Vol. 216, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 2, 1995 Pages 78-83

# CLONING AND CHARACTERIZATION OF THE MURINE ACTIVIN RECEPTOR LIKE KINASE-1 (ALK-1) HOMOLOG +

**Xiying Wu[1], Claudius E. Robinson [1,2], Hon Wa Fong[1,3], Judy S. Crabtree[4], Brenda R. Rodriguez[1], Bruce A. Roe[4] and Jeffrey M. Gimble[1-3,5]**

**[1]Immunobiology & Cancer Program, Oklahoma Medical Research Foundation,** Oklahoma City, OK 73104

**[2]Zoology Department and [4]Chemistry & Biochemistry, University of Oklahoma,** Oklahoma City, OK 73190

**Departments of [3]Microbiology & Immunology and [5]Pathology, University of Oklahoma Health Science Center,** Oklahoma City, OK 73190

Received September 27, 1995

Multiple serine/threonine kinases were detected in a bone marrow stromal cell line. One of these, the murine activin receptor like kinase-1 (ALK-1) homolog was cloned and sequenced. The expressed recombinant protein (62 kDa) was consistent in size with that predicted by the cDNA (58.6 kDa). On Western blots, a goat polyclonal antibody detected the native ALK-1 protein in bone marrow stromal cells, lung, brain, kidney and spleen. Two protein species of 60 kDa and 72-76 kDa were detected. Glycosylation events or alternative splicing may account for the larger protein specie.


Transforming growth factor β (TGFβ) and bone morphogenetic proteins (BMPs) regulate bone marrow stromal cell differentiation **[1]**. *In vivo*, these cytokines promote osteogenesis while inhibiting adipogenesis **[1,2]**. The cellular response is initiated by the interaction of the cytokine with its receptor complex, consisting of a type I and type II transmembrane serine threonine kinase **[3-5]**. Genes encoding a number of type I receptors have recently been cloned based on their conserved kinase domains (I - X) and classified as activin receptor like kinases (ALK) **[3-5]**. The ALK-1 receptor, originally identified in humans, binds to TGFβ and activin in cross-linking experiments **[5]**. However, since the physiologic significance of this finding remains unclear, ALK-1 has been classified as an "orphan" receptor **[6]**. The current experiments were undertaken to determine the type I serine/threonine kinases expressed by the BMS2 bone marrow stromal model **[2]**. In the course of these studies, the murine ALK-1 homolog was identified and cloned. Using a polyclonal

+Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. L48015.

0006-291X/95 $12.00

antibody reagent the native ALK-1 protein has been examined in cell lines and murine tissues. This work provides novel findings concerning the expression and distribution of the native ALK-1 protein.

## MATERIALS AND METHODS

**Materials.** Oligonucleotides and multiple antigenic peptides were synthesized by Dr. Ken Jackson, Oklahoma Center for Molecular Medicine, Oklahoma City, OK. Goat immunizations were performed by Gary Ferrell, of Ferrell Farms, McCloud, OK.

**Cell culture**. The BMS2 cell line **[7]** was cultured and induced as previously described **[2]**.

**Polymerase chain reaction cloning of murine serine/threonine kinases**. Degenerate oligonucleotide primers were synthesized based on conserved sequences within the VIB and VIII kinase domains of the murine Act R-II **[8]** and the C. elegans daf-1 **[9]**:
(a) 5' G(G/C)(C/A)ATTGC(C/T)CG(C/A)GA(C/T)(T/C)T(C/A)3';
(b) 5' GAC(T/C)TC(T/G/A)GG(A/G)GCCAT(G/A)TA 3'.
Specific ALK-1 primers with EcoRI/BamHI restriction sites were prepared based on the human sequences (bp 644-670) and (bp1722-1745) **[10]**. PCR reactions were performed with BMS2 cells or murine lung cDNA for 40 cycles: 94°C for 1 min., 55°C for 2 min, and 72°C for 3 min. Products were subcloned into pBluescript and sequenced using the Sequenase enzyme (U.S. Biochemicals, Cleveland, OH) **[11]**.

**Cloning of the murine ALK-1 cDNA**. A 1.1 kb ALK-1 fragment was radiolabeled **[12]** and used to screen approximately 5 x $10^5$ plaques from a random primed λgt11 murine lung cDNA library (provided by Dr. J. Gitlin, Washington University, St. Louis, MO) according to published methods **[13]**. Inserts were isolated from purified plaques, subcloned into the EcoRI site of pBluescript SKII and sequenced using an Applied Biosystems DNA sequencer (Foster City ,CA) as previously described **[14,15]**.

**In vitro transcription/translation**. The full length murine lung ALK-1 cDNA subcloned into pBluescript was used for in vitro transcription/translation analyses according to the manufacturer's instructions (#L5040, Promega, Madison, WI).

**Transfection.** The full length murine ALK-1 coding region was subcloned into the pEF-BOS expression vector **[16]**. Plasmid DNA (5 μg) was transfected by calcium phosphate precipitation into human kidney 293T cells overnight.

**Antibody preparation and purification**. A 4 chain multiple antigenic peptide (MAP) linked to a lysine core was prepared based on the juxtamembrane domain of the murine ALK-1 protein: RRQEKQRDLHSDLGESSLILKA **[17]**. Goat immunization was performed with 1 mg of MAP in incomplete Freund's adjuvant and an additional injection at week 4 with 1 mg of MAP alone. Serum antibodies were collected after week 5, purified by ammonium sulfate precipitation **[18]** and affinity purified over a column prepared with MAP coupled to cyanogen bromide-activated Sepharose 4B beads (Pharmacia, LKB, Piscataway, NJ) according to the manufacturer's instructions.

**Western blot analysis**. Cell and tissue lysates were analyzed on Western blots as previously described **[19]** using the α-ALK-1 antibody at 7 μg/ml.

**Northern blot analysis**. Northern blots were prepared with 10 μg poly $A^+$ RNA per lane and hybridized with a 1.1 kb ALK-1 or β-actin cDNA probe as previously described **[2]**.

**Table 1. Polymerase Chain Reaction Products from BMS2 Stromal Cell cDNA with Degenerate Kinase Domain Primers**

| PCR Product Size | Sequence Homologue | Per Cent Identity | [Ref.] |
|---|---|---|---|
| 167 bp | Human ALK-1 | 86% | **10** |
| 183 bp | Human Ser/Thr Kinase | 80% | **22** |
| 174 bp | Human Protein Serine Kinase PSK-H1 | 86% | **23** |
| 157 bp | Murine MAP Kinase Activated Protein Kinase-2 | 74% | **24** |

## RESULTS AND DISCUSSION

**Cloning of the Murine ALK-1 Homolog.** Initial studies set out to identify serine/threonine kinase receptors expressed by BMS2 bone marrow stromal cells. Degenerate oligonucleotide primers, based on the conserved VIB and VIII kinase domains, were used to amplify reverse transcribed BMS2 cDNA. Four individual clones were obtained with >75% homology to previously identified human or murine serine/threonine kinases (Table 1). One of these, known as the human activin receptor like kinase-1 (ALK-1) **[10]** and the rat R3 **[20]** protein, encoded a transmembrane protein. A PCR-generated 1.1 kb murine ALK-1 probe was used to isolate nine individual clones from a murine lung cDNA library. The protein encoded by one of these is reported (Figure 1). The current sequence information differed at nine amino acid residues from a recent report **[6]**. Overall, the protein encoded by the murine ALK-1 cDNA exhibited 94.6% and 98% identity with its human and rat homologs, respectively **[10,20]**. Based on in vitro transcription/translation of the murine cDNA, the recombinant ALK-1 protein exhibited a size of 62 kDa on an SDS-PAGE gel (Figure 2).

**Cellular and Tissue Expression of ALK-1 Protein.** A goat polyclonal antibody against an ALK-1 multiple antigenic peptide detected a 58 kDa protein in cells transfected with an ALK-1 expression

```
  1   MTLGSFRRGLLMLSVAFGLTRGDLRRPSKLVNCTCESPHCKRPFCQGSWCTVVLVREQGR   60
 61   HPQVYRGCGSLNQELCLGRPTEFLNHHCCYRSFCNHNVSLMLEATQTPSEEPEVDAHLPL  120
121   ILGPVLALPVLVALGALGLWRVRRRQEKQRDLHSDLGESSLILKASEQADSMLGDFLDSD  180
181   CTTGSGSGLPFLVQRTVARQVALVECVGKGRYGEVWRGSWHGESVAVKIFSSRDEQSWFR  240
241   ETEIYNTVLLRHDNILGFIASDMTSRNSSTQLWLITHYHEHGSLYDFLQRQTLEPQLALR  300
301   LAVSAACGLAHLHVEIFGTQGKPAIAHRDLKSRNVLVKSNLQCCIADLGLAVMHSQSSDY  360
364   LDIGNNPRVGTKRYMAPEVLDEHIRTDCFESYKWTDIWAFGLVLWEIARRTIINGIVEDY  420
421   RPPFYDMVPNDPSFEDMKKVVCVDQQTPTIPNRLAADPVLSGLAQMMRECWYPNPSARLT  480
481   ALRIKKTLQKLSHNPEKPKVIH*    503
```

**Fig. 1. Translated protein sequence from a 2167-bp insert isolated from a murine lung cDNA library.** The N-glycosylation site and putative transmembrane domains are overlined. Single amino acid changes from a recently published murine sequence are highlighted in bold **[6]**.

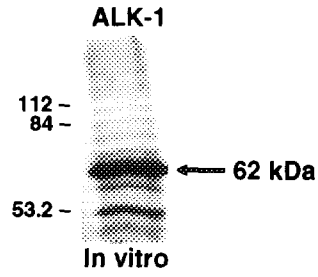


**Fig. 2. *In vitro* expression and detection of ALK-1.** The murine ALK-1 cDNA was transcribed and translated *in vitro* in the presence of [$^{35}$S] methionine using T7 polymerase. Protein size markers (kDa) are indicated.

vector (data not shown). In BMS2 cells, the α-ALK-1 antibody detected native proteins (60 and 76 kDa) that were specifically competed away by the peptide antigen (Figure 3). In murine tissue lysates, the native ALK-1 protein (72 kDa) was most abundant in lung and spleen with lesser amounts in kidney and brain (Figure 4A). A smaller (60 kDa) protein specie was detected in spleen and brain. Little, if any, ALK-1 protein was present in heart, adipose tissues or skeletal muscle. These proteins were not detected on control Western blots probed with pre-immune antibodies (data not shown). Northern blot analysis of polyA$^+$ tissue mRNA confirmed that the ALK-1 mRNA levels were highest in lung, with intermediate levels in heart, kidney, brain, and adipose tissues; spleen and skeletal muscle displayed the lowest detectable mRNA levels (Figure 4B). There are three possible

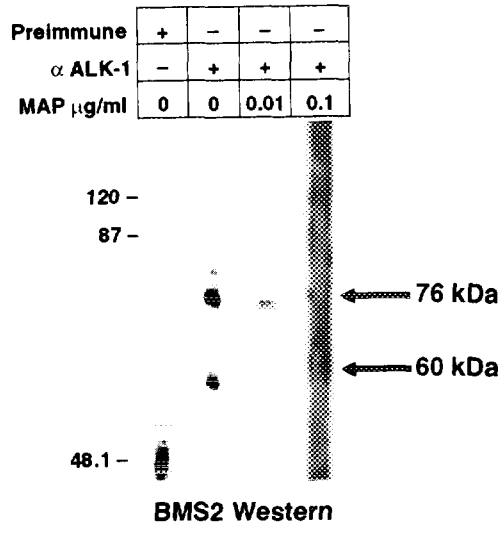


**Fig. 3. Western blot analysis of protein lysates from BMS2 adipocytes.** Western blots were developed with goat pre-immune or affinity-purified α-ALK-1 antibodies in the absence or presence of the multiple antigenic peptide immunogen (MAP). Protein sizes and markers are indicated (kDa).

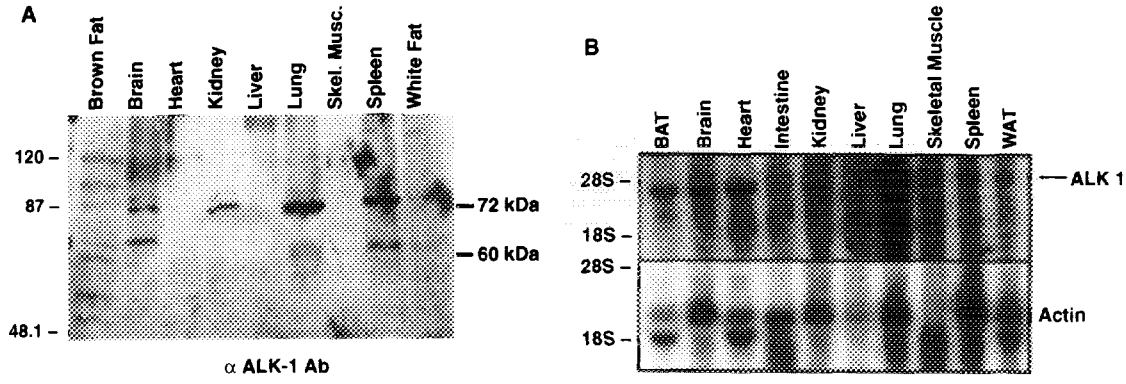


**Fig. 4. Tissue analysis of murine ALK-1 expression. (A)** Western blot analysis. Murine tissue protein lysates (100 μg) were elctrophoresed and analyzed on Western blots with the affinity purified goat anti-ALK-1 antibody. Protein size markers and the major immunoreactive species are indicated (kDa). **(B)** Northern blot analysis. Northern blots prepared with approximately 10 μg of poly $A^+$ mRNA isolated from the indicated murine tissues were hybridized with radiolabeled probes for murine ALK-1 cDNA or actin. The 18S and 28S ribosomal bands are indicated. An arrow indicates the ALK-1 specific mRNA.

explanations for the larger ALK-1 protein specie. First, an N-linked glycosylation site exists in the extracellular domain of the ALK-1 protein (A.A. 97-99, NVS). The addition of oligosaccharides may alter the electrophoretic mobility of the protein. Second, the ALK-1 transcript may be subject to alternative splicing, resulting in a larger sized protein. Third, the 72-76 kDa specie may reflect an antigenically related protein.

**Conclusions.** At this time, ALK-1 remains an "orphan" receptor **[6]**. However, it is postulated that the ALK-1 ligand will prove to be a member of the TGFβ family. When this cytokine is identified, it will be interesting to determine its actions within the bone marrow stroma and other sites of ALK-1 protein expression.

## ACKNOWLEDGMENTS

The authors acknowledge the individuals listed in the Materials and Methods and the following individuals: the staff of the OMRF OASIS for editorial/graphic support; K. Kelly, P.W. Kincade, L. Thompson, and C.F. Webb for valuable discussions; C. Morgan for technical assistance. These studies were supported by NIH grants CA50898 and HG00313 and the Oklahoma Medical Research Foundation. The work serves in partial fullfillment of the University of Oklahoma's M.S. thesis requirements for H.W.F.

## REFERENCES


**1.** Centrella, M., Horowitz, M. C., and Wozney, J. M. (1994) Endocrinol. Rev. 15:27-37.
**2.** Gimble, J.M., Morgan, C., Kelly,K., Wu, X., Dandapani, V., Wang, C.-S., and Rosen, V. (1995) J. Cell. Biochem. 58:393-402.
**3.** Attisano, L., Wrana, J. L., Lopez-Casillas, F., and Massague, J. (1994) Biochim. Biophys. Acta. 1222:71-80.
**4.** Derynck, R. (1994) Trends in Biol. Sci. 19:548-553.
**5.** ten Dijke, P., Franzen, P., Yamashita, H., Ichijo, H., Heldin, C.-H., and Miyazono, K. (1994b) Prog. Growth Factor Res. 5:55-72.
**6.** Dewulf, N., Verschueren, K., Lonnoy, O., Moren, A., Grimsby, S., Vande Spiegle, K., Miyazono, K., Huylebroeck, D., and ten Dijke, P. (1995) Endocrinology 136:2652-2663.
**7.** Pietrangeli, C. E., Hayashi, S. I., and Kincade, P. W. (1988) Eur. J. Immunol. 18:863-872.
**8.** Mathews, L. S., and Vale, W. W. (1991) Cell 65:973-982.
**9.** Georgi, L. L., Albert, P. S., and Riddle, D. L. (1990) Cell 61:635-645.
**10.** ten Dijke, P., Ichijo, H., Franzen, P., Schulz, P., Saras, J., Toyoshima, H., Heldin, C.-H., and Miyazono, K. (1993) Oncogene 8:2879-2887.
**11.** Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467.
**12.** Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132:6-13.
**13.** Gimble, J. M., Dorheim, M.-A., Youkhana, K., Hudson, J., Nead, M., Gilly, M., Wood, Jr., W. J., Hermanson, G. G., Kuehl, M., Wall, R., and Kincade, P. W. (1993) J. Immunol. 150:115-121.
**14.** Chissoe, S. L., Wang, Y. F., Clifton, S. W., Ma, N., Sun, H. J., Lobsinger, J. S., Kenton, S. M., White, J. D., and Roe, B. A. (1991) Methods: A Companion to Methods in Enzymology. 3:55-65.
**15.** Bodenteich, A., Chissoe, S. L., Wang, Y. F., and Roe, B. A. (1994) Automated DNA Sequencing and Analysis Techniques, edit. M.D. Adams, C. Fields, and J.C. Venter. Academic Press, London. Pg. 42-50.
**16.** Mizushima, S., and Nagata, S. (1990) Nucl. Acids Res. 18:5322.
**17.** Tam, J. P., and Shao, J. (1994) In Current protocols in immunology, edit. J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober. Publ. J. Wiley & Sons, New York. 9.6.1- 9.6.19.
**18.** Jensenius, J. C., Andersen, I., Hau, J., Crone, M., and Koch, C. (1981) J. Immunol. Meth. 46:63-68.
**19.** Wu, X., Miyake, K., Medina, K. L., Kincade, P. W., and Gimble, J. M. (1994) Hybridoma 13:409-416.
**20.** He, W. W., Gustafson, M. L., Hirobe, S., and Donohoe, P.K. (1993) Develop. Dyn. 196:133-142.
**21.** ten Dijke, P., Yamashita, H., Ichijo, H., Franzen, P., Laiho, M., Miyazono, K., and Heldin, C.-H. (1994) Science 264:101-104.
**22.** Larsen, F., Solheim, J., Kristensen, T., Kolsto, A. B., and Prydz, H. (1993) Human Molecular Genetics 2:1589-1595.
**23.** Schultz, S. J., and Nigg, E.A. (1993) Cell Growth & Diff. 4:821-830.
**24.** Engel, K., Plath, K., and Gaestel, M. (1993) FEBS Lett. 336:143-147.