Identification of Cell Surface Receptors for the Act-2 Cytokine

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Summary

We have identified cell surface receptors for Act-2, a secreted protein expressed upon activation of T cells, B cells, and monocytes. Although ^{125}I–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.

A ct-2 is a small secreted protein that belongs to a super-family 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 proto-oncogenes 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 super-family 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 α and β chains of murine MIP-1 (macrophage inflammatory protein)[1] (71 and 77%, respectively; reference

7). We therefore hypothesize that Act-2 may represent the human equivalent of MIP-1α or β. 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 foot-pads 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-1α (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 × 10⁵ 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 µg/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

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[1] *Abbreviations used in this paper:* BB, binding buffer; MIP-1, macrophage inflammatory protein 1; RP, reversed-phase; SCI, stem cell inhibitor.

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hAct-2    APMGSDPPTAC-CFSYTARKL-PRNFVVD-Y-YETSSL-CSQP-AVVF-QTKRSKQVCADPSESWVQEYVYDLELN
hG-26     APMGSDPPTAC-CFSYTARKL-PRNFVVD-Y-YETSIL-CSQP-AVVF-QTKRSKQVCADPSETWVQEYVYDLELN
hLD78     ASLAADTPTAC-CFSYTSRQI-PQNFIAD-Y-FETSSQ-CSKP-GVIF-LTKRSRQVCADPSEEWVQKYVSDLELSA
mSISY     APMGSDPPTSC-CFSYTSRQL-HRSFVMD-Y-YETSSL-CSKP-AVVF-LTKRGRQICANPSEPWVTEYMSDLELN
hRANTES   ASPYSSDTTPC-CFAYIARPL-PRAHIKE-Y-FYTSGK-CSNP-AVVF-VTRKNRQVCANPEKKWVREYI--NSLEMS
mTY5      APYGADTPTAC-CFSY-SRKI-PRQFIVD-Y-FETSSL-CSQP-GAIF-LTKRNRQICADSKETWVQEYITDLELNA
mMIP1α    APYGADTPTAC-CFSY-SRKI-PRQFIVD-Y-FETSSL-CSQP-GVIF-LTKRNRQICADSKETWVQEYITDLELNA
mMIP1β    APMGSDPPTSC-CFSYTSRQL-HRSFVMD-Y-YETSSL-CSKP-AVVF-LTKRGRQICANPSEPWVTEYMSDLELN
mTCA3     KSMLTVSNSC-CLNTLKKEL-PLKFIQC-YRKMGSS--CPDPPAVVFRLNK-GRESCASTNKTWVQNH-L-KKVNPC
hMCP-1    XPDAINAPVTC-CYNFTNRKI-SVQRLAS-YRRITSSK-CPKE-AVIF-KTIVAKEICADPKQKWVQDSMDHLDKQTQTP..
mJE       APLTC-CYSFTSKMI-PMSRLES-YKRITSSR-CPKE-AVVF-VTKLKREVCADPKKEWVQTYIKNLDRNQMRS
-------------------------------------------------------------------------------
hPF4      EAEEDGDLQCLCVKTTSQ-VRP-RHITSLEVIKA-GPHCPTA-QLIATL-KNGRKICLDLQAPLYKK-II-KKLLES
bPF4      .EGGEDEDLQCVCLKTTSG-INP-RHISSLEVIGA-GTHCPSP-QLLATK-KTGRKICLDQQRPLYKK-IL-KKLLDGDES
rPF4      ..PEESDGDLSCVCVKTSS-RIHL-KRITSLEVIKA-GPHCAVP-QLIATL-KNGSKICLDRQVPLYKK-II-KKLLES
hPBP      ..DSDLYAELRCMCIKTTSG-IHP-KNIQSLEVIGK-GTHCNQV-EVIATL-KDGRKICLDPDAPRIKK-IVQKKLAGDESAD
hIP-10    VPLSRTVRCTCISISNQPVNP-RSLEKLEIIPA-SQFCPRV-EIIATMKKKGEKRCLNPESKAIKNL-L-KAVSKEMS..
hGRO      ASVATELRCQCLQTLQG-IHP-KNIQSVNVKSP-GPHCAQT-EVIATL-KNGRKACLNPASPIVKK-IIEKMLNSDKSN
haGRO     APVANELRCQCLQTMTG-VHL-KNIESLKVTPP-GPHCTQT-EVIATL-KNGQEACLNPEAPMVQK-IVQKMLKSGIRK
mKC       APIANELRCQCLQTMAG-IHL-KNIQSLKVLPS-GPHCTQT-EVIATL-KNGREACLDPEAPLVQK-IVQKMLKGVPK
hIL-8     ...SAKELRCQCIKTYSKPFHP-KFIKELRV-IESGPHCANT-EIIVKL-SDGRELCLDPKENWVQR-VVEKFLKRAENS
c9E3      ..KMGNELRCQCISTHSKFIHP-KSIQD-VKLTPSGPHCKNV-EIIATL-KDGREVCLDPTAPWVQ-LIV-KALMAKAQLN
mMIP-2    AVVASELRCQCLKTLPRVDF--KNIQSLSVTPP-G...
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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.

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 (acetonitrile containing 0.08% TFA) at 5 ml/min. After 15 min, the percent buffer B was changed linearly in the following order: 10 min, 30%; 40 min, 40%, 45 min, 80%; 55 min, 80%; 60 min, 5%. Act-2-containing fractions from several runs were pooled, concentrated, and purified further using a semipreparative (4.6 × 250 mm) Vydac C4 RP column. The flow rate was 2 ml/min and Act-2 was eluted using the gradient shown in Fig. 2 A. The concentration of Act-2 was determined by quantitative amino acid analysis. Purified Act-2 was hydrolyzed with argon-purged, constant boiling 6 N HCl containing 1% (vol/vol) phenol at 110°C for 20 h using a Waters Associates Pico Tag Work Station. Amino acids were derivatized with phenylisothiocyanate and the PTC-amino acids were separated by RP-HPLC using the general procedures outlined by Waters Associates. Data collection and reduction were performed using a Waters Associates 840 system.

Radiolabeling of Act-2. 1-4 mCi of Na^{125}I (Amersham Corp., Arlington Heights, IL; 3.7 GBq/ml, 10 mCi/100 μl) was added to 1-5 μg of HPLC-purified Act-2 in 100 μl phosphate buffer (PB) pH 7.4, 50 μl of Enzymobeads reagent (Bio-Rad Laboratories, Richmond, CA), and 25 μl of 2% glucose, and incubated for 15-30 min at 22°C. ^{125}I-Act-2 was separated from free label using a G-25 spun column coated with 50 nM PB pH 7.4/1% BSA. The specific activity of ^{125}I-Act-2 was 15-40 × 10^6 cpm/μg.

Binding of ^{125}I-Act-2 to Cells. Cells were washed three times with RPMI 1640 medium/1% BSA (binding buffer, BB) and 2-5 × 10^6 cells resuspended in 100 or 200 μl of BB containing increasing concentrations of ^{125}I-Act-2 for 2 h at 4°C. Nonspecific binding was assessed by incubating samples in the presence of 100-300-fold excess unlabeled Act-2. Each sample was layered on top of a 300-μl FCS cushion and centrifuged to remove the unbound labeled ligand.

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 or absence of 300 nM unlabeled Act-2. At different time points, aliquots were centrifuged through a 300-μl FCS 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
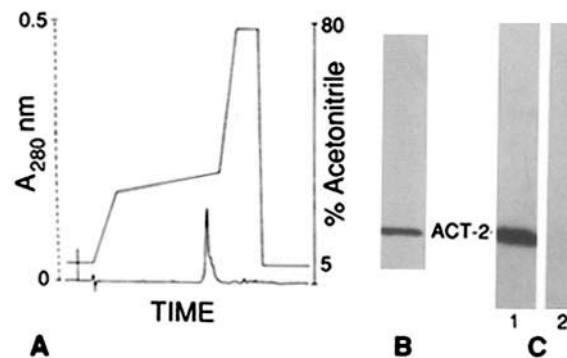

Figure 2. Purification of Act-2. (A) Act-2 was isolated as a single peak on HPLC. (B) Silver stain of Act-2 protein electrophoresed on a 12.5% SDS polyacrylamide gel. (C) Western blot analysis using anti-Act-2 antisera R441 (lane 1) or preimmune antisera (lane 2).
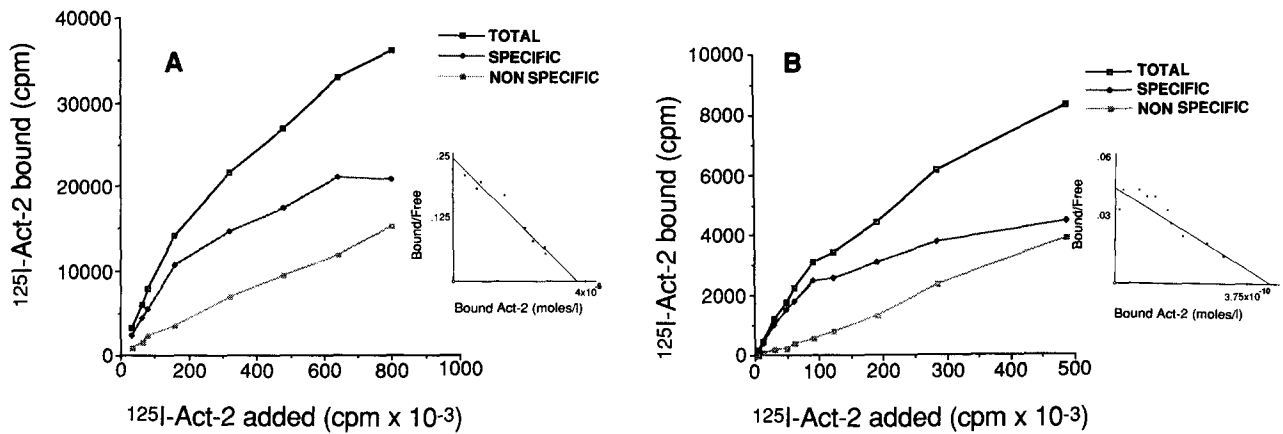
Figure 3. Identification of Act-2 receptors. Saturable binding of ^{125}I-Act-2 to PHA/PMA-activated PBL (*A*) and MT-2 cells (*B*). Scatchard analysis of *A* and *B* are shown in the *insets*. Total, specific, and nonspecific binding are indicated. The binding was performed at 4°C for 2 h. The concentration of Act-2 ranged between 1 and 20 nM. The K_d was 8–12 nM.

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 NH$_2$- and COOH-terminal peptides were synthesized with an N-chloroacetylglycylglycine residue and coupled to BSA that had been reacted with iminotholane (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 $\sim$13 kD, consistent with the size previously reported (1). Act-2 was iodinated to a specific activity of 15–40 $\times 10^6$ 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, K562, 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 (K_d). For MT-2, K562, and PHA/PMA activated

PBLs, Scatchard plots indicated a single class of receptors with a calculated K_d of $7.8–12 \times 10^{-9}$ M and the number of receptors/cell ranging between 7,000 and 10,000 in MT-2 (Fig. 3 *B*, *inset*) and K562 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 $\sim$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
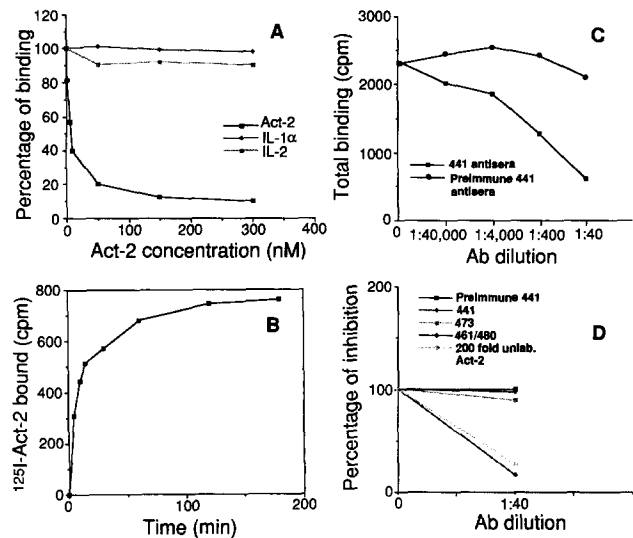

Figure 4. (*A*) Displacement curve using 1.6 nM ^{125}I-Act-2 and increasing concentration of unlabeled Act-2. Unlabeled Act-2, but not IL-1α or IL-2, inhibited binding of ^{125}I-Act-2 to MT-2 cells. (*B*) Time course of association. MT-2 cells were incubated with 2 nM ^{125}I-Act-2, at 4°C for 5–180 min. Samples were tested in duplicate and in the presence and absence of 200-fold excess unlabeled Act-2. (*C*) Pretreatment of ^{125}I-Act-2 with rabbit antisera raised against the entire 69 amino acid long Act-2 peptide (R441) inhibits ^{125}I-Act-2 binding to MT-2 cells at 1:40,000 to 1:40 dilutions. (*D*) R441 antisera, but not R461, R473, and R480 antisera, inhibited Act-2 binding to MT-2 cells at 1:40 dilutions.

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 2.8×10^{-9} M, fairly similar to the K_d of $7.8–12 \times 10^{-9}$ 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_ds 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' $= 3.29 \times 10^{-4}$ s^{-1}) and association (k $= 8.8 \times 10^{4}$ M^{-1}s^{-1}) and to derive the K_d as 3.7×10^{-9} M, using the method of Wang and Smith (12). The binding reached equilibrium by ~2 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. Preincubation 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 antisera 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.

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