shown). No binding was observed with concentrated control media (DMEM 10-fold) to ST2Fc or with various conditioned media to IL-1RFc (black brick bars). Conditioned media from either 3T3 or HUVEC did not show any binding to unactivated chip surface, protein A, or unrelated immobilized Fc fusions. We screened conditioned media from several other cell lines including Jurkat cells for this binding activity, but we were unable to find any other cell lines positive in this assay.

To look for signal transduction by the putative ST2 ligand, we examined CSBP/p38 activation and IL-8 promoter/CAT stimulation in Jurkat cells that have endogenous ST2 receptor.2 The concentrated 3T3 conditioned medium was able to activate CSBP/p38 MAP kinase (Fig. 11) similar to IL-1β. The activation of CSBP could be blocked >80% by preincubation of medium with MST2Fc protein, suggesting that the ST2 ligand is functional. However, the same 3T3-concentrated medium failed to induce transfected IL-1 promoter/CAT or HIV1LTR/CAT reporter genes (data not shown). Since Jurkat cells have endogenous ST2 receptor we could not test the chimeric ST2/IL-1 receptor.
Identification of a Putative Ligand of ST2

DISCUSSION

To identify the ligand(s) of ST2, we have used an ST2Fc fusion protein to assess binding to purified IL-1s and crude cell lysates and media. Our data establish very clearly that none of the IL-1s are ligands for ST2. We did not detect binding via receptor precipitation, cross-linking, BIACore, or signal transduction assays. This is in contrast to a recent report published while this paper was under review, which detected weak binding of rat ST2/Fc-1 to murine IL-1β (40). While we occasionally did detect weak, comparable binding of human IL-1α and IL-1β to high concentrations of ligands and human ST2Fc in receptor precipitation assays, the binding was not saturable. Furthermore, we could not detect any binding of these proteins (including ST2 and IL-1-β) from mouse by the more sensitive BIACore, which can detect affinities in the μM range, so we concluded that ST2 does not bind IL-1. We agree with these authors, however, that IL-1 does not signal through ST2. A second preliminary report is in agreement with our data (41).

In contrast to the negative data with IL-1, we were able to identify a ligand in 3T3 and HUVEC conditioned media using some of the same assays. In both cell media, two proteins of ~18 and 32 kDa were specifically precipitated by ST2Fc. Size exclusion data also indicated a ligand with a molecular mass of ~20 kDa, which, along with the variable appearance of the 32-kDa protein, suggests that the ligand binds as a monomer rather than a heterodimer. Although we do not know the relationship of the 32- and 18-kDa proteins, it is possible that the 32-kDa protein is a precursor of the 18-kDa protein, reminiscent of IL-1. However, we did not detect these proteins in cell extracts. The intensity of the 18-kDa protein also varied, depending upon the presence of endogenous ST2 as evidenced by an increase in signal after acid treatment of conditioned media made from exponentially growing cells. In quiescent cells, where no ST2 is made, there was no effect of acid treatment. These data suggest that ST2 ligand is continuously made, whereas the expression of soluble ST2 is modulated by serum and growth conditions.

Although we have not been able to define a biological activity for this ST2 ligand(s) we have discovered, the ability of this ligand to activate the stress-activated MAP kinase CSBP/p38 and inducing a receptor-associated protein kinase, CSBP/p38, and growth pathways.

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