Serine Phosphorylation, Chromosomal Localization, and Transforming Growth Factor-β Signal Transduction by Human bsp-1

(Received for publication, April 22, 1996, and in revised form, May 23, 1996)

Robert J. Lechleider‡§, Mark P. de Caestecker‡, Anindya Deheja†, Mihael H. Polymeropoulos‡‡, and Anita B. Robert‡

From the Laboratory of Chemoprevention, NCI, National Institutes of Health, Bethesda, Maryland 20892 and the Gene Mapping Unit, Laboratory of Genetic Disease Research, National Center for Human Genome Research, Bethesda, Maryland 20892

The transforming growth factor-β (TGF-β) superfamily regulates a multitude of cellular and developmental events. TGF-β family ligands signal through transmembrane serine/threonine kinase receptors whose downstream effectors are largely unknown. Using genetic data from the fruit fly, we have identified a downstream effector of TGF-β-induced signaling, TGF-β signaling protein-1 (BSP-1) is rapidly phosphorylated in response to TGF-β. Localization of bsp-1 to chromosome 4q28 suggests a role in carcinogenesis. These data suggest that BSP-1 is the prototype of a new class of signaling molecules.

Transforming growth factor-β regulates development, growth, and differentiation. The TGF-β superfamily consists of highly conserved ligands with similar yet distinct biological functions (1–3). Unlike mitogenic ligands such as epidermal growth factor, which signals through transmembrane tyrosine kinase receptors, TGF-β signals through two interacting transmembrane serine/threonine kinase receptors. TGF-β binds to the type II receptor, which then recruits and phosphorylates the type I receptor (8, 9). The mechanism by which the activated receptors then transduce their signal to the nucleus remains obscure. Two proteins, FK506 binding protein-12 (10), a cis-proximal isomerase important in the immunosuppressive response generated by the immunosuppressant drug FK506, and the farnesyl transferase α subunit (11, 12), which is essential for the farnesylation of Ras and other small G-proteins, have been identified as directly interacting with the type I TGF-β receptor cytoplasmic domain. Another protein, TGF-β receptor interacting protein-1 (13), which contains multiple WD repeats, interacts with and is phosphorylated by the type II receptor. However, the involvement of these proteins in mediating downstream events is still unclear. A member of the mitogen-activated protein kinase kinase family (14) identified in a yeast genetic screen has been shown to activate TGF-β responses in mammalian cells, but its role is not known if this represents a final common pathway or a TGF-β-specific signaling event. Several groups have suggested that members of the Ras pathway (15, 16) or Src family kinases (17) are involved, but these results appear to be cell type-specific and not generalizable.

Since biochemical approaches to studying TGF-β-induced signal transduction have yielded little information about the pathways involved, we used dyes from Drosophila genetics to identify TGF-β pathway intermediates. In Drosophila three TGF-β superfamily ligands have been identified, of which decapentaplegic (dpp) is the best studied (18–20). A genetic screen for mutants acting in the dpp pathway yielded two genes, one of which, Mothers against dpp (mad), has null alleles strikingly similar to dpp alleles (21, 22). The cloning of mad revealed a gene with no significant similarity to previously characterized genes. We have cloned a human homolog of mad and characterized its role in TGF-β-induced signal transduction. TGF-β signaling protein-1 (BSP-1) is rapidly phosphorylated in response to TGF-β. Chromosomal localization of bsp-1 suggests a possible role in the development of carcinomas.

MATERIALS AND METHODS

Cloning and Northern Blot Analysis—Polymerase chain reaction (PCR) primers were created from expressed sequence tagged (EST) sequences HSC1GG08 and HSC1GG02 identified by BLAST homology search (23). One set of primers (5′-CCATATGAGAAAGAAATGG3′ and 5′ATCTCTGATCACTGAGTG3′) amplified a 1500-bp fragment from a MDA-MB-231 human breast carcinoma cell cDNA library. This fragment was screened to generate the same library for a full-length clone using standard techniques (24). The longest clone was subcloned into pBluescript (Stratagene) and sequenced in both directions by dideoxy chain termination (Sequenase, U.S. Biochemical Corp.). Sequence analysis was performed using Geneworks. For Northern blot analysis, a human multiple tissue Northern blot (Clontech) was screened using the 1500-bp PCR fragment of bsp-1 as described (24). Glutathione S-transferase (GST) fusion constructs were made by PCR amplification using Pfu (Stratagene) of the relevant genes and subcloning into pGEX-2TK. Physical Mapping—Mapping of HSC1GG08 was performed by PCR analysis of somatic cell hybrids from the NIGMS panel 1 (NIGMS, Camden, N.J.) and the Genebridge 4 radiation hybrid panel (25). Statistical analysis of the data was performed using the RHMAPP software package.2 Oligonucleotide primers used for PCR were as described previously (26). The data vector for HSC1GG08 was:

22002220002220200000000001000102220122100012010200000000202000101122000000020001220000001201

Antibody Production and Immunoprecipitation—To generate stable cell lines, a NotI/Xhol fragment containing the full-length bsp-1 coding region was subcloned into pCPC4 (Invitrogen) and this construct transfected into MDA-MB-231 cells. Cells were selected in hygromycin, and the presence of overexpressing bsp-1 was confirmed by Northern blot

* 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.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) U57456.

2 Supported in part by a Damon Runyon-Walter Winchell Cancer Research Fund physician scientist award. To whom correspondence should be addressed: Laboratory of Chemoprevention, NCI, National Institutes of Health, Bethesda, MD 20892-5055 and Gene Mapping Unit, Laboratory of Genetic Disease Research, National Institute of Human Genome Research, Bethesda, MD 20892.

1 The abbreviations used are: TGF-β, transforming growth factor-β; BSP-1, TGF-β signaling protein-1; EST, expressed sequence tag; GST, glutathione S-transferase; bp, base pair; kb, kilobase(s); BMP-2, bone morphogenic protein-2.

2 D. Slonim, L. Stein, L. Kruglyak, and E. Lander, unpublished software.
analysis. For antibody production, New Zealand White rabbits were inoculated with BSP-1/GST fusion protein using standard techniques. Antibodies from rabbit 367 were affinity-purified as described (27) first on a GST column to eliminate anti-GST antibodies and then on a BSP-1/GST fusion protein column. All cells were maintained in Dulbecco's modified Eagle's medium, 10% fetal bovine serum with antibiotics in a 5% CO2 atmosphere. For methionine labeling experiments, 150-mm plates of subconfluent cells were washed in methionine-free medium and labeled with 50 μCi/ml EXPRE35S35S labeling mix (DuPont NEN) for 2.5 h. Cells were rinsed twice in isotonic buffer and lysed in radiolabeled medium precipitation buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.1% SDS, 0.5% deoxycholate, protease, and phosphatase inhibitors). Lysates were clarified at 100,000 × g in an ultracentrifuge, precipitated with protein A-Sepharose (Pharmacia Biotech Inc.) and normal rabbit serum, and then immunoprecipitated with 10 μg/ml of the indicated antibody for 2 h. Complexes were precipitated with protein A-Sepharose and washed in high salt radiolabeled medium precipitation buffer (as above except 1 M NaCl), and the resulting immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis on 8% gels and visualized by autoradiography or PhosphorImager. For blocking experiments, 367 antibody was used. TGF-β1 was from R & D Systems, Activin from Genentech, and BMP-2 from the Genetics Institute. Phosphoamino acid analyses were performed as described previously (27).

RESULTS

We searched the human EST data base with the Drosophila mad sequence and identified two human clones with significant identity. PCR primers designed from these clones were used to amplify a 1500-bp fragment from a MDA-MB-231 breast carcinoma cell line xenograft library. This fragment was then used to clone the full-length cDNA. The longest clone identified coded for a 465-amino acid protein highly similar to Drosophila Mad (Fig. 1A). We have named this clone bsp-1 for TGF-β signaling protein-1. Upstream in-frame stop codons indicate that this clone contains the entire coding region. Comparison of the amino acid sequence of BSP-1 with other sequences showed two additional genes with high degrees of amino acid identity (Fig. 1A). SMA-2 (28) is the most highly similar member of a Caenorhabditis elegans gene family (named dwarfs) recently identified as acting in the tumour suppression pathway. The previously described DH1 (N terminus) and DH2 (C terminus) domains are underlined. Note that we have extended the DH1 one amino acid in the N-terminal direction and four amino acids in the C-terminal direction, and the DH2 three amino acids in the N-terminal direction and six amino acids in the C-terminal direction. B, a human multiple tissue Northern blot probed with a 1500-bp fragment of bsp-1 showed a single transcript of approximately 3.0 kb in all tissues. No additional transcripts were detected. Expression is highest in heart and skeletal muscle. P: pancreas; K: kidney; S: skeletal muscle; L: liver; L: lung; P: placenta; B: brain; H: heart. Molecular sizes in kilobases are indicated at the left.

3 R. J. Lohleider, data not shown.
overexpressing full-length minithe either TGF-β family ligands, 32P-labeled A549 cells were stimulated for 15 min with either TGF-β, BMP-2 (the mammalian Decapentaplegic homolog), or Activin, and lysates were immunoprecipitated with preimmune IgG (lane P), affinity-purified 367 antibody (lane I), 367 preincubated with a 20-fold molar excess of either glutathione S-transferase (lane G), or GST/BSP-1 fusion protein (lane B). The 52-kDa BSP-1 is indicated (arrow). B, A549 cells were metabolically labeled with 35S-labeled methionine and lysates immunoprecipitated with pre-immune IgG (lane P) or 367 antibody (lane I), 367 preincubated with GST (lane G), 367 preincubated with GST/BSP-1 (lane B), or 367 preincubated with GST/DPC4. BSP-1 is indicated (arrow).

band assignment for HSC1GG08 (31).

To explore the function of BSP-1 in mammalian cells, we first attempted to generate stable transfectants of overexpressing bsp-1 constructs. Several attempts using TGF-β-responsive cell lines were unsuccessful. We then generated stable transfec-
tants overexpressing bsp-1 in a MDA-MB-231 cell clone that is not responsive to TGF-β. Using the overexpressing MDA-MB-231 cells, we were able to identify overexpressed BSP-1 by immunoprecipitation of metabolically labeled cell lysates. Affinity-purified rabbit polyclonal antibodies (367 antibodies) raised against a full-length GST/BSP-1 fusion protein, but not preimmune IgG, immunoprecipitated BSP-1 from 35S-labeled methionine cell lysates (Fig. 2A, compare lanes P and I). BSP-1 migrates as a 52-kDa species by SDS-polyacrylamide gel elec-
rophoresis, in agreement with its predicted molecular weight. Preincubation of antibodies with GST/BSP-1 fusion protein, but not preimmune IgG, immunoprecipitated BSP-1 from 35S-labeled methionine cell lysates (Fig. 2A, lane B), but not with GST alone (Fig. 2A, lane G), eliminated binding, demonstrating the specificity of these antibodies for human BSP-1. In metabolically labeled A549 lung carcinoma cell lysates (Fig. 2B), numerous bands are recognized by un-
blocked antibody (lane I), antibody preincubated with GST only (lane G), or antibody preincubated with GST/DPC4 fusion. Included among these is the 52-kDa endogenous BSP-1 (arrow). Neither preimmune IgG (lane P) nor antibody preincu-
bated with GST/BSP-1 fusion (lane B) precipitates these bands, demonstrating the specificity of our antibody for BSP-1 and related species. The related bands are either strongly associating proteins whose complex formation is not disrupted by the lysis and wash buffers used or closely immunologically related species. The clear absence of the 52-kDa band from the preimmune and BSP-1-blocked lanes and its presence in the DPC4-blocked lane demonstrate the specificity of 367 antibody for BSP-1. The presence of the other proteins does not interfere

**FIG. 2. Immunoprecipitation of BSP-1.** A, MDA-MB-231 cells overexpressing full-length bsp-1 were metabolically labeled with 35S-labeled methionine, and the lysates were immunoprecipitated with preimmune IgG (lane P), affinity-purified 367 antibody (lane I), 367 preincubated with a 20-fold molar excess of either glutathione S-transferase (lane G), or GST/BSP-1 fusion protein (lane B). The 52-kDa BSP-1 is indicated (arrow). B, A549 cells were metabolically labeled with 35S-labeled methionine and lysates immunoprecipitated with pre-immune IgG (lane P) or 367 antibody (lane I), 367 preincubated with GST (lane G), 367 preincubated with GST/BSP-1 (lane B), or 367 preincubated with GST/DPC4. BSP-1 is indicated (arrow).

phosphorylated band running above the 69-kDa marker is also seen, but phosphorylation of this band is not regulated by any ligands. This result suggests that BSP-1 functions in a TGF-β-specific pathway in A549 cells. The BSP-1 band appears as a doublet following orthophosphate labeling, while it is a single band in methionine labeling experiments. It is not known if this represents two different phosphorylation states or immunologically related or physically associated proteins. A time course study of BSP-1 phosphorylation (Fig. 3B) showed a rapid phosphorylation of BSP-1 in A549 cells following TGF-β addi-
tion, with maximal phosphorylation occurring by 15 min and beginning to diminish by 60 min. Phosphoamino acid analysis of immunoprecipitated BSP-1 (Fig. 3C) revealed predominantly serine phosphorylation with some threonine phosphorylation as well. Tyrosine phosphorylation was not detected. These results show that TGF-β, but not BMP-2 or Activin, induces a rapid serine and threonine phosphorylation of endogenous BSP-1 in A549 cells, strongly suggesting a role for BSP-1 in TGF-β-induced signal transduction. The role of phosphoryla-
tion in regulating the function of BSP-1 is not known, but it may serve to regulate association of BSP-1 with other signaling molecules.

**DISCUSSION**

BSP-1 is the first unique component of the TGF-β signaling pathway downstream from the membrane receptors to be identified in mammalian cells. Unlike members of the Ras pathway that have been implicated in TGF-β signaling (15), BSP-1 was identified genetically as being involved specifically in signal transduction induced by TGF-β superfamily ligands. The lack of clear enzymatic motifs in BSP-1 suggests a role as a docking protein, much like GRB-2 or SHC in the Ras pathway. The genetic evidence from C. elegans indicates that three sma genes are required for propagation of a signal from the Daf receptors (28), suggesting that either a cascade or heteromeric complex of BSP-1-like proteins is necessary for signaling. Interestingly, Hs766 pancreatic carcinoma cells, which contain a homozgyously deleted DPC4 gene (29), are not growth inhibited by
TGF-β nor do they exhibit a transcriptional activation response, although they do express BSP-1 protein and TGF-β receptors. The large number of bands we see immunoprecipitated with 367 antibodies in A549 cells may represent tightly associated proteins, suggesting that BSP-1 exists as part of a larger complex. Alternatively, they may represent immunologically related proteins, suggesting a family of BSP-1 proteins. A set of BSP proteins may therefore be necessary for TGF-β induced signal transduction in mammalian cells.

Our data differ significantly from that recently published by Hoodless et al. (32). They demonstrate that BSP-1 (which they call MADR1) is phosphorylated in response to BMP-2 but not in response to TGF-β or other superfamily ligands. They also demonstrate only serine phosphorylation, while we clearly show serine and threonine phosphorylation. These results using epitope tags and overexpressed levels of proteins, including the various ligand receptors in both native and constitutively activated states, contrast markedly with our results obtained by immunoprecipitation of endogenous protein. Perhaps the use of epitope tags and overexpressed levels of proteins in their experiments interferes with some aspect of signaling, blocking phosphorylation induced by TGF-β or phosphorylation on threonine. Another possibility is that the cell types are sufficiently different to exhibit differences in signaling.

The recent identification of DPC4 as deleted or mutated in a large number of pancreatic cancers (29) strongly suggests a role for the bsp-1 family in tumor suppression. As with DPC4 in pancreatic cancer or the TGF-β type II receptor in colon (33, 34) and gastric (35) cancer, it is possible that the loss of bsp-1 contributes to the development of as yet unidentified cancers. A screen of hepatocellular carcinomas using microsatellite markers recently demonstrated loss of heterozygosity at 4q28 in approximately 45% of tumors studied (36). Although bsp-1 has not been identified as the gene lost at this locus, it is an intriguing possibility.

Acknowledgments—We thank Erwin Böttinger for Activin and BMP-2, Seong-J in Kim for DPC4 plasmid, Caroline Crockett and Ravi Ajmera for technical assistance, and the members of the Laboratory of Chemoprevention and Steve Byers for helpful discussions.

REFERENCES
1. Sporn, M. B., and Roberts, A. B. (1992) J. Cell Biol. 119, 1017-1021
2. Roberts, A. B., and Sporn, M. B. (1993) Growth Factors 8, 1-9
3. Alexandrow, M. G., and Kroes, H. L. (1995) Cancor Res. 55, 1452-1457
4. Attisano, L., Wrana, J. L., Lopez-Casillas, F., and Massague, J. (1994) Biochim. Biophys. Acta 1222, 71-80
4 M. P. de Caestecker, P. Hemmati, and R. J. Lechleider, unpublished results.

17620 TGF-β Signal Transduction by BSP-1

5. Massague, J., and Polych, K. (1995) Curr. Opin. Genet. & Dev. 5, 91-96
6. Derynck, R. (1994) Trends Biochem. Sci. 19, 548-553
7. Yingling, J. M., Wang, X. F., and Bassing, C. H. (1995) Biochim. Biophys. Acta 1262, 115-136
8. Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) Nature 370, 341-347
9. Ventura, F., Dody, J., Liu, F., Wrana, J. L., and Massague, J. (1994) EMBO J. 13, 5581-5589
10. Wang, T., Donahoe, P. K., and Zervos, A. S. (1994) Science 265, 674-676
11. Wang, T. W., Danielson, P. D., Li, B. Y., Shah, P. C., Kim, S. D., and Donahoe, P. K. (1996) Science 271, 1120-1122
12. Kawabata, M., Imamura, T., Miyazono, K., Engel, M. E., and Moses, H. L. (1995) J. Biol. Chem. 270, 29628-29631
13. Chen, R. H., Mattinen, P. J., Maruoka, E. M., Choy, L., and Derynck, R. (1995) Nature 377, 548-552
14. Yamaguchi, K., Shirakabe, K., Shihuya, H., Irie, K., Oishi, I., Ueno, N., Takiguchi, T., Nishida, E., and Matsumoto, K. (1995) Science 270, 2008-2011
15. Mulder, K. M., and Morris, S. L. (1992) J. Biol. Chem. 267, 5029-5031
16. Yan, Z., Winawer, S., and Friedman, E. (1994) J. Biol. Chem. 269, 13231-13237
17. Atfi, A., Drobetsky, E., Boissonnault, M., Chapdelaine, A., and Chevalier, S. (1994) J. Biol. Chem. 269, 30688-30693
18. Gelbart, W. M. (1989) Development 107, suppl. 75-64
19. Ferguson, E. L., and Anderson, K. V. (1992) Cell 71, 451-461
20. Letsou, A., Arora, K., Wrana, J. L., Simin, K., Twombly, V., Jamal, J., Staehling-Hampton, K., Hoffmann, F. M., Gelbart, W. M., and Massague, J. (1995) Cell 80, 899-908
21. Rafferty, L. A., Twombly, V., Wharton, K., and Gelbart, W. M. (1995) Genetics 139, 241-254
22. Sekelsky, J. J., Newfeld, S. J., Rafferty, L. A., Chaffoff, E. H., and Gelbart, W. M. (1995) Genetics 139, 1347-1358
23. Altshul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403-410
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning; A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Walter, M. A., Spillet, D. J., Thomas, P., Weissbach, J., and Goodfellow, P. N. (1994) Nat. Genet. 7, 22-28
26. Auffray, C., Behar, G., Bas, F., Bouchier, C., Da Silva, C., Devignes, M. D., Duret, S., Houtte, M., Lamy, B. (1995) C. R. Acad. Sci. Paris Ser. II 318, 263-272
27. Lechleider, R. J., Freeman, R. M., Jr., and Neel, B. G. (1993) J. Biol. Chem. 268, 13434-13438
28. Savage, C. D., Das, P., Finelli, A. L., Townsend, S. R., Sun, C. Y., Baird, S. E., and Padgett, R. W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 790-794
29. Hahn, S. A., Schutte, M., Hogue, A. T., Moskaluk, C. A., da Costa, L. T., Rosenblum, E., Weinstein, C. L., Fischer, A., Yeo, C. J., Hruban, R. H., and Kern, S. E. (1996) Science 271, 350-353
30. Kim, S. J., Park, K., Kooler, D., Kim, K. Y., Wakefield, L. M., Sporn, M. B., and Roberts, A. B. (1992) J. Biol. Chem. 267, 13702-13707
31. Hudson, T. J., Stein, L. D., Gerey, S. S., Maj, J., Castle, A. B., Silva, J., Stolin, D. K., Baptista, R., Kruglyak, L., and Xu, S. H. (1995) Science 270, 1945-1954
32. Hoodless, P. A., Haery, T., Abdollah, A., Stapleton, M., O'Connor, M. B., Attisano, L., and Wrana, J. L. (1996) Cell 85, 489-500
33. Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J. A., Fan, R. S., Zborowska, E., Kinzler, K. W., and Vogelstein, B. (1995) Science 268, 1336-1338
34. Parsons, R., Myeroff, L. L., Liu, B., Willson, J. K., Markowitz, S. D., Kinzler, K. W., and Vogelstein, B. (1995) Cancer Res. 55, 5548-5550
35. Park, K., Kim, S. J., Bang, Y. J., Park, J. G., Kim, N. K., Roberts, A. B., and Sporn, M. B. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8772-8776
36. Berg, V., Laurent-Puig, P., Schmitz, A., Flegio, J. F., Bellosa, P., Blondac-Sage, P., Monges, G., Capron, F., Olschwang, S., and Thomas, G. (1996) Proc. Am. Assoc. Can. Res. 37, 551 (abstr.)