Cybr, a cytokine-inducible protein that binds cytohesin-1 and regulates its activity

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Cytokines regulate lymphocyte development and differentiation, but precisely how they control these processes is still poorly understood. By using microarray technology to detect cytokine-induced genes, we identified a cDNA encoding Cybr, which was increased markedly in cells incubated with IL-2 and IL-12. The mRNA was most abundant in hematopoietic cells and tissues. The predicted amino acid sequence is similar to that of GRP-1-associated protein (GRASP), a recently identified retinoic acid-induced cytohesin-binding protein. Physical interaction, dependent on the coiled-coil domains of Cybr and cytohesin-1, was demonstrated by coimmunoprecipitation of the overexpressed proteins from 293T cells. Cytohesin-1, in addition to its role in cell adhesion, is a guanine nucleotide-exchange protein activator of ARF GTPases. Acceleration of guanosine 5′-*O*-(thiotriphosphate) binding to ARF by cytohesin-1 *in vitro* was enhanced by Cybr. Because the binding protein modified activation of ADP ribosylation factor by cytohesin-1, we designate this cytokine-inducible protein Cybr (cytohesin binder and regulator).

ADP-ribosylation factors | cytohesin-binding protein | PDZ domain | Sec7 domain | T cell

Cytokines regulate lymphoid cells at all stages of their development, differentiation, and activation (1). Some such as IL-2 are required to control and maintain immune system homeostasis. Others are responsible for early lymphocyte development and the differentiation of naive $CD4^+$ helper T (T_H) cells, with IL-12 and IL-4 promoting T_H1 and T_H2 polarization, respectively. T_H1 cells secrete IFN-γ, which enhances cellular immunity against intracellular pathogens, whereas T_H2 cells produce IL-4 and other cytokines that promote antihelminthic immunity and allergic disease (2). Cells of the T_H1 and T_H2 lineages differ not only in their ability to produce different cytokines but have a variety of incompletely understood differences in the expression of receptors and transcription factors and even fundamental differences in the organization of their plasma membranes (3).

Microarray technology has been used effectively to delineate differences between subtypes of cells (4). Because IL-12 is important for the differentiation of naive $CD4^+$ T cells into T_H1 cells, we used this approach to define its effects on gene expression. We identified more than 200 genes that were induced rapidly in response to IL-12 (unpublished data) including several such as *IFN-γ*, *Socs-1*, *IL-12Rβ2*, *IL-18R*, *IRF-1*, *CCR5*, and *GADD45*, known to be regulated by this cytokine. Among the genes of unknown function induced by IL-12, one of the more prominent in multiple microarray experiments was a cDNA variously identified in the database as B3–1/36.3K zipper-containing protein (5), cytohesin-binding protein HE, and pleckstrin homology, Sec7, and Coiled-coil Domains-binding protein (PSCDBP). Because there were no publications describing the function of this gene or its protein product, we investigated it as a potential component of an IL-12-induced differentiation program.

Cytohesins are a family of guanine nucleotide-exchange proteins (GEPs) for the 20-kDa ADP ribosylation factor (ARF) GTPases (6, 7), which also associate with integrins and regulate cell adhesion (8). Cytohesin-1 was isolated in a yeast two-hybrid screen by using the intracellular domain of the integrin CD18 as a bait and was implicated in the regulation of lymphocyte adhesion through LFA-1 (9). Its predicted amino acid sequence was recognized as identical to that of a clone isolated earlier (10) by using subtractive hybridization [natural killer (NK) cells minus T_H Jurkat cells]. Overexpression of cytohesin-1 in Jurkat cells increased their ability to bind ICAM-1, whereas overexpression of a mutant lacking GEP activity decreased adhesion and blocked its stimulation by phorbol myristoyl acetate (8, 9).

We report here some characteristics of a cytohesin-1-binding protein, the expression of which is regulated by cytokines. The mRNA was most abundant in hematopoietic tissues and was increased markedly by incubation of peripheral blood mononuclear cells (PBMCs) with IL-2 and IL-12. The recombinant protein bound cytohesin-1, and the coiled-coil domains of the two proteins were identified as responsible for this interaction. Because the newly identified binding protein modified activation of ARF by cytohesin-1, we designate it Cybr for cytohesin binder and regulator.

Materials and Methods

Cell Lines and Human PBMCs. NK3.3 cells were grown in RPMI medium 1640 with 15% FBS and Lymphocult T (Biotest AG, Dreleich, Germany)/100 units/ml penicillin/100 μg/ml streptomycin and 2 mM L-glutamine/100 units/ml IL-2 at 37°C with 10% CO_2. Human PBMCs were collected by leukopheresis from normal healthy volunteers, purified by using Ficoll/Paque, and incubated in RPMI medium 1640 with 10% FCS and penicillin/streptomycin/glutamine at 37°C with 5% CO_2. For activation, cells were incubated for 3 days in medium containing 2 μg/ml phytohemagglutinin followed by 24 h in growth medium containing 100 units/ml IL-2. Cells were washed with acidified medium (≈pH 4.5) and incubated overnight in RPMI medium 1640 with 1% BSA and then with or without 20 μg/ml cycloheximide for 30 min at 37°C with 5% CO_2, followed by washing with RPMI medium 1640 with 1% BSA before the addition of cytokine.

Microarray Studies. NK3.3 cells and PBMCs were incubated with or without 1,000 units/ml IL-2 and 10 ng/ml IL-12 for 6 h. RNA

Abbreviations: T_H, T helper; GEP, guanine nucleotide-exchange protein; ARF, ADP-ribosylation factor; PBMC, peripheral blood mononuclear cell; Cybr, cytohesin binder and regulator; NK, natural killer; GST, glutathione *S*-transferase; PDZ, PSD-95/Dlg/ZO-1; GRASP, GRP-1-associated protein; PBL, peripheral blood leukocyte.

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was isolated by using the total RNA isolation system (Promega), and mRNA was purified with oligoTex mRNA isolation columns (Qiagen, Chatsworth, CA). Microarray slides containing ≈7,000 cDNAs were hybridized, scanned, and image-analyzed as described (11).

Plasmid Constructs. cDNA encoding full-length human Cybr (GenBank accession no. XM_002443) was amplified by reverse transcription–PCR from NK3.3 mRNA with the primers 5′-ACT CTC GAG TGA TCT TTA TTC ACA *ATG* T-3′ and 5′-ACT GGT *ACC* AAA GCG ACT TTC TTC CTC TT-3′. To generate the X-press-tagged protein, cDNA encoding full-length human Cybr was amplified with the primers 5′-CTG GTA CCA ATG TCT TTA CAA AGG CTC CTG-3′ and 5′-ACT GAA TTC GAA AGG ACA CCA CAA TCC GTC A-3′, and the PCR product was subcloned into pEF-Xpress (Invitrogen) via *Kpn*I and *Eco*RI sites. cDNA encoding full-length human cytohesin-1 was amplified by reverse transcription–PCR using the primers 5′-ACT GAA TTC CAT GGA GGA GGA CGA CAG-3′ and 5′-ACT AGA TCT GCA CGC TCA GTG TCG CTT-3′. The product was subcloned into pFLAG-CMV2 via *Eco*RI and *Bgl*II sites to express cytohesin-1 with an N-terminal Flag tag.

Northern Blot Analysis. Total RNA was extracted by using the Total RNA isolation system (Promega), subjected to electrophoresis, transferred to nylon membrane, and hybridized with ^{32}P-labeled full-length Cybr cDNA (1,080 bp) in ExpressHyb (CLONTECH) following the manufacturer's protocol.

Generation of T_H1 and T_H2 Cell Lines. Naive CD4$^+$ T cells, isolated from umbilical cord blood (purchased from Poietic Technologies, Gaithersburg, MD) by anti-CD4 microbeads (Miltenyi Biotec, Auburn, CA), were incubated (1×10^6 cells per ml) in RPMI medium 1640 with 10% FCS in 24-well plates coated with 5 μg/ml anti-CD3 antibody (OKT3). For T_H1 cells the medium contained 2 ng/ml IL-12 and 2 μg/ml anti-IL-4, and for T_H2 cells the medium contained 20 ng/ml IL-4 and 2 μg/ml anti-IL-12. After stimulation for 4 days, cells were harvested, washed, and incubated with 10 units/ml IL-2 for 4 days. This procedure was repeated a total of three times to achieve complete differentiation of T_H1 and T_H2 cells.

Immunoprecipitation and Immunoblotting. 293T cells were transfected with Fugene (Roche Molecular Biochemicals) by following the manufacturer's protocol, harvested 24 h later, and lysed on ice for 10 min in buffer containing 0.5% Brij, 50 mM Tris·HCl (pH 7.5), 300 mM NaCl, 2 mM EDTA, 200 μM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2.5 μM *p*-nitrophenyl *p*-guanidinobenzoate. Immunoprecipitation, SDS/PAGE, and immunoblotting were performed as described (12). Proteins were detected by enhanced chemiluminescence (Amersham Pharmacia).

Assay of Cytohesin-1 GEP Activity. Procedures for preparation of recombinant human cytohesin-1 (13) and ARF1 (14) are published. To prepare Cybr with an N-terminal His-6 tag, Cybr cDNA was amplified by using forward primer 5′-ATC ATA TGT CTT TAC AAA GGC TCC TGC-3′ and reverse primer 5′-ATC TCA TAT GAG TCA AAA GCG ACT TTC TTC CTC-3′. The product was subcloned in pET-14b (Novagen) using *Nde*I sites. For preparation of glutathione *S*-transferase (GST)-Cybr, the product of amplification of Cybr cDNA using forward primer 5′-GAC AGT CGA CAT ATG TCT TTA CAA AGG CTC CTG C-3′ and reverse primer 5′-ATC TGC GGC CGC AGT CAA AAG CGA CTT TCT TCC TC-3′ was subcloned in pGEX-4T-1 (Amersham Pharmacia) via *Sal*I and *Not*I sites. Expression and purification of the recombinant protein were performed by following the manufacturer's protocol.

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h-Cybr     MSLQRL--LQ  HSS-------  -NGNLADFCA  --------G-  PAYSSYSTLT
m-Grasp    MTLRRLRKLQ  QKEEATAAPD  PAGRAPDSEA  ARAAPLPSGP  PAAAAPPGAP
Consensus  M L RL  LQ              G   D  A         G   PA

h-Cybr     GS---LTMND  ---NRRIQML  ADTVATLPRG  RKQLALTRSS  SLSDFSWSQR
m-Grasp    GEELYAALED  YHPAELYRAL  AVSGGTLPR-  RKGSGF-RWK  NFTQSPEQQR
Consensus  G        D           L  A    TLPR  RK     R           QR

h-Cybr     KLVTVEKQDN  ETFGFEIQSY  RPQNQNACSS  EMFTLICKIQ  EDSPAHCAGL
m-Grasp    KVLTLEKGDN  QTFGFEIQTY  GLHHREEQRV  EMVTFVCRVH  ESSPAQLAGL
Consensus  K  T EK DN   TFGFEIQ Y              EM T  C     E SPA  AGL

h-Cybr     QAGDVLANIN  GVSTEGFTYK  QVVDLIRSSG  NLLTIETLNG  TMILKRTELE
m-Grasp    TPGDTIASVN  GLNVEGIRHR  EIVDIIKASG  NVLRLETLYG  TSIRK-AELE
Consensus    GD  A  N  G   EG        VD I  SG  N L  ETL G  T I K  ELE

h-Cybr     AKLQVLKQTL  KQKWVEYRSL  QLQEHRLLHG  DAANCPS-LE  NMDLDELSLF
m-Grasp    ARLQYLKQTL  YEKWGEYRSL  MVQEQRLVHG  LVVKDPSIYD  TLESVRSCLY
Consensus  A LQ LKQTL   KW EYRSL   QE RL HG     PS           L

h-Cybr     GPLPGPGPAL  VDRNRLSSES  SCKSWLSSMT  MDSEDG-YQT  CVSEDSSRGA
m-Grasp    GAGLLPGSLP  FGP-LLAAPG  SARGGARRAK  GDTDDAVYHT  CFFGGAEPQA
Consensus  G   PG         L        S           D  D  Y T  C        A

h-Cybr     FSRQTSTDDE  CFIPKEGDD-  ----FLR-RS  SSRRNRSISN  TS--SGSMSP
m-Grasp    LP-PPPPPAR  ALGPSSAETP  ASVLFPAPRS  TLSRSASVRC  AGPGGGGGAP
Consensus              P           F   RS     R  S           G   P

h-Cybr     --LW-EGNLS  SMFGTLPRKS  RKGSVRKQLL  KFIPGLHRAV  EEEESRF
m-Grasp    GALWTEAREQ  ALCGAGLRKT  KYRSFRRRLL  KFIPGLNRSL  EEEESQL
Consensus    LW E       G   RK      S R  LL  KFIPGL R      EEEES
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Fig. 1. Amino acid sequences of human (h) Cybr and murine (m) GRASP aligned using the CLUSTALW program. Identical amino acids are indicated in the consensus sequence. Fuchsia denotes PDZ domains, and yellow denotes coiled-coil domains.

Procedures for the assay of guanosine 5′-*O*-(thiotriphosphate) binding and GEP activity (total volume 50 μl) are published (15).

Results

Cloning and Expression of Cybr. To understand better the molecular mechanisms of cytokine action we used glass-slide cDNA microarrays (4, 11) to evaluate the expression of genes induced by IL-2, IL-12, and other cytokines. We compared the expression of ≈6,500 genes in unstimulated NK3.3 cells with those in cells incubated with IL-2 plus IL-12 and found numerous highly up-regulated expressed sequence tags among the several hundred genes that showed altered expression in stimulated cells. Unigene analysis identified a single gene cluster (Hs.270) with sequence very similar to two previously reported cDNAs named B3–1/36.3K zipper-containing protein (GenBank accession no. AAA16575; ref. 5) and cytohesin-binding protein HE (GenBank accession no. AAC19129). The former appears to be a shorter form or a splice variant, and the latter appears to be identical to that described here. Analysis of the predicted sequence revealed that Cybr contains a PSD-95/Dlg/ZO-1 (PDZ) domain (16) spanning amino acids 77–166 and a coiled-coil domain (17) between amino acids 167 and 188. Homology search using the BLAST algorithm identified a murine protein named GRP-1-associated protein (GRASP) as very similar to Cybr (18).

Amino acid sequences of human Cybr and murine GRASP are aligned in Fig 1. Similarity between the PDZ domains (fuchsia) is 62% (identity 48%). Similarity and identity of the coiled-coil domains (yellow) are 88 and 65%, respectively. Overall similarity between the two proteins is 50% and identity 32%. GRASP was reported to be expressed preferentially in brain, lung, and heart, whereas hybridization of multiple-tissue Northern blots with a Cybr cDNA probe (Fig. 2*A*) revealed the largest amounts of a 1.3-kb mRNA in thymus, spleen, lung, peripheral blood leukocytes (PBLs), lymph node, and bone marrow (Fig. 2*B*), although larger transcripts were observed also.

To confirm the microarray findings, we assessed Cybr mRNA levels in primary human PBMCs incubated with IL-2 plus IL-12. In a representative experiment (Fig. 2*C*), unstimulated PBMCs demonstrated little 1.3-kb Cybr mRNA (*Upper*). The amount was increased after incubation for 6 h with either IL-2 or IL-12 (lanes

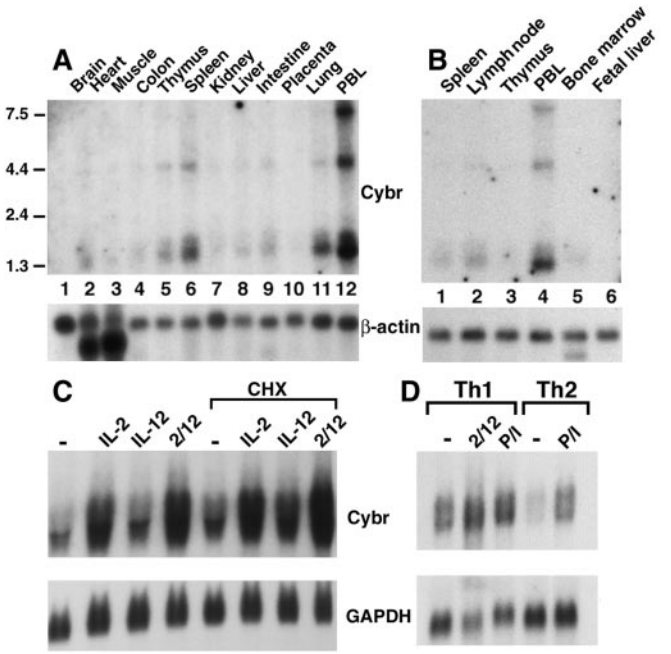

Fig. 2. Distribution of Cybr mRNA in human cells and tissues. (*A* and *B*) Blots with poly(A)$^+$ RNA from the indicated tissues were hybridized with ^{32}P-labeled Cybr cDNA (*Upper*). After stripping, filters were hybridized with β-actin cDNA. (*C*) Each lane contained 20 μg of total RNA from cells incubated for 6 h without additions or with IL-2, IL-12, or IL-2 plus IL-12 (2/12). Some cells were incubated with cycloheximide (CHX) for 30 min before the addition of the indicated cytokines. (*D*) Each lane contained 20 μg of total RNA from Th1 or Th2 lymphocytes untreated or incubated for 6 h with IL-2 and IL-12 (2/12) or phorbol 12-myristate 13-acetate and ionomycin (P/I) as indicated. Blots were hybridized with the Cybr cDNA (*Upper*), and then stripped and hybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (*Lower*). The experiments in *C* and *D* were replicated at least three times.

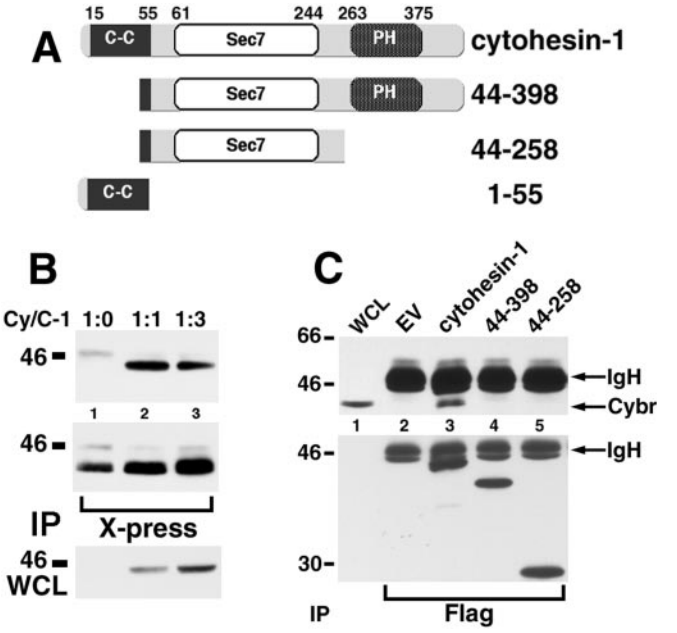

Fig. 3. Coimmunoprecipitation of overexpressed Cybr and cytohesin-1 mutants. (*A*) Diagram of cytohesin-1 mutants. (*B*) 293T cells were transfected with 2 μg of X-press vector containing Cybr cDNA (Cy) and empty Flag vector or 2 or 6 μg of Flag vector containing cytohesin-1 cDNA (C-1) at different ratios (Cy/C-1 = 1:0, 1:1, and 1:3). Proteins were immunoprecipitated (IP) from samples of cell lysates with anti-X-press antibody, separated by SDS/PAGE, and immunoblotted with anti-Flag (*Top*) or anti-X-press (*Middle*) antibodies. Proteins from samples of whole-cell lysates (WCL) were separated also in the same gel as the immunoprecipitates and reacted with anti-Flag antibody (*Bottom*). (*C*) 293T cells were transfected with 2 μg of X-press vector containing Cybr cDNA and 6 μg of empty Flag vector (EV) or vector containing intact cytohesin-1, mutant 44–398, or mutant 44–258. Proteins immunoprecipitated from cell lysates with an anti-Flag antibody were separated by SDS/PAGE before immunoblotting with anti-X-press (*Upper*) or anti-Flag (*Lower*) antibodies. Lane 1 contains 100 μg of whole cell lysate (WCL) from 293T cells transfected with 4 μg of intact Cybr. The arrows indicate the Ig heavy chain (IgH) and Cybr. The experiments in *B* and *C* were replicated at least five times.

2 and 3) and significantly more so by both together. Cybr mRNA also was increased synergistically by IL-12 plus IL-18; IL-18 alone had no effect (data not shown). Because we are most interested in genes directly regulated by IL-2 and IL-12, we examined the effect of prior cycloheximide treatment on Cybr expression by PBMCs (Fig. 2*C*). The absence of cycloheximide inhibition suggests that *de novo* protein synthesis is not required for the increase of Cybr mRNA and argues against it being induced secondarily by other cytokines.

As Cybr was up-regulated by stimuli that are known to induce the differentiation of naive CD4$^+$ T_H cells into the T_H1 subset, we investigated the expression of Cybr in fully polarized T_H1 and T_H2 cells. We found more Cybr mRNA in T_H1 than in T_H2 cells (Fig. 2*D*, lanes 1 and 4). Subsequent incubation of T_H1 cells with IL-2 plus IL-12 increased Cybr expression; phorbol ester plus the calcium ionophore ionomycin increased Cybr mRNA in both subsets of cells.

Cybr Interacts with Cytohesin-1. Yeast two-hybrid studies of GRASP revealed that it interacts with cytohesin-3, and pull-down experiments showed that it also interacts with cytohesin-2 but not cytohesin-1 (18). Considering the similarity between Cybr and GRASP, but also their different tissue distributions, we tried to establish the existence of physical interaction between Cybr and cytohesin. To this end, constructs for expression of Flag-tagged cytohesin-1 and fragments were prepared (Fig. 3*A*). Proteins immunoprecipitated from whole lysates of 293T cells that had been transfected with the indicated construct were separated by SDS/PAGE, transferred to polyvinylidene difluoride, and reacted with anti-Flag (cytohesin-1) or anti X-press (Cybr) antibodies. From cells transfected with Cybr plus different amounts of cytohesin-1 (Fig. 3*B*, *Top*), anti-X-press antibody immunoprecipitated cytohesin-1 as well as the ≈42-kDa X-press-tagged Cybr (Fig. 3*B*, *Middle*). The amounts of expressed cyothesin-1 were confirmed by Western blot of samples from whole-cell lysates before immunoprecipitation (Fig. 3*B*, *Bottom*). Reciprocal immunoprecipitation with anti-Flag antibody of lysates of 293T cells transfected with full-length Cybr plus intact or mutant cytohesin-1 (Fig. 3*C*) confirmed the physical interaction between Cybr and full-length cytohesin-1 (lane 3). In contrast, a fragment containing the Sec7 domain (44–258), responsible for catalysis of guanine nucleotide exchange on ARF, did not precipitate with Cybr (lane 5) nor did a larger polypeptide lacking the N-terminal coiled-coil domain (44–398, lane 4). The amounts of immunoprecipitated intact and mutant cytohesin-1 proteins were assessed by reaction of the stripped membrane with anti-Flag antibody (Fig. 3*B*, *Bottom*).

To delineate the region of Cybr responsible for the association with cytohesin-1, X-press-tagged Cybr or fragments (Fig. 4*A*) were expressed in 293T cells together with full-length cytohesin-1, which was immunoprecipitated from whole-cell lysates with anti-Flag antibody. Full-length Cybr, a mutant (78–359) lacking the first 77 amino acids, and a mutant (166–359) lacking also the PDZ domain were immunoprecipitated with the Flag-tagged cytohesin-1. A mutant Cybr (1–217) lacking the C-terminal 142 amino acids was still immunoprecipitated by the

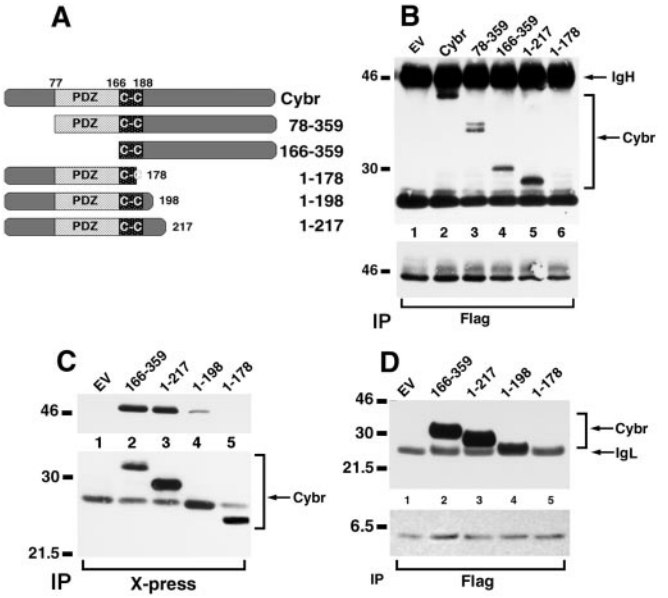

Fig. 4. Coimmunoprecipitation of overexpressed cytohesin and Cybr mutants. (*A*) Diagram of Cybr mutants. (*B*) 293T cells were transfected with 6 μg of Flag vector containing cytohesin-1 cDNA and 6 μg of empty X-press vector (EV) or vector containing intact Cybr or mutant 78–359, 166–359, 1–217, or 1–178. Proteins immunoprecipitated from cell lysates with anti-Flag antibody were separated by SDS/PAGE before immunoblotting with anti-X-press (*Upper*) or anti-Flag (*Lower*) antibodies. The arrows indicate the Ig heavy chain (IgH) and Cybr or Cybr mutants. (*C*) 293T cell were transfected with 6 μg of Flag vector containing cytohesin-1 cDNA and 2 μg of empty X-press vector or vector containing Cybr mutant 166–359, 1–217, 1–198 or 1–178. Proteins immunoprecipitated from cell lysates with an anti-X-press antibody were separated by SDS/PAGE before immunoblotting with anti-Flag (*Upper*) or anti-X-press (*Lower*) antibodies. The arrow indicates Cybr mutants. (*D*) 293T cells were transfected with 6 μg of Flag-tagged cytohesin-1 mutant 1–55 cDNA and 6 μg of empty X-press vector or vector containing Cybr mutant 166–359, 1–217, 1–198, or 1–178. Proteins from samples of cell lysates were immunoprecipitated with anti-Flag antibody separated by SDS/PAGE and immunoblotted with anti-X-press (*Upper*), anti-Flag (*Lower*) antibodies. The arrows indicate Ig light chain (IgL) and Cybr mutants. The experiments in *B–D* were replicated at least five times.

anti-Flag antibody, whereas mutant Cybr (1–178) truncated in the middle of the coiled-coil domain was not (Fig. 4*B*). The amounts of expressed proteins were confirmed by Western blot of samples from whole-cell lysates before immunoprecipitation (data not shown). Reciprocal immunoprecipitation with an anti-X-press antibody of cell lysates of 293T cells transfected with full-length cytohesin-1 and the indicated plasmids (Fig. 4*C*) confirmed the lack of interaction between Cybr mutant (1–178) and cytohesin-1 (*Upper*, lane 5). Interaction of Cybr (1–198) with cytohesin-1 was detectable but limited.

To define a minimal region of interaction between Cybr and cytohesin-1, 293T cells were transfected with the Flag-tagged mutant cytohesin-1 (1–55) containing the coiled-coil domain and mutant Cybr constructs (Fig. 4*D*). Lysates were immunoprecipitated with anti-Flag antibody, and proteins were reacted with anti-X-press antibody (Fig. 4*D*, *Upper*). Cybr mutants 166–359 (lane 2), 1–217 (lane 3), and 1–198 (lane 4) were immunoprecipitated by the Flag antibody. In contrast, Cybr mutant 1–178, truncated in the coiled-coil domain, was not. Blotting the stripped membrane with anti-Flag antibody revealed equivalent immunoprecipitation of the 5-kDa polypeptide containing the cytohesin-1 coiled-coil domain. Taken together these results establish that Cybr binds cytohesin-1 and indicate that this interaction is mediated by the coiled-coil domains of the two proteins. Because of this physical association,

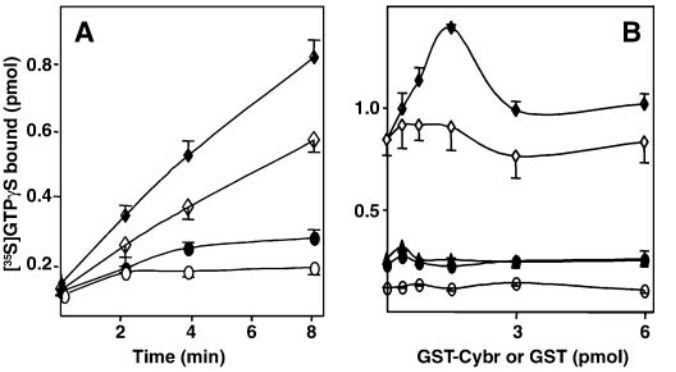

Fig. 5. Effect of Cybr on guanosine 5′-*O*-(thiotriphosphate) (GTPγS) binding by ARF1; modification of cytohesin-1 GEP activity. (*A*) ARF1 (50 pmol) was incubated without additions (○), or with 11 pmol of cytohesin-1 (◇), 2 pmol of His-6-Cybr (●), or both cytohesin-1 and Cybr (◆) for the indicated times at 30°C before measurement of bound [^{35}S]guanosine 5′-*O*-(thiotriphosphate). (*B*) ARF1 (50 pmol) was incubated with 11 pmol of cytohesin-1 and the indicated amounts of GST-Cybr (◆) or GST (◇) for 20 min at 30°C. As controls, ARF1 without cytohesin-1 was incubated with GST-Cybr (●) or GST (▲), and GST-Cybr was incubated alone (○). The data are means ± SD of values from triplicate assays. The findings were replicated at least three times with different preparations of recombinant proteins.

we next sought to determine whether Cybr influenced cytohesin biochemical activity.

Effects of Cybr on GEP Activity of Cytohesin-1. Cytohesin-1 accelerated binding of guanosine 5′-*O*-(thiotriphosphate) by ARF1, and the rate of binding was increased further by Cybr, which, when added alone, was ineffective (Fig. 5*A*). This effect of Cybr occurred over a very narrow concentration range. In Fig. 5*B*, maximal enhancement of the GEP activity of 11 pmol of cytohesin-1 was seen with 1.5 pmol of Cybr, whereas Cybr had no effect on GEP activity of the isolated cytohesin-1 Sec7 domain (data not shown), which is consistent with the absence of demonstrable physical interaction of the two proteins by immunoprecipitation. Cytohesin-2 GEP activity similar to that of cytohesin-1 was increased by Cybr. That of the very different brefeldin A-inhibited BIG1–BIG2 complex, however, was unaffected (data not shown). We have observed repeatedly with different preparations of recombinant proteins that Cybr enhancement of cytohesin-1 GEP activity is seen only in a very limited range of protein concentration, which may be related to involvement of the cytohesin-1 coiled-coil domain in homodimerization as well as in the interaction with Cybr. It most surely is related to having attempted to evaluate the function of Cybr, cytohesin, and ARF1 by using recombinant proteins in an *in vitro* system that lacks all other molecules with which each of these proteins interacts in cells.

Discussion

Cytokines regulate the immune system at all levels. They are critical for lymphocyte development and differentiation but also control events such as adhesion, cytoskeletal rearrangement, and cellular polarization. Considerable progress has been made in understanding how a signal initiated by binding of cytokines to their receptors is transmitted to the nucleus (1). However, exactly which genes cytokines regulate and how they control lymphocyte differentiation and effector functions is still not understood completely. To begin to answer some of these questions, we used microarray technology to compare the regulation of gene expression after incubation of cells with cytokines. We describe here the initial characterization of a cDNA identified in a screen of mRNAs acutely increased in NK cells

and PBMCs by IL-2 and IL-12 stimulation and constitutively expressed in T_H1 but not T_H2 $CD4^+$ T cells, which we designated Cybr.

This cDNA was isolated previously from an NK/T cell library and named B3–1/36.3K leucine zipper-containing protein (5), which was proposed to be a transcription factor based on the presence of the leucine-zipper domain, although its function was not demonstrated. It was found also in GenBank as cytohesin-binding protein HE, or PSCDBP, without functional information. Others already had reported its preferential expression in the T_H1 subset but, based on the initial discovery, assigned it the function of transcription factor (19). To aid in elucidating the function of Cybr, we searched for related proteins and found similarity of Cybr to GRASP, another recently described gene product (18). GRASP is expressed in brain, heart, and lung, whereas we found high levels of Cybr mRNA especially in PBLs, spleen, thymus, and lymph node. These differences might suggest that Cybr is an immune system homologue of GRASP.

Cytohesins are a family of GEPs that activate the 20-kDa ARF GTPases by catalyzing the replacement of bound GDP with GTP. Although this is the only activity demonstrated to be shared by the four cytohesins, their notable similarity in domain structures as well as amino acid sequences is consistent with considerably more extensive similarity of functions (6, 7). ARF GEP activity resides in the Sec7 domain (as it does in all ARF GEPs), which is >77% identical among the cytohesins. This part of the molecule clearly is not involved in the Cybr interaction nor is the pleckstrin homology domain, which likewise is highly conserved among cytohesins and responsible for specificity of their association with polyphosphoinositides. The more divergent C-terminal polybasic region, which in cytohesin-1 and -2 contains potentially regulatory phosphorylation sites, also is apparently unrelated to the Cybr interaction that seemed to require only the coiled-coil domain in the first 55 residues at the N terminus of cytohesin-1.

It was shown also that the interaction involved the coiled-coil domain of Cybr. The PDZ domain of Cybr was not needed, and preliminary findings (data not shown) suggest that it does not mediate homodimerization. PDZ domains are well known participants in protein–protein interactions and the formation of multiprotein complexes (20). Identification of proteins with which the Cybr PDZ domain does interact may provide clues to its intracellular function and/or localization as well as to that of cytohesin-1. After deletion of the N-terminal region, including the PDZ domain of GRASP, the protein was distributed diffusely through the cytoplasm rather than localized at the plasma membrane (18).

Cytohesin-1 has been implicated in lymphocyte adhesion. Kolanus and coworkers reported that cytohesin-1 regulated lymphocyte adhesion by its interaction with the β2 chain (CD18) of integrin LFA-1 (8, 9). Both IL-2 and IL-12 are known to have a role in T cell adhesion and migration (21, 22), possibly related to their effects on the expression of a protein that interacts with cytohesin-1. We found levels of Cybr mRNA undetectable in Jurkat cells (data not shown), which were reported to express cytohesin-1 constitutively (9). Further investigation is necessary to determine whether LFA-1-mediated adhesion is influenced by the Cybr-cytohesin-1 interaction and whether up-regulation of Cybr is necessary for the cytohesin-1 activity.

Other cytohesins also are present in cells and tissues of the hematopoietic system. Cytohesin-4 was found predominantly in PBLs, spleen, thymus, and cultured cell lines of the myeloid lineage (23). Recently, cytohesin-3 was reported in anergic T_H1 cells (24) and was proposed to be important for stability of anergic cells and LFA-1-mediated adhesion. Because multiple members of the cytohesin family are expressed in hematopoietic tissues, it will be important to compare the effects of Cybr on all the immunologically expressed cytohesins.

ARFs are small GTPases that participate in numerous vesicular trafficking pathways. They also activate phospholipase D and phosphatidylinositol-4 phosphate 5-kinase, the reaction products of which, phosphatidic acid and phosphatidylinositol4–5-bisphosphate, can regulate actin-binding proteins and thereby cytoskeletal rearrangement. Among the numerous pathways that cytokines activate, that involving phosphoinositide 3-kinase is one of the most prominent. Although thus far there is no direct link between ARFs and cytokine effects on Cybr expression, Cybr effects on ARF activation may indeed suggest such a connection. Cytokine induction of Cybr provides a potential link between these immunoregulatory molecules and ARF action, another mechanism through which cytokines might regulate lymphocyte functions. It is also important to remember that not all actions of cytohesin-1 require its GEP activity, and the effects of Cybr on other cytohesin functions need to be determined.

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