Identification of Cell Surface Receptors for the Act-2 Cytokine

By Monica Napolitano, Kenneth B. Seamon,* and Warren J. Leonard

From the Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development; and the *Division of Biochemistry and Biophysics, Food and Drug Administration, Bethesda, Maryland 20892

Summary

We have identified cell surface receptors for Act-2, a secreted protein expressed upon activation of T cells, B cells, and monocytes. Although T-Act-2 showed little, if any, specific binding to resting peripheral blood lymphocytes (PBL) receptors were readily detected on PHA/PMA-activated PBL and a variety of cell lines including MT-2, HL60, DMSO differentiated HL60, HeLa, and K562 cells. The equilibrium dissociation constant \( K_d \) is 3-12 nM for MT-2, K562, and PBL activated with PHA/PMA for 40-80 h. We have also identified a rabbit polyclonal antiserum that can block Act-2 binding to its receptors. The ability to detect specific Act-2 receptors and the development of a blocking antiserum should prove valuable in efforts to molecularly clone the Act-2 receptor and to dissect the biological actions of Act-2.

**Act-2** is a small secreted protein that belongs to a superfamily of structurally related proteins (Fig. 1). Act-2 mRNA is induced in T cells, B cells, and monocytes after activation with mitogenic stimuli; however, this gene is not expressed by all proliferating cells, as evidenced by its nonexpression in various cell lines and in serum-stimulated human fibroblasts (1). Thus, its expression may be relatively restricted to certain hematopoietic lineages. Act-2 is an early activation gene with mRNA levels being detected in normal PBL within 15-30 min of activation with PHA with peak expression at 4 h. Act-2 mRNA contains in its 3' untranslated region characteristic A-T rich sequences (1) found in a number of protooncogenes and cytokines, which appear to be involved in regulating mRNA stability (2, 3). Other groups have also identified cDNAs corresponding to the Act-2 gene (denoted nH400 [4], PAT744 [5], and G-26 [6]) and have shown that its expression is inhibited by Cyclosporin A (5), analogous to a number of cytokines and early activation genes. By analogy, therefore, the Act-2 protein may play an important role in immune cell function.

We have previously determined that Act-2 encodes a secreted protein, which we hypothesized to be a cytokine (1). In partial support of this hypothesis, some members of the superfamily of small secreted proteins (Fig. 1) are known to be mediators of inflammation or to have mitogenic activity (7). Particularly striking is the amino acid homology between Act-2 and the \( \alpha \) and \( \beta \) chains of murine MIP-1 (macrophage inflammatory protein) (71 and 77%, respectively; reference 7). We therefore hypothesize that Act-2 may represent the human equivalent of MIP-1\( \alpha \) or \( \beta \). Natural MIP-1 is among the most abundant proteins synthesized after LPS stimulation of macrophages. It has been reported to induce an oxidative burst in neutrophils, to cause local inflammation and neutrophil infiltration if injected subcutaneously in the footpads of mice, to be a prostaglandin-independent endogenous pyrogen, and to have a myelopoietic enhancing activity on bone marrow granulocyte/macrophage progenitor cells (8). Recently it has been reported that a reversible inhibitor of hematopoietic stem cell proliferation, denoted SCI, has the same amino acid sequence as MIP-1\( \alpha \) (9).

To begin to address the potential role of Act-2 in the inflammatory process, we purified to homogeneity recombinant Act-2 protein and performed binding experiments in order to investigate for the presence of cell surface receptors for Act-2. We herein report the identification of specific Act-2 receptors (Act-2Rs) and an antiserum capable of blocking Act-2 binding to cells.

**Materials and Methods**

Cell Cultures. Human cell lines: HL60, K562, MT-2, and HeLa, were grown at 1-5 x 10^6 cells/ml in RPMI 1640 medium (Biofluids, Inc., Rockville, MD) supplemented with glutamine, penicillin, streptomycin, and 10% FCS. MT-2 is an HTLV-1-transformed T cell line. Human PBLs from healthy volunteers were isolated on lymphocyte separation media (LSM, Organon Technika, West Chester, PA). Where indicated, PBLs were stimulated for 40 to 80 h with 0.5 \( \mu \)g/ml PHA (Wellcome Reagent Ltd., Beckenham, England) and 50 ng/ml PMA (Sigma Chemical Co., St. Louis, MO).

**Purification of Act-2.** The Act-2 cDNA was expressed in a baculovirus expression system using infected SF9 insect cells (1). The
cell supernatant was applied to a preparative (10 × 250 mm) Vydac C4 reversed-phase (RP) column in 200-ml aliquots. The column was washed with 95% buffer A (0.1% TFA), 5% buffer B (acetoni-
cel supernatant was applied to a preparative (10 × 250 mm) Vydac
rated by RP-HPLC using the general procedures outlined by Waters
FCScushion and centrifuged to remove the unbound labeled hg and.

Each sample was layered on top of a 300-μl buffer A containing increasing
with RPMI 1640 medium/1% BSA (binding buffer, BB) and 2-5 ×
activity of 125-I-Act-2 was 15-40 × 10^6 cpm/μg.

Figure 1. Family of related proteins. Sequences above the dashed line have two adjacent cysteines, whereas those below have one amino acid between the two cysteines. Conserved cysteines are boxed.

The pellet was washed once with BB and counted in a gamma counter. Within each experiment, all samples were performed in duplicate. Binding data were analyzed using the LIGAND computer program (10).

Time Course of Association. 2.5 × 10^6 MT-2 cells were incubated in BB at 4°C with 2 nM 125-I-Act-2 in the presence of absence of 300 nM unlabeled Act-2. At different time points, aliquots were centrifuged through a 300-μl cushion (8,000 g for 1 min) and the pellets were counted.

Time Course of Dissociation. 2.5 × 10^6 MT-2 cells were incubated for 2 h at 4°C with 2 nM 125-I-Act-2, washed once in supplemented media, and resuspended in the same volume of BB containing 20 μg of unlabeled Act-2 to prevent reassociation of the

Figure 2. Purification of Act-2. (A) Act-2 was isolated as a single peak on HPLC. (B) Silver stain of Act-2 protein electrophoresed on a 12.5% SDS polyacrylamide gel. (C) Western blot analysis using anti-Act-2 antisera R441 (lane 1) or preimmune antisera (lane 2).
labeled ligand after dissociation. At various times, aliquots were centrifuged through an FCS cushion.

**Homologous Displacement Experiment.** 4 × 10^6 cells in 100 μl of BB were bound at 1.6 nM ^{125}I-Act-2 for 2 h at 4°C in the presence of 0.16-300 nM unlabeled Act-2 or 50-300 nM human IL-1α or IL-2. All samples were tested in duplicate in each experiment.

**Identification of Anti-Act-2 Rabbit Antisera.** Act-2 peptides corresponding to the entire 69 amino acids (aa) or to aa 1 to +11 or to aa −56 to +69 of the Act-2 protein were chemically synthesized on an Applied Biosystems (Foster City, CA) Model 430a automated peptide synthesizer using TBOC chemistry. The NH₂- and COOH-terminal peptides were synthesized with an N-chloroacetylglycylglycine residue and coupled to BSA that had been reacted with iminothiolane (11). The full-length Act-2 protein (not coupled to a carrier protein) was injected into rabbits according to standard methods. Antisera from immunized rabbits were evaluated for their ability to recognize Act-2 in Western blotting and immunoprecipitation experiments.

**Results**

**Radiolabeling of Act-2 and Detection of Act-2Rs.** Recombinant Act-2 was purified by HPLC (Fig. 2 A). When electrophoresed and analyzed by silver staining (Fig. 2 B) or Western blotting (Fig. 2 C), the material migrated as a discrete band of ~13 kD, consistent with the size previously reported (1). Act-2 was iodinated to a specific activity of 15–40 × 10^6 cpm/μg and used in binding studies in the presence or absence of 100–300 molar excess of unlabeled Act-2. Saturable binding curves were obtained using PHA/PMA-activated PBL and MT-2 cells (Fig. 3, A, B). Other cell lines such as HeLa, KS562, HL60, and DMSO-differentiated HL60, also expressed specific Act-2 binding (data not shown).

**Scatchard Analyses.** To determine the receptor number and affinity, we performed Scatchard analyses of binding data. Binding of ^{125}I-Act-2 was carried out at 4°C for 2 h (by which time equilibrium is achieved) in the presence or absence of unlabeled Act-2. Specific binding was determined and data were used to derive the equilibrium dissociation constant (Kd). For MT-2, KS562, and PHA/PMA activated PBLs, Scatchard plots indicated a single class of receptors with a calculated Kd of 7.8–12 × 10⁻⁹ M and the number of receptors/cell ranging between 7,000 and 10,000 in MT-2 (Fig. 3 B, inset) and KS562 cells (not shown), and 45,000 in PBL activated with PHA/PMA for 80 h (Fig. 2 A, inset). In two separate experiments, PBL activated with PHA/PMA for 40 or 60 h were found to express ~10,000 receptor sites per cell. This numerical difference may simply reflect the use of different donors in each experiment. Resting PBL instead showed at most only a small percentage of Act-2 binding to PHA/PMA.
activated PBLs; thus the Act-2R, as the Act-2 protein, is induced upon activation.

Displacement Binding Studies. To evaluate whether the affinity of Act-2 for its receptor was modified by the iodination process, we performed binding studies using a constant amount of labeled ligand (1.6 nM) and increasing amounts of unlabeled Act-2. We could then draw a displacement curve (Fig. 4 A, Ligand program, reference 10) and compare the binding patterns generated using the labeled and unlabeled ligands. The $K_d$ derived from Act-2 binding to MT-2 cells in the displacement experiment was $2.8 \times 10^{-9} \text{M}$, fairly similar to the $K_d$ of $7.8-12 \times 10^{-9} \text{M}$ calculated from the Scatchard plots of data derived from using only labeled Act-2 (Fig. 3). We therefore concluded that iodination had little effect on the binding of Act-2 to its receptor(s) and that the calculated $K_d$s were accurate.

The specificity of Act-2 binding was further demonstrated by the inability of IL-1α and IL-2 to inhibit $^{125}$I-Act-2 binding to MT-2 cells (Fig. 4 A).

Kinetic Binding Studies of $^{125}$I-Act-2 to MT2. To determine when the binding of Act-2 to its receptor reached equilibrium and to independently measure the $K_d$, we performed time courses of association (Fig. 4 B) and dissociation (not shown) of Act-2 binding. Natural log plot of the data corresponding to both association and dissociation kinetic studies allowed us to calculate kinetic binding constants for dissociation ($k' = 3.29 \times 10^{-4} \text{s}^{-1}$) and association ($k = 8.8 \times 10^4 \text{M}^{-1} \text{s}^{-1}$) and to derive the $K_d$ as $3.7 \times 10^{-9} \text{M}$, using the method of Wang and Smith (12). The binding reached equilibrium by $\sim 2 \text{h}$.

A Rabbit Polyclonal Antiserum Blocks Act-2 Binding. We next evaluated if anti–Act-2 antisera could block ligand binding. One rabbit antiserum (R441) derived from immunization with the entire 69 aa Act-2 peptide blocked $^{125}$I-Act-2 binding to its receptor. This antiserum could immunoprecipitate and immunoblot the Act-2 protein. The antiserum was preincubated at 1:40,000 to 1:40 dilution for 30 min with 5 nM $^{125}$I-Act-2, followed by binding at 4°C to HeLa cells for 2 h. Precipitation with the R441 antiserum resulted in a concentration dependent inhibition of binding (Fig. 4 C). This antiserum, therefore, may prove to be a valuable tool in elucidating the function of the Act-2 protein. Another antiserum raised against the full-length peptide (R461) and antiser recognizing two shorter peptides corresponding to aa -1 to +11 (R473) and aa 56 to 69 (R480) did not block binding at the concentration tested (Fig. 4 D), despite their ability to recognize the mature Act-2 protein by immunoprecipitation (R461) or Western blotting (R473 and R480) (not shown). These data suggest that the epitopes defined by these antibodies are not part of the Act-2 molecule responsible for binding.

Discussion

Act-2 is a small secreted protein belonging to a "superfamily" of structurally related proteins, some members of which have mitogenic or inflammatory activities. Based on the high degree of homology and by the ability of native MIP-1, MIP-1α, and MIP-1β to displace Act-2 binding to the Act-2R (Napolitano, M., B. Sherry, and W.J. Leonard, unpublished observation), it is attractive to hypothesize that Act-2 may represent the human counterpart of murine MIP-1α or β. It remains to be determined if MIP-1α and/or β individually possess some or all of the functional activities attributed to MIP-1 or whether their association is required for biological activity. By analogy, there may be the need for a coexpression of Act-2 with another factor, to obtain full biological activity. Based on the sequence similarity, LD78 ([13], also denoted PAT464 [5]) is a candidate for such a protein.

In this paper we have identified specific cell surface Act-2Rs ($K_d$ 3-12 nM). These have been detected on MT-2, HeLa, K562, HL60, DMSO-differentiated HL60 cells, and PHA/PMA-activated PBLs but are absent or present at low levels in resting PBL. The marked increase in the specific binding of $^{125}$I-Act-2 following PHA/PMA activation of PBL strengthens the possibility that Act-2 plays a role in the cellular activation process. Furthermore, both PHA/PMA-activated PBL and the MT-2 cell line thus both produce the Act-2 protein and express cell surface receptors for Act-2, suggesting the possibility that autocrine loops may occur following activation.

Although in comparison to a number of other cytokine/lymphokine ligand/receptors systems, the Act-2R identified here appears to have relatively low affinity for its ligand; it is possible that: (a) sufficiently high concentrations of Act-2 are achieved locally in vivo in order to obtain a functional ligand/receptor interaction, (b) a higher affinity receptor not yet identified may be induced on specific subsets of cell types, or (c) an associated protein is required for Act-2 to achieve higher affinity binding.

The multiple related gene products (Fig. 1) may represent commonly evolved proteins, some of which may bind to shared cellular receptors. It is possible that some of these related factors have evolved to be induced in response to different stimuli and may exert similar biological activities. Alternatively, it is also possible that different target specificities may account for diversified ranges of biological actions.

Demonstration of specific cellular receptors for Act-2 provides the opportunity to address the biological function of this cytokine. A more extensive characterization of the Act-2R and its structure will augment our knowledge on the action of Act-2 and on post-receptor events.
We thank Drs. Wilson Burgess, American Red Cross Jerome E. Holland Research Laboratories, Rockville, MD, for purifying Act-2 and performing quantitative amino acid compositions; R. Chizzone, Hoffman La Roche, Inc., Nutley, NJ, for providing IL-1α and IL-2; and M. Baniyash, J.R. Gnarra, R.D. Klausner, L.E. Samelson, and M. Sharon for valuable discussions.

This work was supported in part by NIH grant HL-35762 to Dr. Wilson Burgess and an NIH Intramural AIDS grant to W.J. Leonard.

Address correspondence to Dr. Warren J. Leonard, Cell Biology & Metabolism Branch, NICHD, National Institutes of Health, Bldg. 18T, Room 101, Bethesda, MD 20892.

Received for publication 18 January 1990.

References

1. Lipes, M.A., M. Napolitano, K.-T. Jeang, N.T. Chang, and W.J. Leonard. 1988. Identification, cloning and characterization of an immune activation gene. Proc. Natl. Acad. Sci. USA. 85:9704.
2. Shaw, G., and R. Kamen. 1986. A conserved AU sequence from the 3'-untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell. 46:659.
3. Caput, D., B. Beutler, K. Hartog, R. Thayer, S. Brown-Shiner, and A. Cerami. 1986. Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators. Proc. Natl. Acad. Sci. USA. 83:1670.
4. Brown, K.D., S.M. Zurawski, T.R. Mosmann, and G. Zurawski. 1989. A family of small inducible proteins secreted by leukocytes are members of a new superfamily that includes leukocyte and fibroblast-derived inflammatory agents, growth factors, and indicators of various activation processes. J. Immunol. 142:679.
5. Zipfel, P.F., J. Balke, S.G. Irving, K. Kelly, and U. Siebenlist. 1989. Mitogenic activation of human T cells induces two closely related genes which share structural similarities with a new family of secreted factors. J. Immunol. 142:1582.
6. Miller, M.D., S. Hata, R.D.W. Malefyt, and M.S. Krangel. 1989. A novel polypeptide secreted by activated human T lymphocytes. J. Immunol. 143:2907.
7. Wolpe, S.D., and A. Cerami. 1989. Macrophage inflammatory protein 1 and 2: members of a novel superfamily of cytokines. FASEB (Fed. Am. Soc. Exp. Biol) J. 3:2565.
8. Broxmeyer, H.E., B. Sherry, L. Lu, S. Cooper, C. Carow, S.D. Wolpe, and A. Cerami. 1989. Myelopoietic enhancing effects of murine Macrophage Inflammatory Protein 1 and 2 on colony formation in vitro by murine and human bone marrow granulocytes/macrophages progenitor cells. J. Exp Med. 170:1583.
9. Graham, G.J., E.G. Wright, R. Hewick, S.D. Wolpe, N.M. Wilkie, D. Donaldson, S. Lorimore, and I.B. Pragnell. 1990. Identification and characterization of an inhibitor of hae-mopoietic stem cell proliferation. Nature (Lond.). 344:442.
10. Munson, P.J., and D. Rodbard. 1980. LIGAND: A versatile computerized approach for characterization of ligand-binding systems. Anal. Biochem. 107:220.
11. Lindner, W., and F.A. Robey. 1987. Automated synthesis and use of N-chloroacetyl-modified peptides for the preparation of synthetic peptide polymers and peptide-protein immunogens. Int. J. Peptide Protein Res. 30:794.
12. Wang, H.-M., and K. Smith. 1987. The interleukin 2 receptor. Functional consequences of its bimolecular structure. J. Exp Med. 166:1055.
13. Obaru, K., M. Fukuda, S. Maeda, and K. Shimada. 1986. A cDNA clone used to study mRNA inducible in human tonsillar lymphocytes by a tumor promoter. J. Biochem. 99:885.