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Functional Regions of the Mouse Interleukin-10 Receptor Cytoplasmic Domain

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The functions of wild-type and mutant mouse interleukin-10 receptors (mIL-10R) expressed in murine Ba/F3 cells were studied. As observed previously, IL-10 stimulates proliferation of IL-10R-expressing Ba/F3 cells. Accumulation of viable cells in the proliferation assay is to a significant extent balanced by concomitant cell death. Moreover, growth in IL-10 also induces a previously unrecognized response, differentiation of the cells, as evidenced both by formation of large clusters of cells in cultures with IL-10 and by induction or enhancement of expression of several cell surface antigens, including CD32/16, CD2, LECAM-1 (v-selectin), and heat-stable antigen. Two distinct functional regions near the C terminus of the mIL-10R cytoplasmic domain which mediate proliferation were identified; one of these regions also mediates the differentiation response. A third region proximal to the transmembrane domain was identified; removal of this region renders the cell 10- to 100-fold more sensitive to IL-10 in the proliferation assay. In cells expressing both wild-type and mutant IL-10R, stimulation with IL-10 leads to tyrosine phosphorylation of the kinases JAK1 and TYK2 but not JAK2 or JAK3 under the conditions tested.

Interleukin-10 (IL-10) is a cytokine produced by activated T cells, B cells, monocytes/macrophages, and keratinocytes (15, 24). Mouse and human IL-10s (mIL-10 and hIL-10, respectively) are inhibitors of macrophage activation and inhibit cytokine synthesis by activated T cells and NK cells by blocking the ability of macrophages to act as antigen-presenting or costimulatory cells (15, 24). Like other cytokines, IL-10 has multiple activities, including costimulation of proliferation and differentiation of human B cells (4, 12, 29), mouse thymocytes, T cells (1, 22, 34), and mast cells (39), upregulation of class II major histocompatibility complex expression on mouse B cells (12), and maintenance of viability of mouse mast cell lines, mouse B cells, and human T cells in vitro (12, 35, 41). IL-10 is homologous to an Epstein-Barr virus protein, BCRF1 (25, 41), and BCRF1 exhibits some, but not all, activities of IL-10 (15, 24).

The activities of IL-10 are mediated through cell surface receptors (IL-10R). mIL-10 and hIL-10 receptors (mIL-10R and hIL-10R, respectively) are members of the interferon receptor (IFNR)-like subgroup of the cytokine receptor family (14, 20). Characterization of IL-10R structure, function, and signal transduction should facilitate understanding of the molecular basis for IL-10 activities. We have already shown that recombinant IL-10R expressed in the IL-3-dependent mouse pro-B-cell line Ba/F3 (BaF-IL-10R) can transduce a proliferative signal in response to IL-10 (14, 20). In the current work, we further characterized the response of BaF-mIL-10R cells to IL-10 and found that culture in IL-10 induces or augments expression of several cell surface proteins on these cells. In addition, we expressed modified recombinant mIL-10R and characterized the different events induced in response to mIL-10, thereby identifying regions in the cytoplasmic domain of mIL-10R which mediate different responses.

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MATERIALS AND METHODS

Cells and cell culture. Ba/F3 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 50 μM β-mercaptoethanol, and 10 ng of mIL-3 per ml (BaF medium). Transfection of Ba/F3 cells was done as described previously (14, 17). Ba/F3 transfectants were selected and maintained in BaF medium containing 1 mg of G418 per ml, and cell lines expressing recombinant wild-type and mutant mIL-10R were isolated by repetitive cycles of cell sorting (14, 20). J774 cells were obtained from the American Type Tissue Culture Collection.

Proliferation assay. Cells expressing wild-type and mutant mIL-10R were tested for responsiveness to IL-10 by a cell proliferation and viability assay as described previously (14, 20) with a colorimetric assay using Alamar Blue (Alamar Biosciences, Sacramento, Calif.). The concentration of IL-10 inducing a half-maximal response was defined as 1 U/ml. In some experiments, cells were washed twice and introduced into cultures in which FCS was replaced with a mixture of 0.5 mg of bovine serum albumin (BSA) per ml, 2.5 μg of linoleic acid per ml, 5 μg of insulin per ml, 5 μg of transferrin per ml, and 5 ng of sodium selenite per ml. In cultures in which insulin was also omitted, 0.5 mg of BSA per ml, 2.5 μg of linoleic acid per ml, and 50 μg of transferrin per ml were added.

The number of cells in IL-10-containing cultures was counted. Cells were washed twice and cultured (1×10^5 to 3×10^5/ml) in medium containing IL-3 or IL-10 at concentrations predetermined to give maximal levels of stimulation (20 U/ml for Δ282-414; 80 U/ml for wild type and Δ483-559). Cultures were supplemented with medium or split every 1 to 2 days to maintain cell concentrations below 10^6/ml. At various times, cells were resuspended, and live and dead cells were enumerated in the presence of trypan blue.

RNA preparation. RNA was prepared with the RNAzol reagent (Tel-Test, Friendswood, Tex.).

Detection of mIL-3 mRNA. mIL-3 mRNA was detected by reverse transcriptase PCR with the following primers from R. L. Coffman (DNAX Research Institute of Molecular and Cellular Biology):

5′-CAAGGAGATTATAGGGAAGCTCCCAGAACC-3′ (sense)
5′-TGTAGGCAGGCAACAGTTAAGTTTCTGAAG-3′ (antisense)

WEHI-3 mRNA (provided by C. Grimaldi, DNAX Research Institute of Molecular and Cellular Biology) and an mIL-3 cDNA clone (46) were positive controls for detection of IL-3 mRNA by reverse transcriptase PCR.

Detection of cell surface antigen expression. Cells were grown in medium containing IL-3, IL-10, or both cytokines for 2 days, washed once with fluorescence-activated cell sorting (FACS) buffer (Hanks balanced salt solution, 3% FCS, 0.02% sodium azide), and incubated with the appropriate phycoerythrin-conjugated antibodies (Pharmingen, San Diego, Calif.) or isotype controls for 30

min on ice. For some analyses of J774 cells, it was necessary to block nonspecific binding to Fcγ receptor (FcγR) expressed by these cells with the anti-FcγR antibody 2.4G2 (40) (100 μg/ml). Cells were washed twice with FACS buffer and analyzed with a FACScan (Becton-Dickinson, Milpitas, Calif.).

Immunoprecipitations and immunoblot analysis. After starvation in RPMI 1640 with 0.5% BSA for 4 to 5 h at 37°C, cells were then washed once with RPMI 1640 and resuspended in RPMI 1640 with 0.1% BSA (2×10^7 cells per ml). Cells were incubated with or without IL-10 (180 ng/ml), IL-3 (100 ng/ml), or IL-4 (20 ng/ml) for 10 min at 37°C, pelleted, and lysed (2×10^7/ml) in lysis buffer (1% Triton X-100, 10 mM iodoacetamide, 150 mM NaCl, 10 mM Tris-Cl [pH 7.5], 1 mM sodium orthovanadate, 10 mM sodium fluoride, 2 mM EDTA, 30 mM phosphatase substrate [Sigma Chemical Co., St. Louis, Mo.], 100 μg of tolylsulfonyl phenylalanyl chloromethyl ketone [TPCK] per ml, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, and 10 μg of pepstatin A per ml) by rocking at 4°C for 30 min. Cell debris was removed by centrifugation.

For analysis of total cellular protein tyrosine phosphorylation, aliquots of cell lysate containing 40 μg of protein were boiled for 2 min in 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and then subjected to SDS-PAGE and immunoblotting as described below.

For immunoprecipitations, lysates were incubated with anti-JAK1, anti-JAK2, anti-JAK3 (all three antibodies from UBI, Lake Placid, N.Y.), or anti-TYK2 (Santa Cruz Biotechnology, Santa Cruz, Calif.) antibodies overnight at 4°C. The mixture was then incubated with 100 μl of protein G/A Plus agarose (Santa Cruz Biotechnology) at 4°C for 2 h. After the agarose was washed three times, it was boiled with 2× SDS-PAGE sample buffer for 10 min before being subjected to SDS-PAGE (7.5% polyacrylamide). Proteins were subsequently electrotransferred onto a NitroPlus membrane (Micron Separations, Inc., Westboro, Mass.). After the membranes were blocked with Tris-buffered saline containing 3% BSA at 37°C for 30 min, they were incubated with the antikinase antibodies to visualize protein or with antiphosphotyrosine antibody 4G10 (UBI) in Tris-buffered saline with 0.1% Tween 20 for 2 h. After the membranes were washed several times, the results were visualized by incubation with peroxidase-conjugated anti-rabbit immunoglobulin (Ig) (Promega, Madison, Wis.) or anti-mouse Ig (Amersham, Arlington Heights, Ill.) and subsequent use of the enhanced chemiluminescence detection system (Amersham).

Site-directed mutagenesis. Tyrosine-to-phenylalanine mutants were constructed by following the manufacturer's protocol of the Transformer Site-Directed Mutagenesis kit (Clontech, Palo Alto, Calif.). Tyrosine codons TAT and TAC were changed to TTT and TTC, respectively. The unique restriction site *Cla*I (ATCGAT) in the pJFE14 expression plasmid (5) was changed to *Eco*47III (AGCGCT). Colonies with plasmids that could be digested with *Eco*47III but not *Cla*I were selected, and their open reading frames (ORF) were sequenced to confirm the mutation.

Construction of deletion mutants. Mutants carrying the ORF of mIL-10R and the C-terminal deletion mutants Δ380-559, Δ402-559, and Δ433-559 were constructed by first removing the cytoplasmic domain and 3′ untranslated sequence by *Sma*I (located at Pro-249)-*Not*I digestion of the mIL-10R cDNA pMR29 (14) (see Fig. 5). The excised fragment was then replaced by the appropriate PCR-amplified fragments which had been digested with *Sma*I and *Not*I. The sense PCR primer was 5′-CCA GTG GTA CAT CCG GCA CCC GGG GAA GTT GCC-3′. The antisense PCR primers follow: Δ380-559, 5′-CGT CCG AAG CGC GGC CGC TCA TCA TCA CTG GTC CTG ATG GGT ATA TCC AAG CTG CTG-3′; Δ402-559, 5′-CGT CCG AAG CGC GGC CGC TCA TCA TCA AGA TGC ATC CTG TGT GTA CTT AGG CTG CCC-3′; Δ433-559, 5′-CGT CCG AAG CGC GGC CGC TCA TCA TCA TCT GGT CTG TTT CTG GTA GCC CTG GAA TGT-3′; and ORF, 5′-CGT CCG AAG CGC GGC CGC TCA TCA TCA TTC TTC TAC CTG CAG GCT GGA GAT CAA CGG CAG-3′. The ligation mixture was transformed into *Escherichia coli*, and plasmid from individual colonies was analyzed. Each selected clone was then sequenced to confirm the deletion. For one C-terminus deletion mutant (Δ483-559) and all N-terminus deletion mutants (Δ282-389, Δ282-414, and Δ282-458), the *Dra*III site (Val-281) in mIL-10R was used instead of *Sma*I. The sense primer was 5′-CCC GAT GCC ATT CAC ATC GTG GAC CTG GAG GTT TTC CC-3′. The antisense primers for each construction follow: Δ282-389, 5′-CGT CCG AAG CAC ATC GTG TCT CCA GGG CAG CCT AAG TAC ACA CAG GAT GCA TCT GCC-3′; Δ282-414, 5′-CGT CCG AAG CAC ATC GTG GAG GAG AAA GAC CAA GTC ATG GTG ACA TTC CAG GGC TAC CAG-3′; Δ282-458, 5′-CGT CCG AAG CAC ATC GTG GGG GTA CAC CTG CAG GAT GAT TTG GCT TGG CCT CCA CCA GCT-3′; and Δ483-559, 5′-CGT CCG AAG CGC GGC CGC TCA TCA TCA AGA CTC CTG TTT CAA ATA ACC TGC GGC CAG-3′. PCR amplification was performed as follows: 2 min at 94°C and then 30 cycles, with each cycle consisting of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C.

Scatchard plot analysis. Cells (1×10^5 to 5×10^5 in 200 μl) were incubated as triplicate samples with 4 to 530 pM ^{125}I-hIL-10 (94 μCi/μg; Dupont-NEN, North Billerica, Mass.) in the presence or absence of 100 nM hIL-10 (T. Nagabhushan, Schering-Plough Research Institute, Kenilworth, N.J.) for 4 h at 4°C in RPMI 1640 with 2% BSA and 0.02% sodium azide (binding buffer) with shaking and then pelleted through a mixture of phthalate oils as described previously (14, 20, 36). The cell pellet and supernatant were assessed for bound and free ^{125}I counts per minute, respectively. Nonspecific binding counts per minute in samples containing unlabelled hIL-10 were subtracted to obtain specific binding counts per minute. Values for K_d and receptor number were obtained by Scatchard analysis as described previously (14, 20).

Internalization of IL-10. Cells ($\sim 1.5 \times 10^6$ to 2×10^6 in 150 to 250 μl) were incubated with 700 pM ^{125}I-hIL-10 in binding buffer without sodium azide for 2 to 60 min at 37°C. A 50-μl aliquot of the binding reaction mixture was added to 150 μl of 0.1 M NaCl–80 mM sodium citrate (pH 4.0) for 10 min at room temperature, conditions predetermined to maximize cell viability (>90%) and removal of receptor-bound IL-10 yet minimize nonspecific binding of ^{125}I-hIL-10. The cells were then pelleted through an oil pad and assessed for cell-associated and free ^{125}I counts per minute as described above. Nonspecific cell-associated counts per minute in samples containing unlabelled hIL-10 competitor were subtracted to obtain specific cell-associated counts per minute. The resulting values were compared with the amount of ^{125}I-hIL-10 bound at 4°C in 80 to 90 min in the presence of sodium azide (no internalization), with or without excess hIL-10 competitor, and expressed as a percentage of the specific counts per minute bound during the 4°C incubation.

RESULTS

Ba/F3 cells expressing mIL-10R proliferate in response to IL-10. We previously reported that Ba/F3 cells expressing recombinant IL-10R exhibit a proliferative response to IL-10, whereas the parent Ba/F3 cells do not (14, 20). Both mIL-10 (Fig. 1A and B) and hIL-10 (not shown) stimulated BaF-mIL-10R (BaMR29a1) cells with indistinguishable specific activities of 0.5×10^7 to 1×10^7 U/mg, similar to that observed for IL-10's macrophage deactivating factor or cytokine synthesis inhibitory factor activity (8, 15, 24). Using BSA, transferrin, linoleic acid, sodium selenite, and insulin instead of FCS in the culture medium did not detectably alter the cells' response to IL-10 under the conditions described. However, the elimination of insulin or prior culture for 3 to 5 h in the absence of serum and growth factor significantly diminished the capacity of the cells to respond to IL-10 (not shown). Because Ba/F3 cells proliferate in response to IL-3, we determined whether IL-10 induced expression of IL-3 or its mRNA in BaMR29a1 cells. We could not detect production of either IL-3 protein (by an enzyme-linked immunosorbent assay) or IL-3 mRNA (by PCR) by BaMR29a1 cells stimulated with IL-10 (not shown). Furthermore, no evidence of synergism between suboptimal concentrations of IL-3 and IL-10 was observed.

The response measured in the colorimetric assay was also reflected by an increase in cell numbers in cultures containing IL-10 (Fig. 1C to E). The increase in viable cell number was to a significant extent counterbalanced by ongoing cell death, with an apparent net doubling time of viable cells of 2 or 3 days during the culture period. This result contrasted with the doubling time of ~12 h observed for IL-3-induced proliferation (Fig. 1F), where little or no concomitant cell death occurs.

IL-10 and IL-3 induce different patterns of protein tyrosine phosphorylation in BaMR29a1 cells. BaMR29a1 cells were cultured in the absence of cytokine and serum for 5 h, washed, and then either not stimulated or stimulated with IL-10 or IL-3 for 10 min. Cytoplasmic cell lysates were analyzed by SDS-PAGE and antiphosphotyrosine Western blotting (immunoblotting). The results (Fig. 1G) show that the patterns of protein tyrosine phosphorylation induced by the two cytokines were different. IL-10 stimulation induced prominent bands at 80, 120, and 130 kDa, while IL-3 induction revealed tyrosine-phosphorylated proteins at 55, 70, 95, 120, 130, 140, and 150 kDa, as observed previously (30). These results suggested that the signal transduction pathways induced by IL-10 and IL-3 are different.

Modulation of expression of cell surface antigens by IL-10. In the presence of IL-3, BaMR29a1 and parent Ba/F3 cells grow as single cells with occasional small clusters observed (Fig. 2A). In contrast, BaMR29a1 cells grown in IL-10 form large aggregates visible within 24 h (Fig. 2B), suggesting induction of expression of new cell surface proteins by IL-10. We

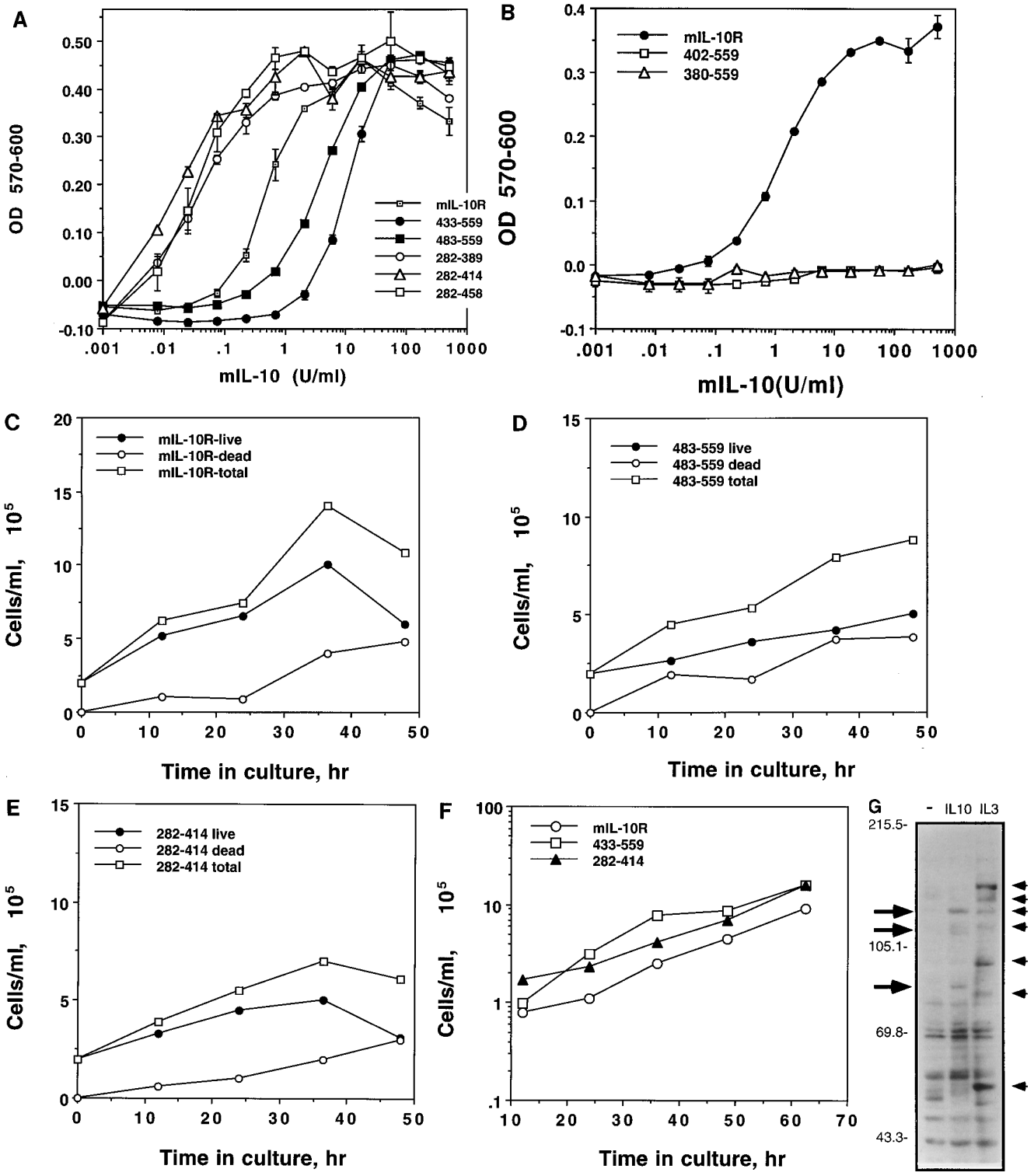


FIG. 1. (A and B) Proliferative responses of Ba/F3 cells expressing wild-type mIL-10R and cytoplasmic domain mutants (A) and wild-type mIL-10R and two deletion mutants which did not respond to mIL-10 (B). Each error bar represents the range of two samples. (C to F) Increase in cell number by Ba/F3 cells expressing mIL-10R (C) and two mIL-10R mutants in response to IL-10 (D and E) or in response to IL-3 (F). (G) protein tyrosine phosphorylation induced by IL-10 and IL-3. Specific bands induced by IL-10 (large arrows to the left of the gel) and IL-3 (small arrows to the right of the gel) are indicated. The mutant designations in the figure are shown without the delta symbols. OD 570-600, optical density at 600 nm subjected from optical density at 570 nm.

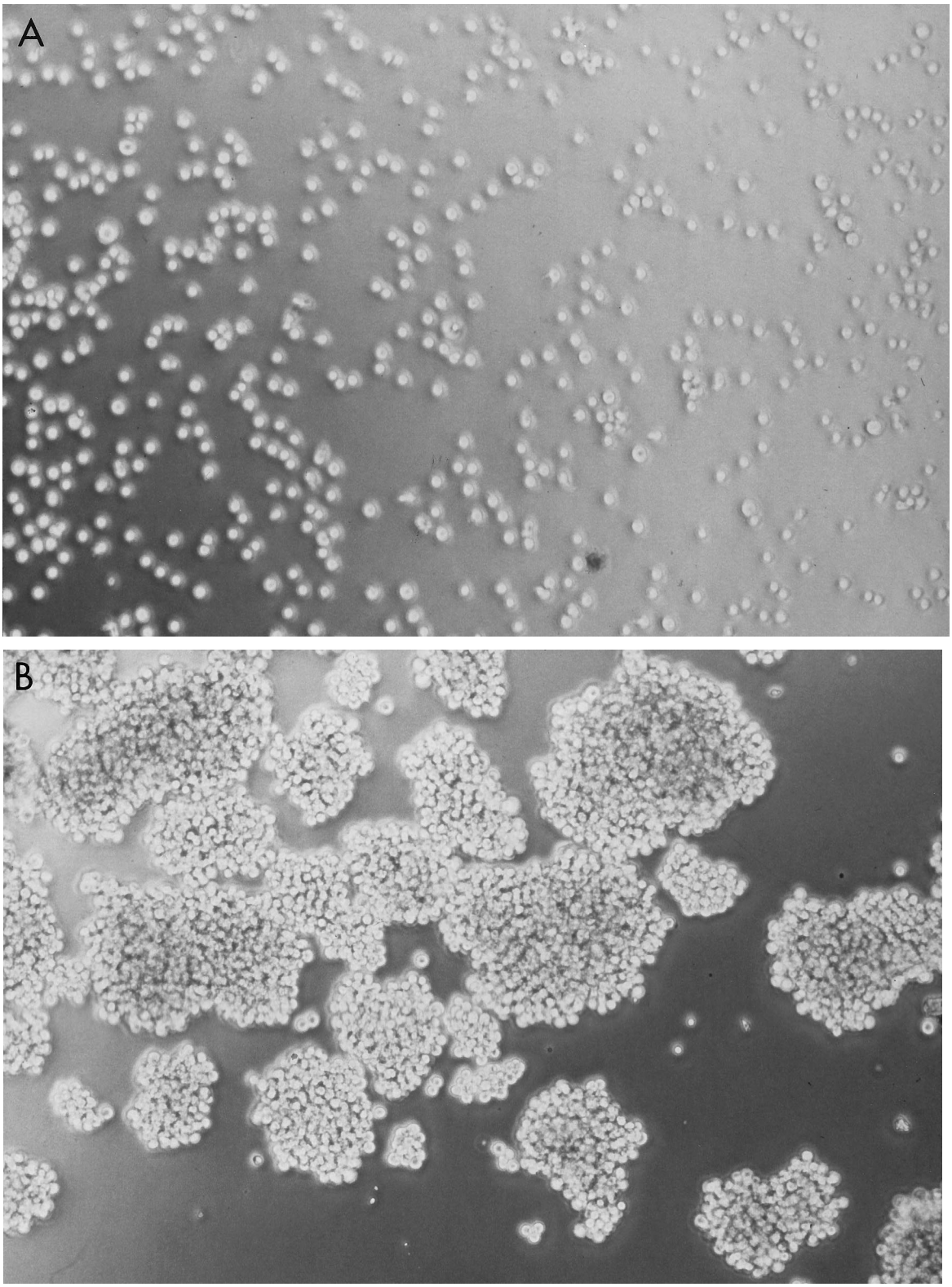


FIG. 2. BaMR29a1 cells growing in IL-3 (A) and after 48 h of culture in IL-10 (B).

tested for effects on expression of the following antigens and were unable to detect modulation of expression by IL-10: LPAM1, T200 (CD45), I-A, CD18, CD23, LFA-1, 6C3, TSA1 (Ly6 relative [21]), Ly1 (CD5), CD22, transferrin receptor, ICAM1, and CD43. Neither BaMR29a1 nor BaF3 was stained by antibodies against the following antigens: B7, CD38, CD40, IgA, and IgM. However, BaMR29a1 cells grown in IL-10 do exhibit induced or enhanced expression of CD32/16 (FcγRII/

III), CD2, LECAM-1, and heat-stable antigen (antibody M1/69; Pharmingen) compared with expression by cells grown in IL-3 (Fig. 3A to C), although inclusion in the cultures of antibodies against these four antigens, singly or in combination, did not significantly inhibit formation of aggregates in response to IL-10 (not shown). IL-10 also enhanced expression of CD32/16 by the IL-10R$^+$ macrophage cell line J774 (14) (not shown).

Addition of IL-3 to cultures containing IL-10 inhibited induction or enhancement of expression of these cell surface antigens (Fig. 3D). Similar observations were made by Fukunaga et al. (10) who reported that, in FDC-P1 cells expressing recombinant granulocyte colony-stimulating factor receptor, induction by granulocyte colony-stimulating factor of myeloperoxidase mRNA was inhibited by IL-3.

IL-10 induces tyrosine phosphorylation of the tyrosine kinases JAK1 and TYK2, but not JAK2 or JAK3. IL-10R is structurally related to interferon receptors (IFNR) and may therefore share signalling mechanisms with the IFNR family (14, 20). Like alpha interferon (IFN-α) and IFN-γ, IL-10 activates Stat1 and related molecules (18, 19, 44), including Stat3 (43, 44a). We thus examined IL-10-induced tyrosine phosphorylation of members of the Janus kinase family, which are required for signal transduction by IFNs (3, 26, 42). Figure 4 shows that stimulation of BaMR29a1 cells led to tyrosine phosphorylation of the JAK1 and TYK2 tyrosine kinases. In contrast, we could not detect phosphorylation of JAK2 or JAK3 induced by IL-10, although stimulation by IL-3 or IL-4, respectively, was effective (Fig. 4) as reported previously (23, 33). Similar results have been obtained with the mast cell line MC/9 (25a) which expresses IL-10R (14) and responds to IL-10 (41).

Proliferation signals are mediated by at least two different regions of the mIL-10R cytoplasmic domain. Mutant mIL-10R containing various deletions of the cytoplasmic domain (Fig. 5) were prepared and stably expressed in Ba/F3 cells, along with individual Y $\rightarrow$ F mutations of the four tyrosines in the cytoplasmic domain (14) (Y374F [Y at position 374 replaced with F], Y396F, Y427F, and Y477F; Y-427 and Y-477 are conserved in hIL-10R [20]). Two independent DNA clone isolates and their stable transfectant cell lines were characterized for each mutant. Ba/F3 transfectants stably expressing mutant IL-10R were tested for induction of proliferation by IL-10.

Proliferative responses mediated by mIL-10R mutants reproducibly differed in sensitivity to IL-10 as discussed below, but the magnitudes (plateau levels) of the responses were all similar, suggesting that none of the mutants was partially activating with respect to the measured activity. Responses were observed (Fig. 1A, B, D, and E) for all mutants tested except two of the C-terminal deletions, Δ380-559 and Δ402-559. These mutant mIL-10R were not detectably impaired in the ability to bind ligand, and the levels of expressed mutant IL-10R were not significantly reduced from those of BaMR29a1 (wild-type mIL-10R) cells (Fig. 6A and B).

In view of IL-10-induced proliferation observed with mutant mIL-10R Δ433-559 and Δ483-559, we inferred that the region S401-R432 (the region from S-401 to R-432) was required to mediate this response to IL-10. Further definition of regions required for this response was derived from observations that all mutant mIL-10R with deletions from the membrane-proximal end of the cytoplasmic domain (Δ282-389, Δ282-414, and Δ282-458 [Fig. 5]) were active (Fig. 1A and B). These data together implied the existence of two domains, S401-R432 and G459-E559, which mediate proliferative responses to IL-10. Furthermore, only one of these domains was required for proliferation to occur (Fig. 1 and 5). None of the Y $\rightarrow$ F mutants was detectably altered in the ability to stimulate proliferation; their response curves were virtually superimposable on that of BaMR29a1 (not shown).

Ba/F3 transfectants expressing membrane-proximal deletion mutants displayed the striking and unexpected property of sensitivity to IL-10 greater by 1.5 to 2 log units than that of wild-type mIL-10R (Fig. 1A) and were thus termed superactivating mutants. This property was not associated with increased mIL-10R number or binding affinity for IL-10, since these mutant mIL-10R exhibited ligand binding properties similar to those of both a nonsuperactivating mutant (Δ483-559) and wild-type mIL-10R (Fig. 6A and B), and only one superactivating mutant (Δ282-389) had as much as a threefold-enhanced IL-10R number compared with that of BaMR29a1 cells. Also, superactivation was not due to impaired mIL-10R internalization, since mIL-10R Δ282-389 and Δ282-458 internalized ^{125}I-hIL-10 at least as proficiently as wild-type mIL-10R and in fact better than the nonsuperactivating Δ433-559 mutant (Fig. 6C).

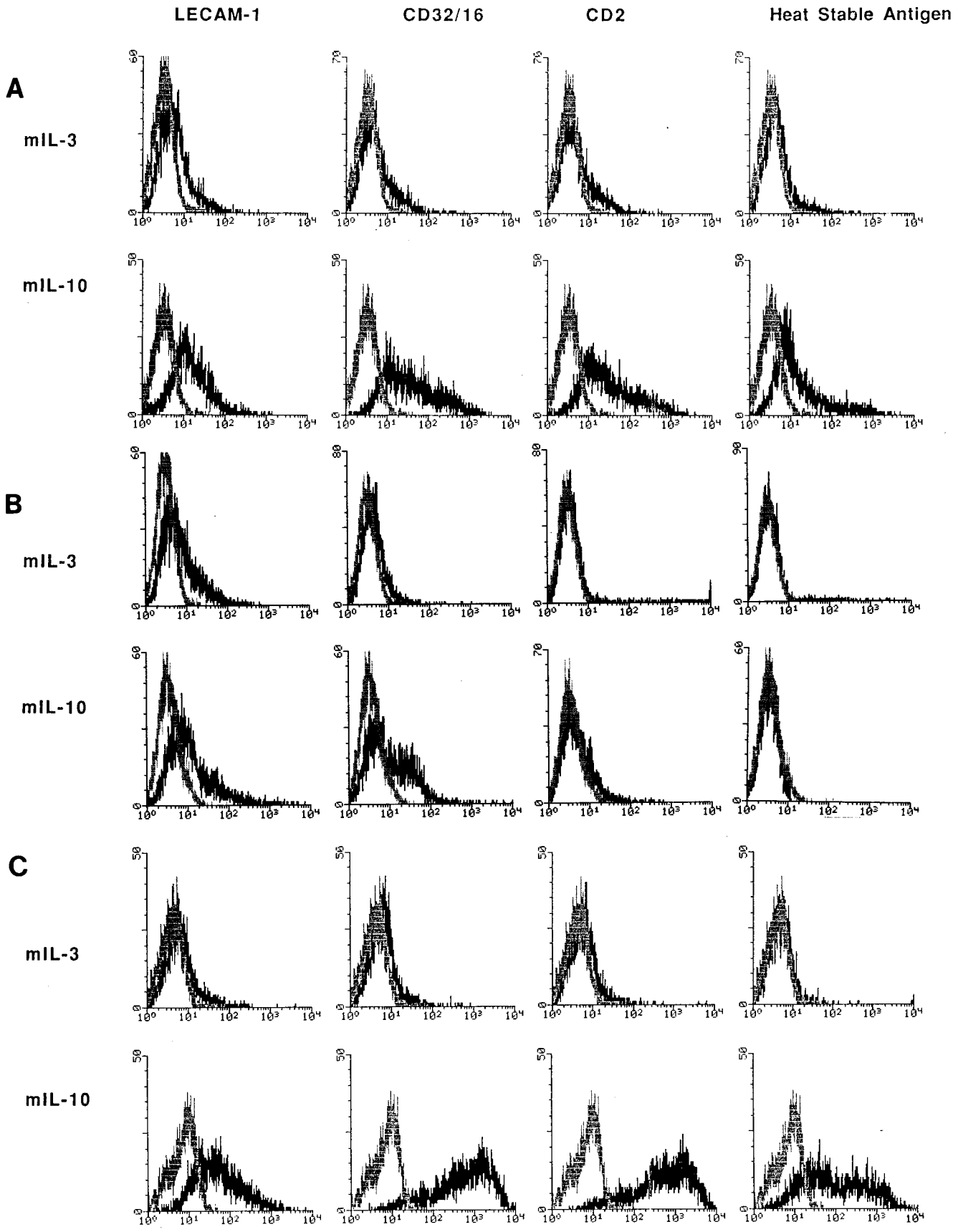
A C-terminal region of the mIL-10R cytoplasmic domain mediates modulation of cell surface antigen expression. mIL-10R mutants were examined for aggregate formation and induction or enhancement of expression of cell surface antigens in response to IL-10. All mutants except Δ483-559 (Fig. 7B), Δ433-559, Δ402-559, and Δ380-559 (not shown) formed aggregates when grown in IL-10, suggesting a requirement for the region S482-E559 in mediating this response. We also tested induction of CD32/16, CD2, LECAM-1, and heat-stable antigen by IL-10 in these cell lines and found that only Δ433-559, Δ402-559, and Δ380-559 were markedly deficient in this response (Fig. 3), also implicating the region G459-S482 in modulating expression of these molecules. Since these two regions are adjacent, we infer that a domain contained within the region G459-E559 is required for modulation or induction of cell surface antigen expression by IL-10. This functional domain overlaps one but not both of the domains involved in proliferation (see Fig. 9). Furthermore, these results suggested that one or more cell surface proteins in addition to the four examined may be responsible for mediating the aggregation induced by IL-10.

We also observed that cells expressing superactivating mutant mIL-10R identified above, especially Δ282-458, consistently formed smaller aggregates in IL-10 than those expressing wild-type mIL-10R (not shown). Furthermore, compared with wild-type mIL-10R, cells expressing any of the Y $\rightarrow$ F mutants formed aggregates which were much larger than those of the wild type and were observed earlier in culture; they also exhibited enhanced levels of CD32/16, CD2, LECAM-1, and heat-stable antigen expression (Fig. 3C). We do not presently understand the reason for these findings.

The panel of mutant IL-10R (Fig. 5) was tested for the ability to mediate IL-10-induced tyrosine phosphorylation of JAK1 and TYK2. The results (Fig. 8) indicated that, despite their different functional properties, the mutant IL-10R were all able to induce tyrosine phosphorylation of JAK1 and TYK2. In addition, we did not observe consistently detectable differences in the overall patterns of protein tyrosine phosphorylation induced by IL-10 in cells expressing IL-10R mutants (not shown) versus wild-type mIL-10R (Fig. 1G).

DISCUSSION

IL-10 induced proliferation of Ba/F3 cells expressing recombinant mIL-10R. Stimulation of proliferation appeared to be independent of IL-3, since IL-10 did not detectably induce production of IL-3 by BaMR29a1 cells. The response was associated with a measurable increase in the number of viable


LECAM-1
CD32/16
CD2
Heat Stable Antigen
A
mIL-3
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B
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C
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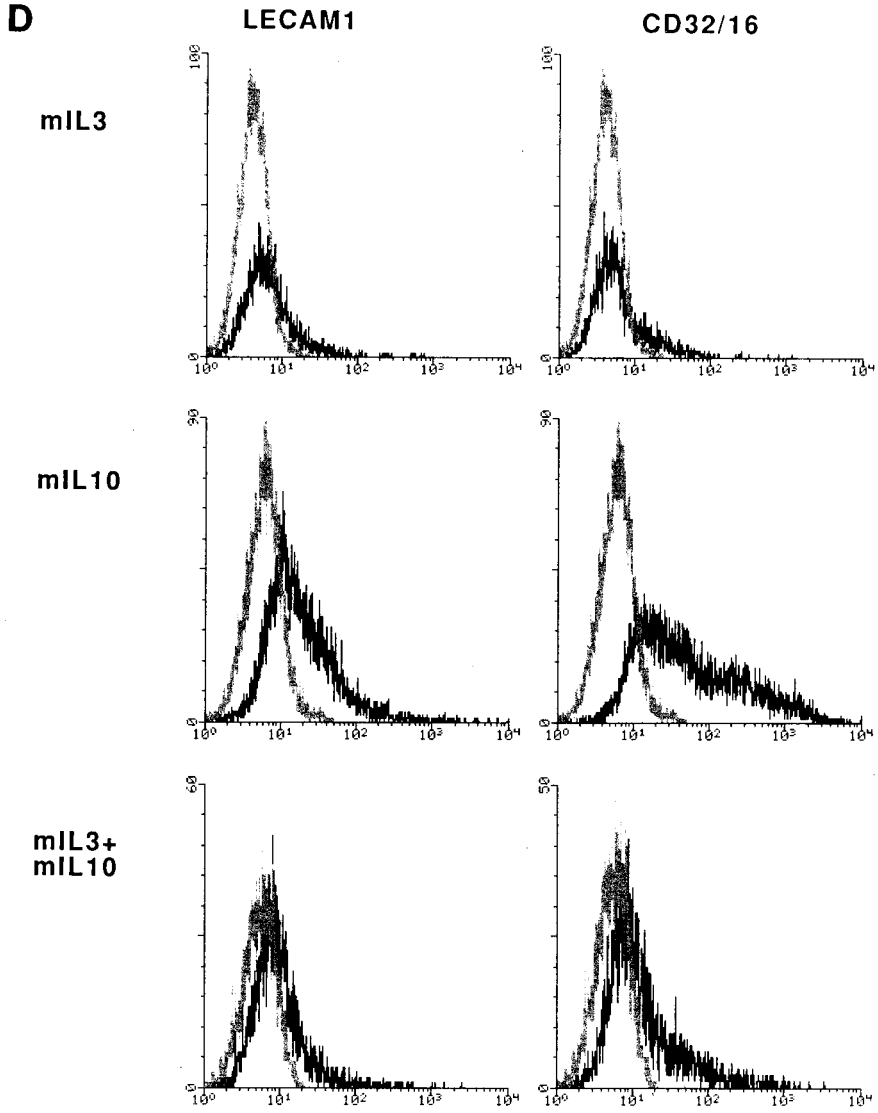

FIG. 3. Induction by IL-10 of cell surface antigen expression in cells expressing wild-type (A), Δ433-559 (B), and the Y → F mutant Y374F (C) mIL-10R. (D) IL-3 inhibits IL-10-induced expression of LECAM-1 and CD32/16 by BaMR29a1 cells.

cells in culture, although accumulation of viable cells was substantially inhibited by ongoing cell death (Fig. 1C to E), suggesting that, unlike IL-3 (30–32), IL-10 does not completely block cell death (apoptosis) which occurs in the absence of IL-3. This observation is consistent with findings by others that

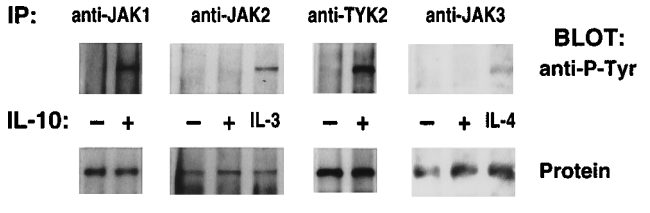

FIG. 4. IL-10 induces tyrosine phosphorylation of tyrosine kinases JAK1 and TYK2, but not JAK2 or JAK3, in BaMR29a1 cells. Immunoprecipitates (IP) using the indicated antikinase antibodies were assessed by Western blotting for tyrosine phosphorylation and protein. anti-P-Tyr, antibody against phosphorylated tyrosine.

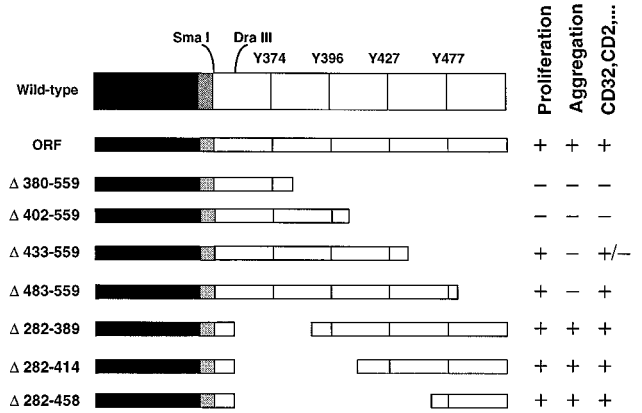

FIG. 5. Schematic diagram of cytoplasmic domain deletion mutants of mIL-10R. The abilities of each mutant to mediate proliferative or differentiation responses are indicated to the right of the diagram. The extracellular domain (black bar) and transmembrane domain (stippled bar) are indicated.

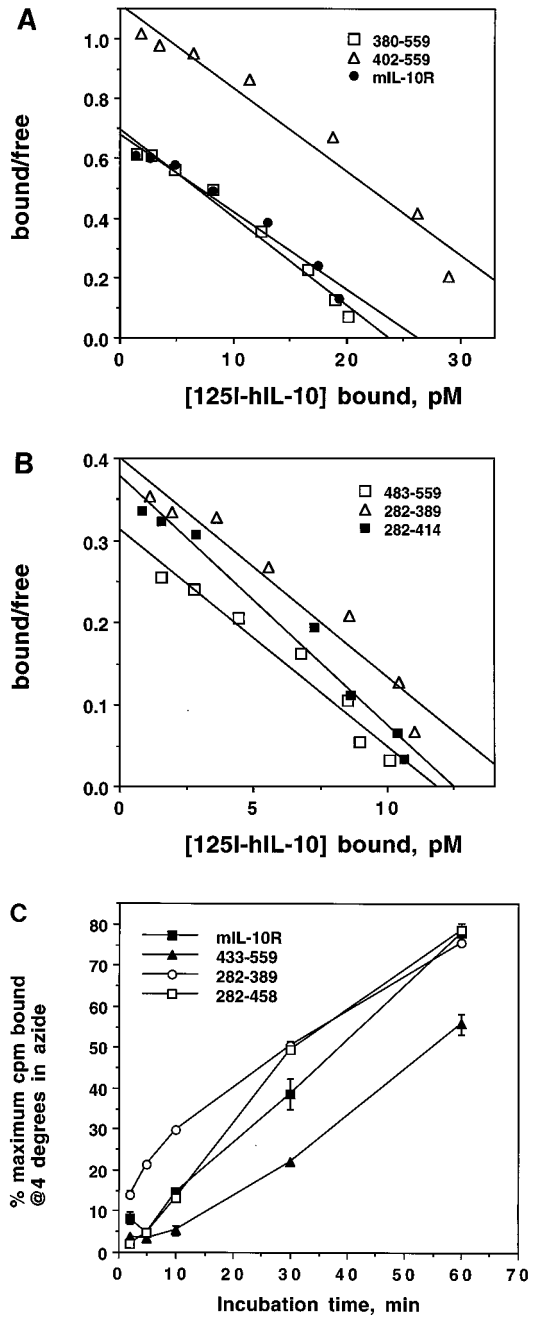

FIG. 6. Binding of ^{125}I-hIL-10 to cells expressing wild-type, Δ380-559, or Δ402-559 mIL-10R (A) or Δ282-414, Δ282-389, or Δ483-559 mIL-10R (B). The data are presented as Scatchard plots; the calculated values of K_d and IL-10R number follow: 39 pM and 6,300 IL-10R per cell for BaMR29a1, 34 pM and 5,700 IL-10R per cell for Δ380-559, 36 pM and 9,600 IL-10R per cell for Δ402-559, 38 pM and 7,200 IL-10R per cell for Δ483-559, 37 pM and 18,000 IL-10R per cell for Δ282-389, and 33 pM and 6,300 IL-10R per cell for Δ282-414. In panel A, 5×10^5 cells per sample were used; in panel B, 10^5 (Δ282-389), 2×10^5 (Δ483-559), or 2.4×10^5 (Δ282-414) cells per sample were used. (C) Internalization of ^{125}I-hIL-10 by Ba/F3 cells expressing wild-type and mutant IL-10R. ^{125}I-hIL-10 internalized after the indicated time at 37°C is depicted as a percentage of the total amount bound to the same cells at 4°C in the presence of azide in 90 min.

IL-10 does not activate the Ras/Raf/MAP kinase pathway (11, 25a). The response may therefore be regarded as short-term proliferation. Consistent with its ability to stimulate BaMR29a1 cells, IL-10 is a cofactor for proliferation of mouse mast cells (39, 41) and thymocytes (22) and is a potent growth factor for human B cells activated by anti-CD40 monoclonal antibody (29).

IL-10 also modulated expression of cell surface antigens, as evidenced by marked aggregation of cells in culture with IL-10 and induction or augmentation of expression of CD32/16, CD2, LECAM-1, and heat-stable antigen (Fig. 2). IL-10-enhanced expression of CD32/16 was also observed on the IL-10R$^+$ mouse macrophage cell line J774, and induction by IL-10 of FcγR expression by human monocytes has been reported (37). Aggregation was not due to receptor ligand-receptor-mediated cross-linking of cells because (i) the Δ433-559 and Δ483-559 mutants had substantially inferior aggregation responses (Fig. 7) but did not exhibit less IL-10 binding or lower IL-10R numbers (Fig. 6) and (ii) Ba/F3-hIL-10R transfectants aggregated very poorly in response to hIL-10 (20a).

Finally, as observed in ex vivo mouse and human monocytes/macrophages (18, 19, 43) and a macrophage cell line (43, 44), IL-10 activated the Stat1 transcription factor as well as Stat3 (25b, 43) in BaMR29a1 cells. Thus, the effects of IL-10 on BaMR29a1 cells are similar to those observed on various IL-10R$^+$ cells and cell lines.

IL-10R is structurally related to IFNR, and we found that IL-10 induced tyrosine phosphorylation of the tyrosine kinases JAK1 and TYK2, but not JAK2 or JAK3. In this respect, the pattern of JAK or TYK phosphorylation induced by IL-10 resembles more closely that induced by IFN-α, rather than IFN-γ. However, other evidence (18, 25c, 43) suggests that, unlike IFN-α, IL-10 does not activate Stat2. Thus, the IFN-α and IL-10 signalling pathways likely diverge to an extent between activation of the Janus kinases and of the Stat transcription factors, activation of the latter being thought to occur immediately downstream of JAK or TYK activation (3). The specificity determining this divergence may reside to some degree in the structures of the IL-10R and IFNR cytoplasmic domains, which could govern the arrays of Stat and related molecules that they recruit. Evidence suggesting a direct physical interaction between Stat molecules and the cytoplasmic domains of IFN-γR (13), IL-4R (16), and IL-10R (43) has been obtained.

We identified three distinct functional regions of the mIL-10R cytoplasmic domain (Fig. 9). A domain near the C terminus of mIL-10R (G459-E559) mediates both a proliferative response and modulation of cell surface antigen expression in these cells. A distinct, neighboring domain (S401-R432) can also transduce a proliferative response; only one of these two regions must be present for the response to occur. Distinct receptor domains mediating different cytokine responses have also been demonstrated for granulocyte colony-stimulating factor receptor (10), IL-3R/granulocyte-macrophage colony-stimulating factor receptor (IL-3R/GMCSFR) (28, 30, 32), and IFN-γR (6, 7, 13). Finally, we defined a third membrane-proximal domain, elimination of which resulted in a superactivating IL-10R; cells expressing these mutant IL-10R were 30- to 100-fold more sensitive to IL-10 than cells with wild-type receptor. This property of superactivation was not associated with dramatically altered ligand affinity or IL-10R number, or with detectably impaired ability to internalize ligand (in contrast to epidermal growth factor receptor [38]). We therefore suggest that this region may interact with an intracellular protein(s) which negatively regulates IL-10R activation. Possibilities could include a phosphatase or other inhibitor of JAK, TYK, or Stat activation, or alternatively one or more additional subunits of a possible IL-10R complex, as suggested earlier (14, 20). Mutations which result in qualitatively similar functional changes (superactivation) were also described for

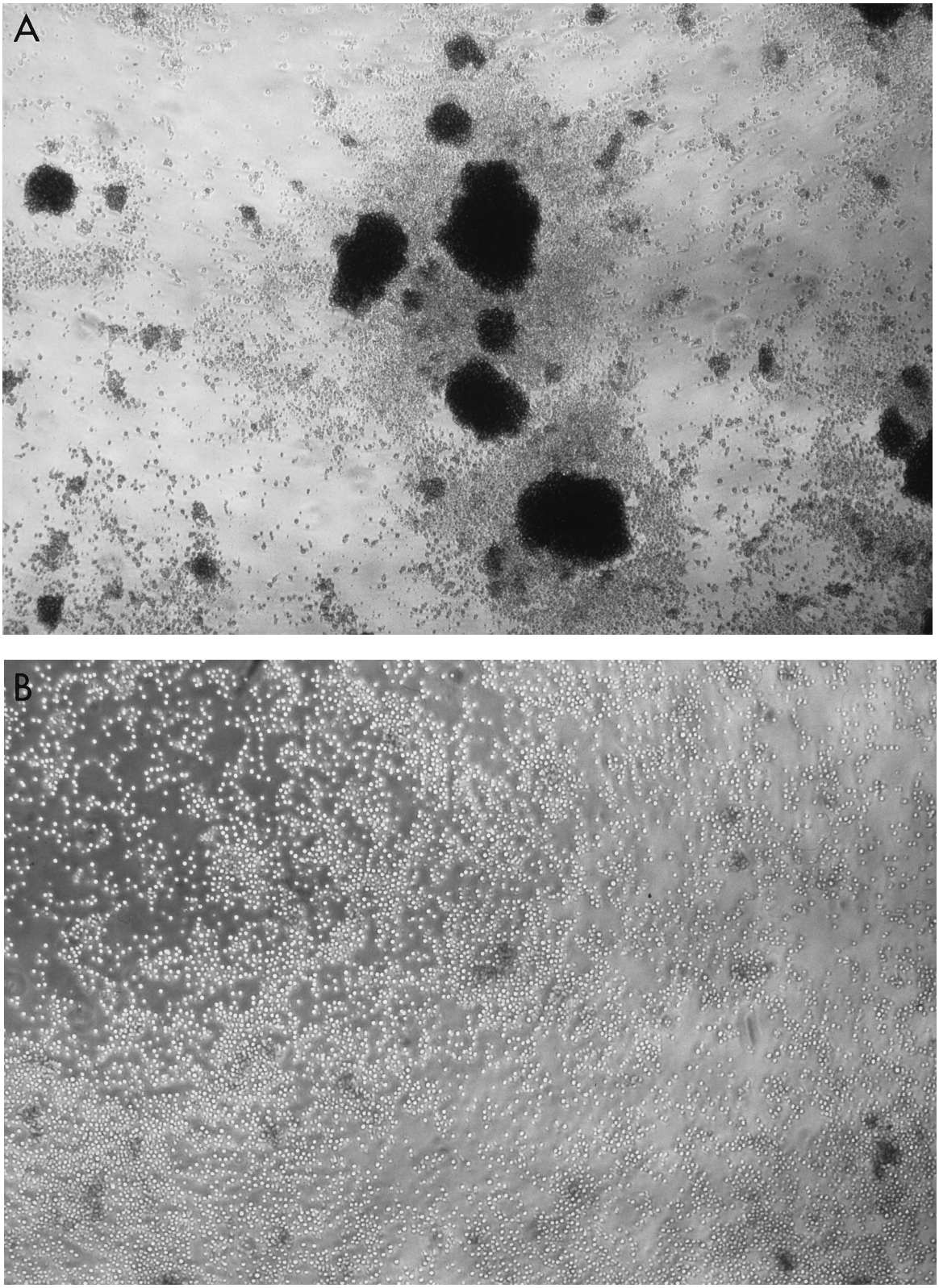

FIG. 7. Comparison of aggregates formed in response to IL-10 by cells expressing wild-type IL-10R (A) and the Δ483-559 mutant IL-10R (B).

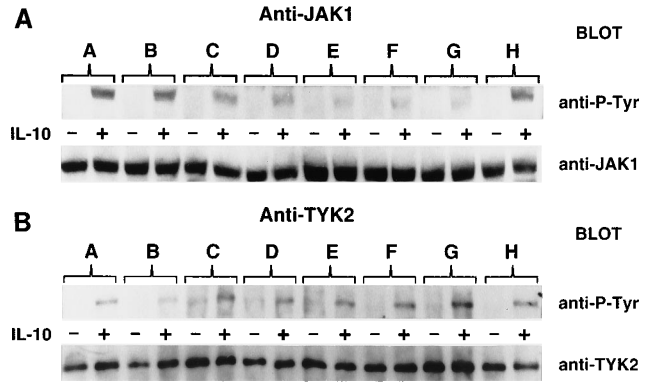

FIG. 8. Activation of JAK1 (A) and TYK2 (B) by IL-10 in cells expressing mutant IL-10R. Lanes: A, Δ380-559; B, Δ402-559; C, Δ433-559; D, Δ483-559; E, Δ282-389; F, Δ282-414; G, Δ282-458; H, mIL-10R. anti-P-Tyr, antibody against phosphorylated tyrosine.

the erythropoietin receptor (EpoR) (2) and IL-3R/GMCSFR (30, 32).

Data indicating segregation of functional regions of the cytokine receptor cytoplasmic domain were also reported for another IFNR family member, IFN-γR (6, 7, 13). These studies identified a membrane-proximal region of approximately 47 amino acids which was required for biologic responses and ligand-receptor internalization. A C-terminal region necessary for biologic responses was also identified, including a functionally critical tyrosine residue (Y-440) which was phosphorylated upon receptor activation. In mIL-10R, deletion of the region containing both Y-427 and Y-477 abolished IL-10-induced proliferation and differentiation (Fig. 1B and 5; also Results) and also appeared to inhibit activation of Stat1 and Stat3 (43). However, IL-10R mutants lacking only one of these tyrosines or containing single Y → F mutations could mediate proliferation (Fig. 1, 5, and 9) and Stat1 and Stat3 activation (43), although mutants lacking the C-terminal 76 amino acids were at least partly impaired in inducing differentiation (Fig. 7).

In contrast to these findings, none of the mIL-10R deletions abolished IL-10-induced tyrosine phosphorylation of JAK1 and TYK2. The membrane-proximal 36-amino-acid segment present in all of the mutants contains a fairly proline-rich sequence (five Pro residues between K-260 and D-276 [14]) which is somewhat similar to the so-called Box I region implicated in activation of Janus kinases by other cytokines (27, 28, 45). The IL-10R mutants described here thus separate activation of the Janus kinases and the Stat proteins (43). Further

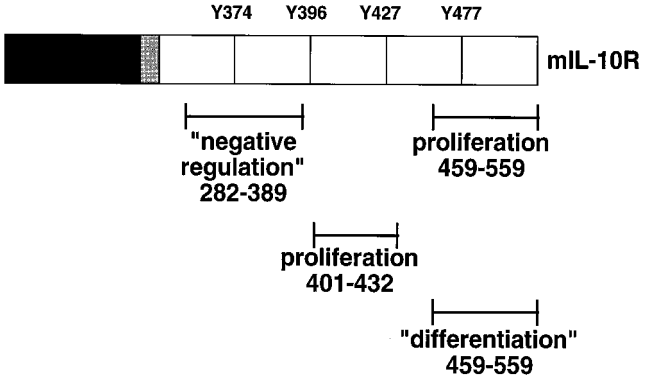

FIG. 9. Functional regions of the mIL-10R cytoplasmic domain.

studies will determine the particular roles of these signal transducers in IL-10 signal transduction.

The activities, IL-10R mutants, and cell lines characterized in this report should enable a detailed analysis of IL-10R function and signal transduction. The superactivating IL-10R cell lines currently provide the most sensitive known bioassay for mouse and human IL-10 and may facilitate detection of IL-10 in ex vivo biologic fluids. Furthermore, in view of IL-10's ability to induce apoptosis of B chronic lymphocytic leukemia cells (9), superactivating IL-10R mutants might be useful in gene transfer experiments to render B chronic lymphocytic leukemia or other cells hypersensitive to IL-10.

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A. S.-Y. Ho and S. H.-Y. Wei contributed equally to this work.

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