Identification of a Novel Immunoreceptor Tyrosine-based Activation Motif-containing Molecule, STAM2, by Mass Spectrometry and Its Involvement in Growth Factor and Cytokine Receptor Signaling Pathways*

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In an effort to clone novel tyrosine-phosphorylated substrates of the epidermal growth factor receptor, we have initiated an approach coupling affinity purification using anti-phosphotyrosine antibodies to mass spectrometry-based identification. Here, we report the identification of a signaling molecule containing a Src homology 3 domain as well as an immunoreceptor tyrosine-based activation motif (ITAM). This molecule is 55% identical to a previously isolated molecule designated signal transducing adaptor molecule (STAM) that was identified as an interleukin (IL)-2-induced phosphoprotein and is therefore designated STAM2. Tyrosine phosphorylation of STAM2 is induced by growth factors such as epidermal growth factor and platelet-derived growth factor as well as by cytokines like IL-3. Several of the deletion mutants tested except the one containing only the amino-terminal region underwent tyrosine phosphorylation upon growth factor stimulation, implying that STAM2 is phosphorylated on several tyrosine residues. STAM2 is downstream of the Jak family of kinases since coexpression of STAM2 with Jak1 or Jak2 but not an unrelated Tec family kinase, Etk, resulted in its tyrosine phosphorylation. In contrast to epidermal growth factor receptor-induced phosphorylation, this required the ITAM domain since mutants lacking this region did not undergo tyrosine phosphorylation. Finally, overexpression of wild type STAM2 led to an increase in IL-2-mediated induction of c-Myc promoter activation indicating that it potentiates cytokine receptor signaling.

A large number of cell surface receptors transmit their signals by pathways involving tyrosine phosphorylation. The receptor may themselves possess tyrosine kinase activity such as receptor protein-tyrosine kinases (e.g. epidermal growth factor receptor (EGFR)) or may not have any catalytic activity (cytokine receptors such as the erythropoietin receptor) (1–6). Signaling by cytokine receptors is mediated by cytoplasmic kinases such as the Jak or Src family of kinases (4, 7). In both of these receptor systems, a cascade of events involving tyrosine phosphorylation is initiated eventually leading to the recruitment of multiprotein complexes and activation of downstream target proteins or genes. Most of the substrates that are recruited are not specific to the receptor protein-tyrosine kinases or the cytokine receptor signaling pathways. Adapter proteins such as Shc and p85 subunit of PI 3-kinase are utilized by almost all of these signaling pathways (8, 9).

Mass spectrometry-based proteomics has recently emerged as a powerful tool to analyze protein-protein interactions in general as well as in the study of receptor-mediated signaling pathways (10). We have initiated a systematic study to identify all substrates of the EGFR that are tyrosine-phosphorylated in HeLa cells. Using a combination of affinity purification using anti-phosphotyrosine antibodies and one-dimensional electrophoresis followed by mass spectrometry-based identification, we have previously reported Vav-2 as a novel substrate of the EGFR (11). In this report we have identified another molecule that undergoes tyrosine phosphorylation in response to EGF. This molecule contains an SH3 domain as well as an immunoreceptor tyrosine-based motif (ITAM) domain and is approximately 55% identical to STAM, a molecule originally identified downstream of the IL-2 receptor complex (12). Based on its relatedness to STAM, it is appropriate to designate this molecule as STAM2.

STAM2 is phosphorylated on tyrosine residues upon addition of growth factors including EGF and PDGF. Since STAM was shown to undergo tyrosine phosphorylation in response to cytokines, we tested if STAM2 was downstream of cytokine receptors as well (13). Indeed, we found that STAM2 was induc-

* This work was supported in part (at the Protein Interaction Laboratory) by a grant from the Danish National Research Foundation to the Center for Experimental BioInformatics, University of Southern Denmark. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; ITAM, immunoreceptor tyrosine-based activation motif; MS/MS, tandem mass spectrometry; PI 3-kinase, phosphatidylinositol 3-kinase; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; SH3, Src homology 3; STAM, signal transducing adaptor molecule; IL, interleukin; WT, wild type; mAb, monoclonal antibody; PBS, phosphate-buffered saline.

This paper is available on line at http://www.jbc.org
ibly tyrosine-phosphorylated by IL-3 in a growth factor-dependent cell line, BaF3. To determine the regions critical for the tyrosine phosphorylation of STAM2, a deletion analysis was carried out. We found that mutants lacking the ITAM domain or the amino terminus and SH3 domain were still capable of being phosphorylated on tyrosine residues, suggesting that these regions were not required for its tyrosine phosphorylation by EGF. When STAM2 was cotransfected with Jak1 or Jak2, it underwent tyrosine phosphorylation but not by Etk that belongs to the Tec family of kinases (14–16). This suggests that STAM2 is downstream of the Jak family of kinases and may mediate some of the effects of Jak kinases. Mutational analysis also showed that presence of the ITAM domain was critical for its tyrosine phosphorylation by Jak1 or Jak2 since a mutant lacking the COOH terminus but not one lacking the ITAM domain underwent tyrosine phosphorylation. These results indicate that different upstream pathways may mediate the tyrosine phosphorylation of STAM2 by EGFR as compared with that of the Jak family of kinases. Finally, when STAM 2 was overexpressed in a reconstitution system in HepG2 cells, it led to increased induction of c-Myc promoter activation by IL-2. Analysis with several deletion mutants of STAM2 showed that several regions were required for this effect. A mutant containing the ITAM domain but lacking the carboxyl terminus (DC) did not enhance IL-2-mediated c-Myc induction. This mutants still undergoes efficient tyrosine phosphorylation, suggesting that residues in the carboxyl terminus are critical for c-Myc induction. A similar result was obtained when several other mutants were tested, indicating that several regions may cooperate for its downstream signaling effects.

EXPERIMENTAL PROCEDURES

Cell Culture, Growth Factors, and Antibodies—HeLa and 293T cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum plus antibiotics. BaF3 and 32D cells were grown in RPMI containing 10% conditioned supernatant from WEHI cell line, 10% fetal bovine serum, and antibiotics. NIH 3T3 fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and antibiotics. HCD 57 cells were grown in RPMI containing 10% fetal bovine serum supplemented with erythropoietin and antibiotics. HepG2 cells were grown normally and then serum-deprived for approximately 24 h before treating half of the cells with EGF for 5 min. The cell lysates were immunoprecipitated (IP) with anti-phosphotyrosine antibodies (anti-pTyr) and the immune complexes washed and resolved by SDS-polyacrylamide gel electrophoresis. The gel was then silver-stained and is shown in the figure. Molecular mass markers are indicated on the left. The arrow indicates the position of the band that was analyzed in this study. B, MS spectrum from nanoelectrospray MS/MS analysis of the peptides derived from the band shown in panel A. The peptides corresponding to m/z values of 596.35, 701.35, and 810.9 were subsequently fragmented. T refers to trypsin autolysis products. C, mass spectrometric identification of STAM by nanoelectrospray tandem mass spectrometry. The figure shows the fragmentation pattern of the NH2-terminal peptide derived from STAM at m/z value of 810.9 (indicated as [M+2H]+), one of the tryptic peptides shown in panel B. The Y0 series of fragment ions (COOH terminus-containing fragments) as well as those from a and b fragment ion series (NH2 terminus-containing fragments) are labeled according to convention (30, 31). The sequence of the peptide derived from this spectrum is shown at the top. D, identification of a STAM2, a molecule related to STAM. The figure shows the fragmentation pattern of one of the tryptic peptides shown in panel B (m/z = 596.35) (indicated as [M+2H]+). The Y0 series of fragment ions as well as those from a and b fragment ion series are labeled. The sequence of the peptide derived from this spectrum is shown at the top.
cells were grown in Eagle's minimal essential medium supplemented with 2 mM l-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. Anti-phosphotyrosine mAb (4B10), anti-Shc, anti-Jak1, anti-Jak2, and anti-Grb2 antibodies were from Transduction Laboratories (Lexington, KY). Anti-phosphotyrosine mouse mAb (4G10), anti-EGFR mAb, EGF, and PDGF were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-Myc monoclonal antibody (9E10) was from Covance (Denver, PA). Interleukin 3 was from Sigma, and recombinant erythropoietin was a gift from AMGEN.

**cDNAs and Constructs**—The cDNA clone (DKFZp564C047) corresponding to STAM2 was obtained from the German Cancer Research Center (DKFZ) (17). Wild type STAM2 and the various deletion mutants were obtained by subcloning the polymerase chain reaction fragments into the vector pEF-1/myc-His (Invitrogen, Carlsbad, CA). Wild type EGFR was obtained by subcloning the EGFR cDNA (kindly provided by Dr. Stuart Decky) into pcDNA3 vector. Wild type PDGFRβ cDNA (18) was kindly provided by Dr. Andrius Kazlauskas and was subcloned into pcDNA3.

**Transfection Experiments**—293T cells were cotransfected with 7.5 μg of wild type STAM2 expression vector along with 7.5 μg of the plasmids encoding EGFR, PDGFR, Jak1, Jak2, or Etk using the calcium phosphate method. 48 h after transfection, the cells were serum-starved for 24 h. Cells were then lysed in lysis buffer and processed as described below. For electroporation experiments, BaF3 cells were washed two times in PBS, resuspended in PBS, and mixed with 40 μg of STAM2 plasmid. The cells were then electroporated using Gene Pulser II (Bio-Rad). The settings used were 250 V and 950 microfarads. After electroporation, the cells were resuspended in complete medium and allowed to grow for 24 h. Subsequently, the cells were washed and resuspended in serum-free medium overnight.

**Immunoprecipitation and Western Blotting**—For experiments shown in Fig. 1, approximately 106 serum-deprived HeLa cells were left untreated or treated with 1 μg/ml EGF for 5 min and processed essentially as described previously (11). Cells were lysed in 25 ml of lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, and 1 mM sodium orthovanadate in the presence of protease inhibitors. Cell lysates were cleared by centrifugation and then incubated with 100 μg of 4G10 mAb coupled to agarose beads and 50 μg of biotin-conjugated RC20 mAb bound to streptavidin-agarose beads for 4 h at 4 °C. Precipitated immune complexes were then washed and the bound proteins eluted by 100 μl phenylphosphate in lysis buffer. A protocol describing cell biological and mass spectrometric aspects in greater detail is available (19). The gels were silver-stained by a method compatible with mass spectrometric analysis as described previously (20).

In 293T and BaF3 cell transfection experiments, the cells were starved as described above and then were treated with EGF (1 μg/ml) or PDGF (50 ng/ml) or IL-3 (100 ng/ml) for 5 min and lysed in lysis buffer. Cleared cell lysates were immunoprecipitated with 10 μg of antibody against the Myc epitope, for 2 h at 4 °C. Following incubation, the beads were washed three times in lysis buffer, boiled in sample buffer, resolved by SDS-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose. The membrane was blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS) containing 0.1% Tween 20 overnight at 4 °C and then incubated with 1 μg/ml 4G10 anti-phosphotyrosine or corresponding Western blotting antibody for 1 h. The membranes were incubated with secondary antibodies followed by chemiluminescent detection according to manufacturer's instructions (ECL, Amersham Pharmacia Biotech).

**Luciferase Assays**—HepG2 cells were grown in complete medium to 60% confluence and transfected by the calcium phosphate method with expression vectors in the following amounts: 1 μg of pHXLuc, 1 μg of IL-2Rβ, 1 μg of γc, 1 μg of STAT5a, 0.1 μg of β-galactosidase, and different amounts of STAM2pEF1 or pEF1 vector alone as indicated. After 24 h cells were split into two, and 12 h later the medium was replaced with serum-free medium and IL-2 added to 100 units/ml. Cells were harvested after an additional 24 h incubation, and relative luciferase and β-galactosidase activities were measured on a MicroLumat luminometer (E&G Berthold, Bad Wildbad, Germany).

**Mass Spectrometry**—Identification of proteins by mass spectrometry was done essentially according to a strategy described previously (21). The band indicated in Fig. 1 was excised from one-dimensional silver-stained polyacrylamide gel and processed as described (21). After re-
RESULTS AND DISCUSSION

Identification of a Novel Signaling Molecule, STAM2, in the EGFR Signaling Pathway by Mass Spectrometry—We performed anti-phosphotyrosine affinity purification of HeLa cell lysates that were stimulated with EGF. In parallel, unstimulated cell lysates were subjected to the same protocol in order to identify proteins that were specifically observed in EGF-stimulated immunoprecipitates. Fig. 1A shows a silver stained gel after these immunoprecipitates were resolved by one-dimensional electrophoresis. The band indicated by the arrow was excised and subjected to in-gel trypsin digestion as described earlier (21). The peptides were then extracted from the gel and subjected to nanoelectrospray tandem mass spectrometry.

Several peptides were observed in the mass spectrum of this band as shown in Fig. 1B. Sequencing of one of these peptides (m/z = 810.9) by fragmentation showed that it was the NH2-terminal peptide derived from STAM, an SH3 and an ITAM domain-containing protein, lacking the initiation methionine as described earlier (Fig. 1C) (12). STAM was originally identified as a 70-kDa phosphoprotein that was tyrosine-phosphorylated by IL-2 in a human T cell line (12). It was shown to be associated with Jak2 and Jak3 kinases via its ITAM domain and was phosphorylated by these kinases (13). Another peptide derived from STAM was also sequenced, confirming the identification (data not shown). Sequencing of the peptide corresponding to m/z value of 596.35 yielded the sequence VLE-ALELYNK as shown in Fig. 1D. Upon searching the data bases with this sequence, it was found to correspond to a GenBank entry (accession no. CAB63735) submitted by the German Human Genome Project that was labeled as a hypothetical protein (17). Two other peptides including a tyrosine-phosphorylated peptide that corresponded to this protein were also identified from this band by tandem mass spectrometry (data not shown).

Comparison of the open reading frame of this clone to the known proteins in the data base confirmed that it was a novel molecule that was 55% identical to STAM. Because of this sequence relatedness, it is appropriate to refer to this molecule as STAM2. The open reading frame of STAM2 encodes a protein of 525 amino acids with a predicted molecular mass of approximately 58 kDa. It contains a single SH3 domain as well as an ITAM domain that is found in several signaling proteins and immune receptors, respectively (Fig. 2A) (26, 27). It contains 16 tyrosine residues, 12 of which are conserved between STAM and STAM2. Most of these tyrosine residues are contained within the COOH terminus or the ITAM domains. A pair of conserved tyrosines is present in the SH3 domain and the region immediately amino-terminal to it. An alignment of ITAM domains of STAM and STAM2 shows that they contain the classic YXXL spaced by nine non-conserved residues (Fig. 2B) (28). The ITAM domains of these two proteins are about 72% identical to each other, suggesting that they may possess similar functions in these two proteins. A Northern blot analysis showed that STAM2 was expressed in almost all tissues examined (data not shown).

Induction of Tyrosine Phosphorylation of STAM2 by Growth Factors and Cytokines—In order to test whether STAM2 is tyrosine-phosphorylated by growth factors, we set up a reconstitution assay in 293T cells. 293T cells were cotransfected with wild type STAM2 expression vector along with the plasmids as shown. 48 h after transfection, the cells were serum-starved for 24 h. Cell lysates were then immunoprecipitated (IP) with anti-Myc antibody followed by Western blotting with anti-phosphotyrosine antibody (anti-pTyr) to determine the phosphorylation status of STAM2. The bottom panel shows reprobing of the blot with anti-Myc antibody.

Phosphorylation of STAM2 by Jak but Not Tyk2 Family Kinases—Since STAM2 is phosphorylated by cytokine receptors that do not contain any catalytic activity, we wished to ascertain whether the Jak family kinases were capable of phosphorylating STAM2. We therefore cotransfected

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293T cells with expression vectors for STAM2 and Jak1, Jak2, or Etk. As shown in Fig. 4, STAM2 underwent tyrosine phosphorylation when Jak1 or Jak2 were cotransfected. Etk is a tyrosine kinase that belongs to the Tec family and is unable to phosphorylate STAM2 (16).

**Requirement of ITAM Domain for Tyrosine Phosphorylation**

**Fig. 5. Requirement of different domains of STAM2 for its tyrosine phosphorylation.**

A, schematic of the deletion mutants of STAM2. The tyrosine residues are indicated as Y, and the SH3 and ITAM domains are shown as black boxes. All the constructs were tagged with a Myc epitope at the COOH terminus. B, tyrosine phosphorylation of STAM2 deletion mutants by EGFR. 293T cells were cotransfected with vector control (Mock) or EGFR and the various deletion mutants of STAM2 as shown. 48 h after transfection, the cells were serum-starved for 24 h and treated with EGF. Cell lysates were immunoprecipitated (IP) with anti-Myc antibody followed by Western blotting (WB) with anti-phosphotyrosine antibody (anti-pTyr) as shown. The bottom panel shows reprobing of the blot with anti-Myc antibody. C, requirement of ITAM domain of STAM2 for tyrosine phosphorylation by Jak1. 293T cells were cotransfected with Jak1 expression vector along with the indicated deletion mutants of STAM2. 48 h after transfection, the cells were serum-starved for 24 h. Cell lysates were then immunoprecipitated with anti-Myc antibody, followed by Western blotting with anti-phosphotyrosine antibody to determine the phosphorylation status of STAM2. The bottom panel shows reprobing of the blot with anti-Myc antibody.
Cloning of a Novel ITAM-containing Molecule, STAM2

by Jak Kinases but Not by EGFR—Since STAM2 is downstream of both EGFR and Jak family kinases, we wished to determine which regions were essential for it to act as a substrate of these kinases. To this end, we constructed a series of mutants lacking various regions as shown by the schematic in Fig. 5A. As shown in Fig. 5B, the deletion mutants underwent efficient tyrosine phosphorylation in response to EGF, suggesting that it is tyrosine-phosphorylated on multiple tyrosine residues in the SH3 and ITAM domains as well as in its carboxyl terminus. However, when a mutant expressing a deleted ITAM domain was coexpressed with Jak1, no tyrosine phosphorylation was detected (Fig. 5C). This suggests that the ITAM domain is required for its tyrosine phosphorylation by Jak1. Identical results were obtained when these mutants were cotransfected with Jak2 (data not shown). Finally, if a construct containing only the amino-terminal region (N only) was coexpressed with EGF, no tyrosine phosphorylation was detectable (data not shown), suggesting that the SH3 domain and the region between the SH3 and ITAM domains may be important for this phosphorylation event.

Involvement of STAM2 in c-Myc Promoter Activation—Cytokines such as IL-2 up-regulate immediate early response genes such as those for c-Myc and c-Fos. We therefore decided to test the role of STAM2 in IL-2-mediated induction of c-Myc in a reconstitution system in HepG2 cells. As shown in Fig. 6A, overexpression of WT STAM2 led to an increase in IL-2-mediated c-Myc induction. This effect was dependent upon the amount of STAM2 DNA used in the transfection as shown in the figure. STAM has previously been shown to have a similar effect (13). This implies that STAM2 plays a similar positive role in cytokine signaling as STAM. This effect of STAM was demonstrated to require the ITAM domain since mutants lacking the ITAM domain were unable to cooperate in IL-2-induced c-Myc induction (13). We tested all of the mutants that we have generated in the reconstitution assay. Fig. 6B shows that most of the mutants lost the ability to up-regulate IL-2-induced c-Myc promoter activation. Most surprisingly, the mutant lacking the carboxyl terminus (labeled ΔC) but containing the ITAM domain could had no positive effect on c-Myc induction. Similarly, a mutant lacking the amino terminus plus the SH3 domain (labeled Δ(N+SH3)) also lost the ability to up-regulate c-Myc induction. It is important to note that both of these mutants undergo efficient tyrosine phosphorylation by Jak kinases, suggesting that tyrosine phosphorylation is not sufficient for its effect on c-Myc induction.

Conclusion—We have identified a novel molecule that is involved in signaling events downstream of receptor protein tyrosine kinases as well as cytokine receptors. STAM like molecules have been found in Drosophila (29) as well Caenorhabditis elegans (data not shown), suggesting an evolutionarily conserved function. Cloning of STAM2 expands this family of cytoplasmic molecules containing ITAM domains that are otherwise only found in immune receptors. Since STAM2 does not contain any phosphotyrosine binding motifs such as Src homology 2 or phosphotyrosine binding domains, it is likely that it does not interact directly with the receptor tyrosine kinases. The ITAM region is dispensable for the tyrosine phosphorylation of STAM2 by EGF but not by Jak family kinases, suggesting that there is at least one distinct kinase in the EGFR signaling pathways capable of phosphorylating STAM2. Overexpression of WT STAM2 led to an increase in IL-2-mediated c-Myc induction, suggesting that it plays a positive role in cytokine signaling in a manner analogous to STAM. It was found that several regions of STAM2 were required for this effect as evidenced by a deletion analysis. Specifically, deletion of the carboxyl terminus or the unique amino terminus plus the SH3 domain resulted in STAM2 being unable to up-regulate c-Myc induction. One possibility is that multiple protein-protein interactions are required for this effect and the loss of some of them causes STAM2 to become inactive in this assay. Cloning of STAM2 will permit a detailed analysis of signal transduction pathways downstream of cytokines as well as growth factor receptors.

Acknowledgments—We thank Drs. Stuart Decker and Andrius Kazlauskas for providing cDNA constructs and Dr. Kazuo Sugamura for providing pHXLuc plasmid.

FIG. 6. Involvement of STAM2 in c-Myc promoter activation induced by IL-2. A, overexpression of WT STAM2 enhances c-Myc promoter activation. HepG2 cells were transfected with expression vectors for c-Myc promoter cloned upstream of luciferase (pHXLuc), IL-2Rβ, γc, STAT5a, 0.1 μg of β-galactosidase vector, and the indicated amounts of STAM2/pEF1 or pEF1 vector. After 24 h, cells were split into two, and 12 h later the medium was replaced with serum-free medium and IL-2 added to 100 units/ml. Cells were harvested after an additional 24-h incubation, and relative luciferase and β-galactosidase activities were measured. A dose response showing relative luciferase activities after normalizing for β-galactosidase with increasing amounts of WT STAM2 is shown. The experiment shown is representative of four experiments that were performed. B, requirement of various domains of STAM2 for c-Myc promoter activation. HepG2 cells were transfected with expression vectors as in panel A except that 8 μg of the indicated wild type or mutant constructs of STAM2 were used. Relative luciferase activities after normalizing for β-galactosidase are shown in the figure. The experiment is representative of six experiments that were performed.
Note Added in Proof—While this manuscript was under review, another report describing human STAM2 was published (32).

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J. Biol. Chem. 2000, 275:38633-38639.
doi: 10.1074/jbc.M007849200 originally published online September 18, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M007849200

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