Chicken Macrophage Stimulating Protein Is a Ligand of the Receptor Protein-tyrosine Kinase Sea*

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Affinity chromatography, employing the extracellular domain of the Sea receptor, was used to enrich Sea-binding proteins from chicken serum. One isolated protein bound both a Sea-immunoglobulin fusion protein and an antiserum raised against murine macrophage stimulating protein. Amino-terminal sequencing of the dual-reactive protein yielded sequences which were identical to the predicted \(\alpha\) and \(\beta\) subunits of chicken macrophage stimulating protein. The partially purified chicken macrophage stimulating protein caused auto-phosphorylation of the Sea receptor. Previous work showed that recombinant expression of fully activatable human or mouse macrophage stimulating protein required a specific Cys to Ala substitution (Wahl, R. C., Costigan, V. J., Batac, J. P., Chen, K., Cam, L., Courchesne, P. L., Patterson, S. D. Zhang, K., and Pacifici, R. E. (1997) \textit{J. Biol. Chem.} 272, 1–4). Therefore, we expressed both the wild type and the specific Cys to Ala form of chicken macrophage stimulating protein as recombinant proteins. After proteolytic activation, only conditioned media from COS cells transfected with the C665A chicken macrophage stimulating protein, but not from wild type chicken macrophage-stimulating protein, or control vector, was detected by the Sea-immunoglobulin fusion protein in Western blotting experiments. Conditioned media containing the C665A chicken macrophage-stimulating protein readily caused Sea phosphorylation, while conditioned media containing the wild type chicken macrophage-stimulating protein was only effective at inducing receptor phosphorylation at high concentrations. In addition to receptor phosphorylation, the C665A chicken macrophage-stimulating protein induced phosphorylation of Shc, Erk1, and Erk 2. We conclude that macrophage-stimulating protein is a ligand of the Sea receptor protein-tyrosine kinase.

The receptor protein-tyrosine kinases are a structurally related family of transmembrane proteins which regulate a wide variety of cellular responses to extracellular stimulation (2). The binding of ligand to the extracellular domain of these receptors results in rapid intracellular autophosphorylation followed by the phosphorylation of multiple downstream effector proteins (2).

The Met receptor protein-tyrosine kinase (3, 4) is the prototype of a subfamily of receptor protein-tyrosine kinases. Other members of this subfamily include the RON\(^2\)Stk (5–7) and Sea receptors (8). A common feature of this subfamily is that the mature version of the receptor consist of a heterodimer between a small \(\alpha\) chain (\(-35 \text{ kDa}\)) and a larger \(\beta\) chain (\(-160 \text{ kDa}\)) which arise from proteolytic cleavage of a precursor protein. The gene encoding Met has been identified in both mammalian and chicken species (3, 4, 9). However, RON/Stk has only been identified in mammalian species, while Sea has only been found in chicken species, despite repeated efforts to find orthologs in other species (10).\(^2\)

The Sea receptor was originally identified as the cellular homologue of the avian retroviral oncprotein v-sea. This avian erythroblastosis virus (S13) induced sarcomas, erythroleukosis, and anemia in infected birds (11, 12). Transformation of erythroid cells with the sea oncogene rendered them erythropoietin independent (13, 14). The Sea receptor is a heterodimeric protein composed of a 35-kDa \(\alpha\) and a 160-kDa \(\beta\) chain and is expressed at low levels in a variety of tissues including kidney, intestine, liver, stomach, white blood cells, and allantochorion (8, 15). Protein comparisons indicate that the extracellular domain of the Sea receptor is more similar to RON (48% identity) or stk (44% identity) than to Met (39% identity). Analysis of the extracellular domain of chicken Met indicates 72% identity to human MET, but only 37% identity to RON. Because a mammalian analog of Sea has not yet been identified, and since a chicken Met gene but not a chicken Ron gene has been identified, it is possible that RON and Sea are orthologous receptors. However, definitive data has not yet been obtained to support or refute this hypothesis. Analysis of downstream signaling events indicated that activation of the Sea intracellular domain (fused to the extracellular domain of Trk and activated by nerve growth factor) triggered similar biological responses to those of RON or Met when these receptors were stimulated by their respective ligands (16).

A family of related proteins serve as ligands for these receptors. Hepatocyte growth factor/scatter factor (HGF/scatter factor) 

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1 The abbreviations used are: RON, receptor d’origine nantaise; stk, stem cell tyrosine kinase; Sea, sarcoma, erythroleukosis, and anemia; HGF, hepatocyte growth factor; MSP, macrophage-stimulating protein; Sea-Fc, Sea receptor-immunoglobulin fusion; PBS, phosphate-buffered saline; anti-Tyr(P), anti-phosphotyrosine; PCR, polymerase chain reaction; chMSP, chicken MSP; mMSP, murine MSP; b, base pair(s); CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

2 A. A. Welcher, unpublished observations.
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The Sea-Fc-binding proteins from chicken serum—The Sea-Fc construct (14) was transfected into Chinese hamster ovary cells and recombinant expression was induced with 10 μg/ml of polyethylene imine (29). The Sea-Fc was purified with a protein G-Sepharose HiTrap column (Mab Trap G II, Amersham Pharmacia Biotech) following the manufacturer’s instructions.

One liter of undialyzed chicken sera (Life Technologies, Gaithersburg, MD), containing ~20 g of protein, was chromatographed on a 80-ml heparin-Sepharose column (Amersham Pharmacia Biotech) that was equilibrated with 0.15 M NaCl, 20 mM sodium phosphate, pH 7.0 (PBS). The column was washed with PBS to remove unbound proteins, and bound proteins were eluted with 2 × NaCl, 20 mM sodium phosphate, pH 7.0. The 2 m salt eluate, containing ~600 mg of protein in 150 ml, was dialyzed overnight at 4 °C versus PBS, and then serine proteases were inactivated with 1 mM Pefabloc (Roche Molecular Biochemicals, Indianapolis, IN). Sea-Fc was coupled to cyanogen bromide-activated Sepharose at 1 mg of Sea-Fc/ml gel, according to the manufacturer’s instructions (Amersham Pharmacia Biotech). The heparin-Sepharose eluate was chromatographed on a 1-ml column of Sea-Fc-Sepharose equilibrated with PBS. After sample application, the column was washed with PBS until the A280 returned to baseline, then washed with 5 ml of PBS + 0.35 M salt, followed by 10 ml of a high pH elution buffer consisting of 50 mM CAPS, pH 10.4, 0.5 M NaCl, 1 mM CHAPS, 0.005% Tween 20. Both elution buffers eluted well defined peaks of absorption at 280 nm containing 1–2 A280 units. Fraction 15 (Fig. 1) contained about 1 mg of protein. Unless otherwise stated, chemical reagents came from Sigma, while the molecular biology reagents came from Roche Molecular Biochemicals.

**Gel Electrophoresis, Western Blot, and Sequence Analysis of Purified and Reduced Chicken MSP Proteins—SDS-PAGE gels were from Novex (San Diego, CA). Nonreduced samples were mixed with sample buffer containing 2% SDS but not β-mercaptoethanol, and were not heated.**

For Western blot analyses, separated proteins were transferred to nitrocellulose or Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with either 5% nonfat dry milk or 5% bovine serum albumin and 1% ovalbumin (for anti-phosphotyrosine (anti-Tyr(P)) blots only) in 20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 for 1 h at room temperature. For MSP detection, the membranes were incubated with 4–20 μg/ml anti-murine MSP antibodies (raised against murine pro-MSP (13)) for 1 h at room temperature, followed by donkey anti-rabbit IgG conjugated with horseradish peroxidase (Amersham) for 20 min at room temperature. For Sea detection, the membranes were incubated with 4 μg/ml of the 4E10 antibody (15) for 1 h at room temperature, followed by donkey anti-rabbit IgG conjugated with horseradish peroxidase (Amersham) for 20 min at room temperature. For anti-Tyr(P) detection, the membranes were incubated with 1 μg/ml anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) for 1 h at room temperature, followed by sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham) for 20 min at room temperature. For anti-Tyr(P) detection, the membranes were incubated with 1 μg/ml rabbit anti-ERK1/2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, followed by donkey anti-rabbit IgG conjugated with horseradish peroxidase (Amersham) for 20 min at room temperature. For the Shc and ERK detection experiments, the membranes were incubated either with 1 μg/ml rabbit anti-human Shc antibody (Upstate Biotechnology, Lake Placid, NY) or with 1 μg/ml rabbit anti-ERK1/2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature, followed by sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham) for 20 min at room temperature. Bound immune complexes were detected with ECL reagents (Amersham). Amino-terminal sequencing was performed on an Applied Biosystems 477A (Perkin-Elmer, Norwalk, CT). The 354-bp product generated by oligonucleotides 5 and 6 was digested with the restriction enzymes Bgl II and Nar I to 936 bp; and the 986-bp product generated by oligonucleotides 1 and 2 was digested with the restriction enzymes Bgl II and Nar I to 975 bp. These three fragments were immunoprecipitated with either the monoclonal anti-Sea antibody SC3 (14) or anti-Tyr(P) (Upstate Biotechnology, Lake Placid, NY) at 4 °C overnight. Antibody-antigen complexes were then incubated with 50 μl of protein G-Sepharose beads (Amersham Pharmacia Biotech) at 4 °C for 30 min. Immune complexes were pelleted, washed, and resuspended in sample buffer.

**Cloning of Chicken MSP Gene**—A full-length cDNA of chMSP was constructed from three fragments generated by polymerase chain reaction (PCR) using as template an adult chicken liver 5′-stretch cDNA library in Agt10 (CLONTECH Laboratories, Palo Alto, CA). The primers listed in Table I were designed according to the sequence of GenBank accession number X84043 (9). Five μl (~5 × 10⁵ plaque forming units) of phase library was used for each PCR. Amplification was carried out for 35 cycles of 94 °C (15 s), 60 °C (15 s), and 72 °C (90 s) using AmpliTaq DNA Polymerase with the GeneAmp kit (Perkin-Elmer, Norwalk, CT). The 354-bp product generated by oligonucleotides 1 and 2 was digested with the restriction enzymes XhoI and NarI to 270 bp; the 1245-bp product generated by oligonucleotides 3 and 4 was digested with restriction enzymes NarI and BglII to 936 bp; and the 666-bp product generated by oligonucleotides 5 and 6 was digested with restriction enzymes BglII and EcoRI to 975 bp. These three fragments were ligated into the mammalian expression vector pBJS (30) prelinearized with restriction enzymes XhoI and EcoRI and the resulting construct was transformed into DH10B cells (Life Technologies, Gaithersburg, MD). The confirmed sequence of this full-length cDNA construct was identical to the published sequence (9) except for a silent nucleotide change (C instead of G) located at nucleotide 1981.

**Construction of C665A Chicken MSP—Cys665** was converted to Ala using an overlap amplification process. Wild type chicken MSP plasmid and oligonucleotides 8 and 9 containing the Cys to Ala change at nucleotides 2005 and 2006 were used to generate mutated chicken MSP cDNA. The 67-bp product was generated by carrying out 5 cycles at 94 °C (15 s), 60 °C (15 s), and 72 °C (30 s), followed by another 20 cycles...
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TABLE I
Sequences and positions of oligonucleotide primers (sequences written in lowercase represent linkers that were added to the chMSP primers)

| Sequence | Nucleotide position |
|----------|---------------------|
| 1. caacactcgaGGGCCATGCGGCGG | 7–27 |
| 2. CCATGGCCACGAGTCATC | 350–331 |
| 3. caacactcgaACCACGAGTCATC | 10–27 |
| 4. CAGGTGGTTCGCGGGGTGG | 1242–1224 |
| 5. CAGCGAAGGCTACACGCGGTCC | 1156–1174 |
| 6. caacagagctcttaTCACACCATC | 2127–2106 |
| 7. CTCATACGATC | 1951–1969 |
| 8. CAGGCACCGGCCCGGTGCTGGT | 2017–1995 |
| 9. CCGCGACGCCTGGGTGCTGG | 1997–2017 |
| 10. caacagagctcttaTCACACCATC | 2127–2118 |

at 94 °C (15 s), 64 °C (15 s), and 72 °C (30 s) with oligonucleotides 7 and 8. The 146-bp product was generated by carrying out 5 cycles at 94 °C (15 s), 58 °C (15 s), and 72 °C (30 s), followed by another 20 cycles of 94 °C (15 s), 68 °C (15 s), and 72 °C (30 s) with oligonucleotides 6 and 9. Both amplified fragments were isolated from a polyacrylamide gel and eluted with MAXAM buffer (0.5 μM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS, pH 8.0). Equal molar ratios of the two isolated fragments were re-amplified with oligonucleotides 7 and 10 for 25 cycles at 94 °C (15 s), 64 °C (15 s), and 72 °C (30 s) to generate the 192-bp mutated product. The isolated 192-bp fragment was digested with restriction enzymes Apol and EcoRI to a 149-bp fragment. Wild type chicken MSP plasmid was digested with restriction enzymes EcoRI and BglII to generate a 4.6-kilobase fragment, and BglII and Apol to generate the 775-bp fragment. The mutated fragment was then ligated with the 4.6-kilobase and 775-bp fragments to generate the mutated chMSP cDNA in the pBJ5 expression vector.

Production of Recombinant MSP in COS Cells—COS-7 cells were transfected as described previously (30). Conditioned media of transfected cells were concentrated by Centriprep-10 (Amicon, Beverly, MA). In order to activate recombinant pro-MSP, concentrated conditioned media was incubated at 37 °C for 1 h with human kallikrein (Enzyme Research Laboratories, South Bend, IN) at a final concentration of 20 μg/ml followed by addition of Pefabloc-SC (Roche Molecular Biochemicals, Indianapolis, IN) to a final concentration of 0.1 mM to stop the proteolytic activation.

For some experiments, conditioned media was concentrated 100× by chromatography on a 1-ml HiTrap heparin-Sepharose column (Amersham Pharmacia Biotech) and eluted with 2 M NaCl. The buffer was exchanged to PBS using Centricron-10 (Amicon Corp.) and activated by kallikrein as above.

Sea-Fc Competition Studies—30× concentrated, activated conditioned media from COS cells transfected with C665A chMSP was either mock-treated (PBS incubation) or preincubated with 10 or 100 μg/ml of either the Sea-Fc protein or the leptin receptor-Fc protein (an unrelated Fc fusion protein containing the extracellular domain of the leptin receptor). The mixtures were incubated at 4 °C for 1 h, then treated with the 32DSe5-2 cells as above. The final concentration of the C665A chMSP used to treat the cells represented 12× concentrated conditioned media.

RESULTS

During the course of our work with recombinant murine wild type and C677A variant MSP (1), we observed that the Sea-Fc fusion protein could be used as the primary probe in a Western blot analysis. As such, it detected active murine MSP with high sensitivity (see below), while neither pro-MSP nor reduced MSP nor any species other than the intact heterodimer were detected (data not shown). These observations suggested that the Sea-Fc might be a useful reagent for affinity purification of the Sea ligand. Since the known ligands for this family of receptors are detected in normal sera (22, 23), we investigated whether there were Sea-binding proteins present in chicken sera.

Both HGF and MSP bind strongly to heparin-Sepharose at neutral pH and physiological salt concentration (0.15 M), and can be eluted with increasing salt concentrations of about 0.8 M salt for MSP (31) and 1.2 M salt for HGF (32). Since it was anticipated that the Sea ligand might have a similar tight heparin-binding property, a heparin-Sepharose binding step with a single high salt elution step was employed as an initial purification step. One liter of chicken serum was chromatographed on a heparin-Sepharose column and bound proteins were eluted with high salt. The interaction of plasminogen-related growth factors with their receptors requires proteolytic activation of the ligands (21). According to literature reports, the heparin-Sepharose chromatography step used here was expected to purify both plasma kallikrein (33), an activator of human MSP (21), and hepatocyte growth factor activator (34). Indeed, incubation of the high salt eluate from the heparin-Sepharose column with the kallikrein substrate benzoyl-Pro-Phe-Arg-nitroanilide (35) resulted in considerable hydrolysis of the nitroanilide (data not shown), while the unfractionated chicken serum was inactive. This observation suggested that endogenous protease inhibitors had been removed and serum proteases had been activated during purification and were present in the eluate. Accordingly, the heparin-Sepharose eluate was incubated overnight at 4 °C in order that the endogenous, active serum proteases would activate potential plasminogen-related growth factors that were present. After incubation, the endogenous proteases were inactivated with Pefabloc, and the high salt heparin-Sepharose eluate was dialyzed versus PBS and then chromatographed on a Sea-Fc column. Bound proteins were eluted with a pH 10.5 buffer. Both the heparin-Sepharose and the Sea-Fc column eluted material showed well defined peaks of absorbity at 280 nm.

Analysis of the four peak fractions (numbers 13–16), as indicated by A280, to contain proteins eluted from the Sea-Fc-Sepharose column, is shown in Fig. 1. Staining with Coomassie Blue revealed that this two-step purified material contained multiple protein bands, but the overall protein complexity was reduced considerably relative to the starting sera (fraction 15 contains 1 mg of protein compared with 800 mg in the heparin pool, and 20 g in the starting serum). It was expected that isolation of some of the proteins resulted from nonspecific interactions with either the Sepharose resin or the Fc portion of the Sea-Fc, a result observed with other affinity purifications (data not shown). To characterize the proteins eluting from the Sea-Fc column further, Western blot analyses were performed. When the Sea-Fc fusion protein was used as the primary probe, a protein band with an apparent mobility of 84 kDa was readily detected in all the lanes, but especially in fraction 15 (Fig. 1B). Although, several protein species were eluted from the Sea-Fc column (Fig. 1A), the subsequent Western blot result implies a particularly strong interaction between the Sea-Fc and the 84-kDa species. For comparison, the same gel contained a standard of 100 ng of mMSP, which also reacted with the Sea-Fc. When a similar blot was probed with an anti-murine MSP antiserum, only the lane containing the largest amount of the 84-kDa protein was detected (Fig. 1C). On the same gel, 1 ng of mMSP was readily detected. Comparison of Fig. 1, B and C, reveals that the 84-kDa band reacts stronger with the Sea-Fc than 100 ng of mMSP; but reacts weaker with the anti-murine MSP than 1 ng of mMSP. To summarize, the partially purified preparation contained a discrete Coomassie-staining doublet band of about 84 kDa that bound the Sea-Fc.

Based on sequences already in SWISS-PROT, the Cys residue number in human, murine, and chicken is 672, 677, and 665, respectively.
purified protein responsible for Sea phosphorylation. However, using an anti-Sea antibody indicated that equivalent amounts of the protein mixture resulted in phosphorylation of the Sea receptor. The ligand was present in limiting amounts as an endogenous residue and Met and Ile in the fifth and fifteenth position of the subunit sequence, and Met and Ile in the fifth and fifteenth position of the β subunit are characteristic of chMSP and differ from mouse and human MSP (8). The NH$_2$ terminus of the α subunit of chMSP is different from that predicted by Thery et al. (9). By comparing the intensity of the 84-kDa band in fraction 15 to the intensity of a known amount of mMSP, we estimate that about 50 μg of chMSP was recovered from a liter of chicken serum. Rechromatography of the Sea-Fc-Sepharose flow-through on a preparative column and eluting fractions 13, 14, 15, and 16 were used for analysis. In parallel by 10% nonreducing SDS-PAGE, the end lane contains 100 ng of purified, recombinant mMSP. Panel B, parallel samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the Sea-Fc protein. The end lane contains 100 ng of purified, recombinant mMSP. Panel C, same as panel B except the nitrocellulose was probed with an anti-murine MSP antisera. The end lane contains 1 ng of purified mMSP. The isolated chMSP migrates slightly faster than the recombinant mMSP as indicated on the right.

and anti-murine MSP antisera.

To further characterize the reactive protein(s), NH$_2$-terminal sequencing was performed on the 84-kDa reactive protein from fraction 15 of the Sea-Fc-Sepharose eluate and on an additional preparation of chicken serum. The resulting residues and yield, listed in Table II, identify the 84-kDa doublet band as chMSP. Sequences from both the α and β subunits were detected. The presence of the amino acid residues His, Arg, and Leu in the first, tenth, and eleventh position of the α subunit sequence, and Met and Ile in the fifth and fifteenth position of the β subunit are characteristic of chMSP and differ from mouse and human MSP (8). The NH$_2$ terminus of the α subunit of chMSP is different from that predicted by Thery et al. (9). By comparing the intensity of the 84-kDa band in fraction 15 to the intensity of a known amount of mMSP, we estimate that about 50 μg of chMSP was recovered from a liter of chicken serum. Rechromatography of the Sea-Fc-Sepharose flow-through on the Sea-Fc-Sepharose, resulted in the recovery of only 1/10 of the amount of chMSP found in the first passage. This suggested that the amount of Sea-Fc-Sepharose was not limiting in this purification and the majority of protein capable of binding to Sea-Fc was recovered by the process. This overall purification was repeated five times with similar results (data not shown).

Having recovered Sea-binding proteins which included chMSP, we determined if they were capable of inducing Sea receptor phosphorylation. A stably transfected 32D cell line expressing the Sea receptor (15) was treated for 15 min with two amounts of the partially purified protein mixture from above. As shown in Fig. 2A, either 9 μl (lane 1) or 50 μl (lane 2) of the protein mixture resulted in phosphorylation of the Sea receptor. The ligand was present in limiting amounts as an increased amount of protein resulted in an increase in Sea phosphorylation. Treatment of the cells with PBS containing 1% bovine serum albumin (lane 3) failed to induce phosphorylation. Analysis of a parallel set of samples by Western blot using an anti-Sea antibody indicated that equivalent amounts of Sea receptor were present in all samples (Fig. 2B).

The preceding results strongly implied that chMSP is the purified protein responsible for Sea phosphorylation. However, since the protein mixture was not homogeneous, it remained possible that another protein was responsible for the observed phosphorylation. To directly address this, we produced chMSP as a recombinant protein in a mammalian expression system. Based on the published chMSP sequence (8), primers were used to amplify the coding region from a chicken cDNA library. Comparison of the isolated sequence to the published sequence revealed identity except for a single conservative nucleotide change, a C instead of a G, at position 818. Analysis of a variety of clones from several PCR experiments confirmed the isolated sequence. Previous work on mouse and human MSP indicated that a Cys (residue 672 in the human and 677 in the murine sequence) to Ala mutation was required for maximal potency of the recombinant protein (1). The presence of a homologous Cys residue in the chMSP (residue 665 in the chicken sequence) indicated that a similar amino acid residue change would likely be required, so the corresponding C665A MSP cDNA was produced and expressed in parallel to the wild type chMSP. Based on previous experiments with mouse or human MSP (1), it was anticipated that conditioned media would require treatment with kallikrein to convert pro-MSP to active MSP. Therefore parallel batches of conditioned media were either untreated or treated with kallikrein and incubated with Sea-expressing 32D cells. As shown in Fig. 3A, none of the nonactivated conditioned media were able to induce Sea phosphorylation (lanes 1–9). In contrast, activation of the wild type or C665A MSP containing conditioned media resulted in Sea phosphorylation (lanes 15–18). Only the highest concentration of wild type MSP-conditioned media led to detectable phosphorylation, while a clear dose-response was obtained with the C665A chicken MSP-conditioned media (lanes 16–18). Neither nonactivated nor activated conditioned media from mock-transfected COS cells induced phosphorylation at any concentration (lanes 1–3 and 10–12). Analysis of similar blots probed with anti-Sea antisera indicated that all treated cell samples contained equivalent amounts of the Sea receptor (Fig. 3B). These results clearly demonstrate that both wild type and C665A chicken MSP can induce Sea phosphorylation.

The ability of mMSP, purified natural chMSP, and conditioned media containing recombinant chMSP (wild type and C665A) to interact with either the Sea-Fc or the anti-MSP antisera in a Western analysis were compared in Fig. 4. As shown previously, Sea-Fc detected ~84-kDa bands in the murine C677A MSP and purified chMSP preparations (Fig. 4A). Sea-Fc detected the C665A chicken MSP only after activation (compare lanes 4 and 7). Therefore, the proteolytic cleavage-induced activation is required for the interaction of chMSP with the Sea-Fc. Sea-Fc did not detect bands in conditioned media from either wild type recombinant chMSP nor mock-transfected COS cells. The inability to detect recombinant chMSP with the Sea-Fc was likely a matter of sensitivity because similar amounts of the protein were able to induce Sea phosphorylation (Fig. 3A). The observation of similar results with the phosphorylation studies (Fig. 3) indicated that chMSP activation is necessary for both binding to the Sea-Fc and inducing receptor phosphorylation. The observed ~160-kDa reactive band(s) stem from the kallikrein treatment and were present in the kallikrein mixture alone used to treat the conditioned media (lane 3). We have never observed Sea-Fc to detect any band other than the 84-kDa heterodimer.

Fig. 4B shows the results when the same set of proteins were probed with anti-MSP antisera. As shown previously, both the C677A mMSP and the purified chMSP were recognized by the antisera (lanes 1 and 2). Although the C665A chMSP was recognized by the antisera regardless of activation, activation of the wild type recombinant chMSP caused the disappearance.
TABLE II
Edman sequencing of chicken serum MSP

| Residue no. | chMSP β chain | Seq 1a   | pmol | Seq 2   | pmol | chMSP α chain | Seq 1b | pmol |
|------------|---------------|---------|------|---------|------|---------------|--------|------|
| 1          | V             | V (2.7) |      | V (1.8) |      | H             | H (1.6) |      |
| 2          | V             | V (2.8) |      | V (1.8) |      | R             | R (1.2) |      |
| 3          | G             | G (1.7) |      | G (1.9) |      | S             | S (0.8) |      |
| 4          | G             | G (1.8) |      | G (1.6) |      | P             | P (0.7) |      |
| 5          | M             | M (0.9) |      | M (0.5) |      | L             | L (0.7) |      |
| 6          | P             | P (1.8) |      | P (1.7) |      | N             | N (0.7) |      |
| 7          | G             | G (1.1) |      | G (1.5) |      | D             | D (0.6) |      |
| 8          | N             | N (1.6) |      | N (1.8) |      | F             | F (0.7) |      |
| 9          | S             | S (0.5) |      | S (1.6) |      | Q             | Q (0.3) |      |
| 10         | P             | P (0.6) |      | P (1.1) |      | R             | R (0.4) |      |
| 11         | W             |         |      |         |      |               |        |      |
| 12         | T             | T (0.3) |      | T (1.4) |      |               |        |      |
| 13         | V             | V (0.4) |      | V (0.7) |      |               |        |      |
| 14         | S             | S (0.1) |      | S (0.4) |      |               |        |      |
| 15         | I             | I (0.3) |      | I (0.5) |      |               |        |      |

Fig. 2. Partially purified chMSP from sera causes Sea receptor phosphorylation. Proteins isolated in fraction 15 (lane 1, 9 μl; lane 2, 50 μl; lane 3, 1% bovine serum albumin) were incubated with 32D cells expressing the Sea receptor, and the receptor was immunoprecipitated and analyzed by Western blot. Panel A, the membrane was incubated with an anti-Tyr(P) antibody. Panel B, the membrane was incubated with an anti-Sea antibody. Molecular weight markers (Amersham Pharmacia Biotech) are shown on the right.

of the ~84-kDa reactive band. Prolonged exposures of the lower panel showed a lower, ~50-kDa reactive band in the wild type chMSP-conditioned media after treatment (data not shown).

In order to directly compare C677A mMSP to C665A chMSP, both were produced in COS cell-conditioned media. Varying amounts of activated COS cell-conditioned media containing either C665A chicken or C677A murine MSP were used to treat Sea-expressing cells. As before, the chMSP-conditioned media readily caused Sea phosphorylation, while none of the amounts of mMSP were able to induce phosphorylation (Fig. 5A). Analysis of parallel blots probed with anti-Sea antisera indicated that all tested samples contained the same amount of Sea receptor (Fig. 5B). A separate Western blot analysis indicated that both proteins were present in conditioned media, but because of their different reactivity (murine greater than chicken) it was not possible to directly compare expression levels. In a parallel experiment, purified, recombinant C677A mMSP, at concentrations as high as 5 μg/ml, failed to induce Sea phosphorylation (data not shown) even though this material was active on the murine receptor (1). Thus, Sea receptor phosphorylation induced by MSP is species specific.

The Sea-Fc fusion protein was able to block the ability of recombinant C665A chMSP to stimulate autophosphorylation of the Sea receptor (Fig. 5, C and D). Preincubation with either 10 μg (Fig. 5C, lane 3) or 100 μg of Sea-Fc (Fig. 5C, lane 4) inhibited subsequent phosphorylation of the Sea receptor. However, neither 10 μg (Fig. 5C, lane 6) nor 100 μg (Fig. 5C, lane 7) of an unrelated Fc fusion protein (comprising the extracellular domain of the leptin receptor) had any effect on chMSP-induced receptor phosphorylation. Therefore, the chMSP is binding specifically to the Sea receptor, and inducing phosphorylation.

To begin to understand the biological significance of the interaction of MSP with the Sea receptor, we examined whether potential downstream targets of the Sea receptor were phosphorylated in a ligand-dependent manner. Activated conditioned media from COS cells either mock-transfected or transfected with the C665A chMSP were used to treat Sea-expressing 32D cells. Cells were lysed and various proteins immunoprecipitated and analyzed by Western blot. Only treatment of the cells with C665A chMSP-conditioned media led to the specific phosphorylation of Erk 1 and Erk 2 (Fig. 6A). Treatment with mock-transfected COS cell-conditioned media failed to cause an increase in phosphorylation above the level seen with cells treated without COS cell-conditioned media (compare lanes 1 and 3). Shown for comparison are results from Sea-expressing 32D cells treated with interleukin 3, a cytokine known to cause phosphorylation of similar signal transduction molecules and required for the survival and growth of 32D cells. Attempts to grow Sea-expressing 32D cells with chicken C665A MSP-conditioned media in the absence of interleukin 3 were not successful (data not shown), however, purified, active recombinant hMSP was also unable to abrogate the interleukin 3 dependence of 32D cells expressing the RON receptor (data not shown).

DISCUSSION

The biochemical purification that resulted in the isolation of chMSP was designed for the generic purification of plasminogen-related growth factors, as the known ligands in this family are detected in normal sera (22, 23), and bind strongly to heparin-Sepharose (31, 32). Likewise, the MSP-activating en-
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**Recombinant chMSP causes Sea receptor phosphorylation.** Wild type and C665A chMSP were produced as recombinant proteins in the conditioned media of COS cells and tested for their ability to cause Sea receptor phosphorylation. Conditioned media from mock-transfected (mock), wild type (wt) chMSP (Wt chMSP), or C665A chMSP (C665A chMSP) were tested as is (1×), concentrated 5-fold (5×), or 20-fold (20×). The conditioned media were either untreated (lanes 1–9) or kallikrein-treated (lanes 10–18) and incubated with 32D cells expressing the Sea receptor and analyzed as in Fig. 2, Panel A, transferred proteins were detected with an anti-Tyr(P) antibody. Lanes 1 and 10, mock 1×; lanes 2 and 11, mock 5×; lanes 3 and 12, mock 20×; lanes 4 and 13, wild type chMSP 1×; lanes 5 and 14, wild type chMSP 5×; lanes 6 and 15, wild type chMSP 20×; lanes 7 and 16, C665A chMSP 1×; lanes 8 and 17, C665A chMSP 5×; lanes 9 and 18, C665A chMSP 20×. Panel B, transferred proteins (same order as in panel A) were detected with an anti-Sea antibody. Molecular weight markers (Amersham Pharmacia Biotech) are shown on the right.

**Fig. 3.** Recombinant chMSP causes Sea receptor phosphorylation. Wild type and C665A chMSP were produced as recombinant proteins in the conditioned media of COS cells and tested for their ability to cause Sea receptor phosphorylation. Conditioned media from mock-transfected (mock), wild type (wt) chMSP (Wt chMSP), or C665A chMSP (C665A chMSP) were tested as is (1×), concentrated 5-fold (5×), or 20-fold (20×). The conditioned media were either untreated (lanes 1–9) or kallikrein-treated (lanes 10–18) and incubated with 32D cells expressing the Sea receptor and analyzed as in Fig. 2, Panel A, transferred proteins were detected with an anti-Tyr(P) antibody. Lanes 1 and 10, mock 1×; lanes 2 and 11, mock 5×; lanes 3 and 12, mock 20×; lanes 4 and 13, wild type chMSP 1×; lanes 5 and 14, wild type chMSP 5×; lanes 6 and 15, wild type chMSP 20×; lanes 7 and 16, C665A chMSP 1×; lanes 8 and 17, C665A chMSP 5×; lanes 9 and 18, C665A chMSP 20×. Panel B, transferred proteins (same order as in panel A) were detected with an anti-Sea antibody. Molecular weight markers (Amersham Pharmacia Biotech) are shown on the right.

zyme, kallikrein, and HGF activator, are also detected in normal sera and bind strongly to heparin-Sepharose (33, 34). We noted that our post-heparin purified pool was active in hydrolyzing a kallikrein substrate. Thus, since MSP was shown to interact with Sea-Fc by our Western blot procedure, it is not surprising that activated chMSP was purified from chicken serum by our two-step purification. It should be reiterated that this Western blot technique did not work when the disulfide bonds of MSP are reduced prior to SDS-PAGE.

Our results clearly demonstrate that chMSP is capable of acting as a ligand for the Sea receptor. Chicken MSP bound to a Sea-Fc protein, and caused both the autophosphorylation of Sea as well as the phosphorylation of downstream target proteins including Shc, Erk 1, and Erk 2. These activities were seen with chMSP derived from two different sources, either as a natural purified protein or as a recombinantly produced protein. It has been reported that a Cys to Ala variant of mMSP is the most active recombinant form, but the recombinant MSP with the complete wild type sequence showed activity at high concentrations (1). Similar results are shown here for the chMSP. The wild type sequence showed activity at high concentrations, while the C665A variant showed much greater activity. Preincubation of the C665A variant with the Sea-Fc soluble protein blocked the chMSP from inducing Sea receptor phosphorylation thus confirming receptor-specific binding.

Although mMSP was detected readily by Sea-Fc in a Western blot, it was unable to induce receptor phosphorylation, even using concentrations as high as 5 μg/ml of purified recombinant material (data not shown). This reinforces the concept that receptor binding is necessary, but not sufficient for causing receptor dimerization necessary for autophosphorylation. There is only 60% identity between chicken and mouse MSP. The species selectivity was also manifest in the better reaction of mMSP with the anti-murine antisera, and the better reactivity of Sea-Fc with chMSP.

As shown previously for hMSP and mMSP (1), a conserved Cys residue has a deleterious effect on recombinant expression of activatable chMSP. Without activation, both the wild type and C665A variant are readily detected as ~84-kDa proteins which bind anti-MSP antisera (Fig. 4). However, after activation, the wild type recombinant protein is no longer detected as an ~84-kDa protein. Long exposures detected the presence of a much smaller ~50-kDa protein which could bind the anti-MSP antisera. We interpret these results to indicate that the wild type recombinant chMSP contained misfolded disulfide bonds which result in a loss of the intersubunit disulfide, and an increased lability to kallikrein. It has been reported that the RON-binding domain of MSP is located in the β chain (36). However, we never observed Sea-Fc binding via the Western blot technique to any species other than the 84-kDa species. With regard to the partially purified naturally occurring chMSP, if any disulfide bond variants are present in vivo, the Sea-Fc affinity chromatography employed here presumably selected for MSP in which the intersubunit disulfide was present. Therefore, this material behaved more like the C665A variant. By Western blot analysis, the Sea-Fc is quite efficient at discriminating between different forms of the chMSP. This is particularly evident in the results shown in Figs. 3 and 4, where conditioned media containing recombinant wild type chMSP failed to bind the Sea-Fc even though it could weakly...
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cause Sea phosphorylation. We interpret this to mean that a small percentage of the recombinant wild type chMSP was folded correctly, but was present in too low a concentration to cause the production of only properly folded MSP.

Although the current data clearly show that MSP is a ligand for the Sea receptor, it remains unclear if Sea and RON/Stk are corresponding orthologs. Protein comparisons indicate that the extracellular domain of the Sea receptor is more similar to RON (48% identity) than to Met (39% identity). Because a mammalian analog of Sea has not yet been identified, and since a chicken Met gene but not a chicken Ron gene has been isolated due to its effect on hematopoiesis. The Sea receptor was identified as the cellular homologue of the avian retroviral oncprotein v-Sea. This avian erythroblastosis virus (S13) induced sarcomas, erythroblastosis, and anemia in infected birds (11, 12). Transformation of erythroid cells with the sea onco-
gene rendered them erythropoietin independent (13, 14). Likewise, MSP, through the RON/Stk receptor, has been shown to have a variety of biological activities, particularly on cells of the hematopoietic lineage. It induced macrophage shape change and chemotactic migration (25), apoptosis of an erythroid cell line (26), and megakaryocytopenesis (27). The demonstration of the involvement of MSP, Ron/Stk, and Sea in hematopoiesis strongly implies that chicken MSP will likewise play a role in chicken hematopoiesis. To date, there is limited information on the tissue distribution of Sea and chicken MSP. Sea protein has been detected at low levels in several tissues including the kidney, intestine, liver, stomach, white blood cells, and allanto-chorion (15). Chicken MSP mRNA has been detected at all stages of chicken development, with expression at particular stages found in the neural tube, notochord, floor plate, myocyte, and aortic arches (9). Our purification studies indicate that adult chicken sera is a rich source of MSP. Further work will be required to compare Sea and MSP expression in development and adult chicken tissues to try to gain clues as to where they may interact. Since MSP is a circulating ligand, it could interact with receptor(s) in a number of tissues, and thus there may not be a one-to-one correspondence in the expression of the Sea receptor and the chMSP. The production and purification of significant quantities of active recombinant chMSP will allow for a determination of the full range of its in vivo activities.

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REFERENCES
1. Wahl, R. C., Costigan, V. J., Batac, J. P., Chen, K., Cam, L., Courchesne, P. L., Patterson, S. D., Zhang, K., and Pacifici, R. E. (1997) J. Biol. Chem. 272, 15055–15056
2. van der Geer, P., Hunter, T., and Lindberg, R. A. (1994) Annu. Rev. Cell Biol. 10, 251–337
3. Park, M., Dean, M., Kaul, K., Braun, M. J., Gonda, M. J., and Vande-Woude, G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6379–6383
4. Chan, A. M., King, H. W., Deakin, E. A., Tempst, P. R., Hilkens, J., Kroezen, V., Edwards, D. R., Wills, A. J., Brookes, P., and Cooper, C. S. (1988) Oncogene 2, 593–599
5. Roncin, C., Muscatelli, F., Mattei, M. G., and Breathnach, R. (1983) Oncogene 2, 1195–1202
6. Gaudino, G., Follenzi, A., Naldini, L., Collesi, C., Santero, M., Gallo, K. A., Godowski, P. J., and Comoglio, P. M. (1994) EMBO J. 13, 3524–3532
7. Iwama, T., Okano, K., Toda, Y., Matsudo, Y., and Suda, T. (1994) Blood 83, 3160–3169
8. Huff, J. L., Jelinek, M. A., Borgman, C. A., Lansing, T. J., and Parsons, J. T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6140–6144
9. Theur, C., Sharpe, M. J., Batley, S. J., Stern, C. D., and Gherardi, E. (1995) Dev. Genet. 17, 990–101
10. Ohashi, K., Mizuno, K., Kuma, K., Miyata, T., and Nakamura, T. (1994) Oncogene 9, 699–705
11. Stubb, E. L., and Firth, J. (1935) J. Exp. Med. 61, 593–616
12. Beug, H., Hayman, M. J., Graf, T., Benedict, S. H., Wallbank, A. M., and Vogt, P. K. (1985) Virology 145, 141–153
13. Knight, J., Zenke, M., Disela, C., Kowenz, E., Vogt, P., Engel, J. D., Hayman, M. J., and Beug, H. (1988) Genes Dev. 2, 247–258
14. Kain, P., Frykberg, L., Brady, C., Stanley, I., Beug, H., Vennstrom, B., and Graf, T. (1986) Cell 45, 349–356
15. Huff, J. L., Jelinek, M. A., Jamieson, T. A., and Parsons, J. T. (1996) Oncogene 12, 299–307
16. Medico, E., Mongiorgi, A. M., Huff, J., Jelinek, M. A., Follenzi, A., Guadino, G., Parsons, J. T., and Comoglio, P. M. (1996) Mol. Biol. Cell 7, 495–504
17. Bottaro, D. P., Rubin, J. S., Faletto, D. L., Chan, A. M. L., Kmiecik, T. E., Vande Woude, G. F., and Aaronson, S. A. (1991) Science 251, 802–804
18. Naldini, L., Vigna, E., Narisunman, R. P., Guadino, G., Zarrnegar, R., Michalopoulos, G. K., and Comoglio, P. M. (1991) Oncogene 4, 501–504
19. Gaudino, G., Follenzi, A., Naldini, L., Collesi, C., Santero, M., Gallo, K. A., Godowski, P. J., and Comoglio, P. M. (1984) EMBO J. 13, 3524–3532
20. Wang, M.-H., Ronsin, C., Gesnel, M. C., Coupey, L., Skeel, A., Leonard, E. J., and Breathnach, R. (1994) Science 266, 117–119
21. Zarnegar, R., and Michalopoulos, G. (1989) Cancer Res. 49, 3314–3320
22. Wang, M.-H., Skeel, A., Yoshimura, T., Copeland, T. D., Sakaguchi, K., and Leonard, E. J. (1993) J. Leukocyte Biol. 54, 289–296
23. Zarrnegar, R., and Michalopoulos, G. K. (1993) J. Cell Biol. 5, 1177–1180
24. Leonard, E. J., and Skeel, A. (1976) Exp. Cell Res. 102, 434
25. Iwama, A., Yamaguchi, N., and Suda, T. (1996) EMBO J. 15, 5866–5875
26. Bana, P., Price, D. J., London, R., Deng, B., Mark, M., Godowski, P. J., and Avraham, H. (1996) J. Immunol. 156, 2903–2904
27. Gaudino, G., Avantaggiato, V., Follenzi, A., Acampora, D., Simeone, A., and Comoglio, P. M. (1995) Oncogene 11, 2627–2637
28. DeClerck, Y. A., Yeat, T. D., Lu, H. S., Jing, X., and Langley, K. E. (1991) J. Biol. Chem. 266, 3893–3899
29. Welcher, A. A., Bitter, C. M., Radeke, M. J., and Shooter, E. M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 159–163
30. Shimamoto, A., Kimura, T., Kato, M., and Nakamura, T. (1993) FEBS Lett. 333, 61–66
31. Ohida, E., Teboubuchi, H., Nakayama, H., Hirose, S., Sakiyama, O., Takahashi, K., Miyazaki, H., Hashimoto, S., and Daikuhara, Y. (1988) J. Clin. Invest. 81, 414–419
32. Heimark, R. L., and Davie, E. W. (1988) Methods Enzymol. 80, 157–172
33. Shimomura, T., Kondo, J., Ochisi, M., Naka, D., Miyazawa, K., Morimoto, Y., and Kitamura, N. (1993) J. Biol. Chem. 268, 22927–22932
34. Silverberg, M., and Kaplan, A. P. (1988) Methods Enzymol. 163, 85–95
35. Wang, M.-H., Julian, F. M., Breathnach, R., Godowski, P. J., Takehara, T., Yoshikawa, W., Hagiya, M., and Leonard, E. J. (1997) J. Biol. Chem. 272, 16999–17004