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Anergic T Lymphocytes Selectively Express an Integrin Regulatory Protein of the Cytohesin Family

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It has been proposed that the maintenance of T cell anergy depends on the induction of negative regulatory factors. Differential display of reverse transcribed RNA was used to identify novel genes that might mediate this function in anergic Th1 clones. We report that anergic Th1 clones do indeed express a genetic program different from that of responsive T cells. Moreover, one gene, the general receptor of phosphoinositides 1 (GRP1), was selectively induced in anergic T cells. The GRP1, located in the plasma membrane, regulated integrin-mediated adhesion and was invariably associated with unresponsiveness in multiple models of anergy. T cells expressing retrovirally transduced GRP1 exhibited normal proliferation and cytokine production. However, GRP1-transduced T cells were not stable and rapidly lost GRP1 expression. Thus, although GRP1 may not directly mediate T cell anergy, it regulates cell expansion and survival, perhaps through its integrin-associated activities. The Journal of Immunology, 2000, 164: 308–318.

Peripheral immune tolerance is an essential biological process to prevent autoaggression against self protein. Several mechanisms in the periphery ensure tolerance to self, including clonal deletion (1, 2), TCR down-modulation (3), cytokine-mediated immune suppression (4–6), ignorance of the antigen (7, 8), and clonal anergy (9, 10). The latter is a status of functional paralysis to a usually mitogenic stimulus (11). At the molecular level, the hallmark of anergy is the inability of the T cell to produce the T cell growth factor IL-2 (11). The three phases of anergy, namely induction, maintenance, and reversal, have been investigated in various in vitro as well as in vivo models (12). The majority of the in vitro data have been generated using Th1 clones. Th2 clones have been difficult to inactivate because the production of their autologous growth factor, IL-4, is not affected by a typical Th1 anergy protocol (13). In Th1 clones, anergy can be induced via two different pathways: 1) inactivating the T cell with a TCR agonist in the absence of costimulation (14, 15) and 2) triggering the TCR with an antagonistic peptide ligand on costimulation-competent APC (16). The proximal signaling events generated by the different pathways are dissimilar. A blockade or absence of costimulatory signals during T cell activation does not interfere with TCR-associated phosphorylation events (17), but affects Ras activation (18) and transcription factor induction (19). In contrast, TCR antagonism is characterized by a reduced tyrosine phosphorylation of CD3ζ and CD3ε and a lack of activation of the tyrosine kinases p56lck and ZAP-70. (17, 20, 21). Although there are differences, both signaling pathways converge at the level of IL-2 gene expression, resulting in reduced IL-2 mRNA levels and IL-2 protein production (21, 22). Impaired signaling through the IL-2R is the key event in the induction of anergy (23, 24). Thus, despite the different signaling events in the costimulation-deficient vs the TCR-antagonistic pathway, both treatments result in anergy. By comparison, a common requirement for anergy induction through either pathway is a dependence upon an intracellular calcium flux, because EGTA or cyclosporin A blocks anergy induction in either model (16, 25, 26).

In an attempt to understand how anergic T cells maintain their unresponsive state, several groups have analyzed the molecular defects in anergic Th1 clones. The results have been summarized in recent reviews (27, 28). The current models suggest that T cell anergy is a consequence of an alteration in the normal cascade of signaling events, ultimately leading to impaired IL-2 production. However, previous studies have not addressed a role for novel proteins that might be induced during the establishment of anergy and regulate cell function. We reasoned that the existence of proteins that are specifically induced in anergic T cells is likely, because anergy is long lasting and results in multiple changes in T cell function (27, 28). Genes associated with the anergic state could be important in the maintenance of anergy. Alternatively, these cells may express proteins that regulate cell survival or migration, because anergic T cells persist for weeks in vivo or in vitro (29–31), but are unable to home toward germinal centers (32). Thus, we employed the differential display technique (33) to compare the gene expression in anergic vs responsive murine Th1 clones and to identify novel molecules that might be crucial to the function of these cells.

In this report we describe the identification and characterization of a gene that is specifically induced in anergic T cells, namely...
general receptor of phosphoinositides 1 (GRP1) (34), a recently published nucleotide exchange factor for the small G protein ADP-ribosylation factor-1 (ARF1) (35). We demonstrate that GRP1 localizes to the plasma membrane in lymphoid cells and regulates LFA-1/ICAM-1-mediated adhesion. Finally, we show that T cells expressing a retrovirally transduced GRP1 construct proliferate normally and display no altered cytokine production, but are not stable and rapidly lose GRP1 expression.

Materials and Methods

Mice

DO11.10 mice, transgenic for an OVA323–339-specific αβ-TCR, were a gift from Drs. D. Loh and K. Murphy (Washington University, St. Louis, MO) (36) and maintained at the age of 6–8 wk from the National Cancer Institute, Frederick Cancer Research and Developmental Center (Frederick, MD). The animals were bred and maintained in a specific pathogen-free barrier facility.

Medium and Abs

All murine cells were maintained and stimulated in complete DMEM containing 5% FCS. Human cell lines were maintained in supplemented RPMI 1640 containing 10% FCS. The hybridoma secreting anti-MHC class I (H-2Ld) mAb, 30–5–75, was obtained from the American Type Culture Collection (ATCC, Manassas, VA). 145-2C11, a hamster anti-mouse CD3ε mAb (37), was purified on protein A-Sepharose from hybridoma supernatant and dialyzed in PBS. The anti-CD3-IgG3 is a chimeric Ab combining the Ag specificity of 145-2C11 with a murine IgG3 Fc portion with weak affinity for murine Fc receptors (38). The mAb was used as an ascites. A rabbit anti-GRP1 reactive polyclonal antiserum was generated by HTI BioProducts (Ramona, CA) using recombinant GRP1 obtained by PreScission Protease (Pharmacia, Uppsala, Sweden)-mediated cleavage of a GST-GRP1 fusion protein harvested from transformed Escherichia coli. The purified hyperimmune rabbit Ab were purified on protein A-Sepharose and used for Western blotting. A purified rabbit anti-actin mAb was used by Sigma (St. Louis, MO). Blocking anti-CD11a and anti-CD18 Abs (MEM-30 and MEM-48) (39) were gifts from Dr. V. Horejsi (Institute of Molecular Genetics, Prague, Czech Republic). The anti-IL-4 Ab-secretion was obtained from the American Type Culture Collection (Frederick, MD). The animals were bred and maintained at the age of 6–8 wk from the National Cancer Institute, Frederick Cancer Research and Developmental Center (Frederick, MD). The animals were bred and maintained in a specific pathogen-free barrier facility.

Cells

The OVA-reactive murine Th1 clones pGL2 and pGL10 and the Th2 clones pl3 and pl104 have been described previously (41, 42). The CD8+CD4+ T cell line, 3.7.2, was provided by P. Fields (University of Chicago, Chicago, IL). The 3.7.2 cell line was generated by multiple restimulation of lymph node T cells from 2C TCR transgenic mice (43) as described previously (44). The CD8+ T cells were found by ELISA to secrete IFN-γ upon activation, but not IL-4 (data not shown).

The OVA-reactive clones were maintained as follows. T cells (3 x 10⁶) were expanded on 10⁴ irradiated (20 Gy) E-depleted splenocytes from DBA/2 mice in the presence of 0.2 mg/ml OVA (Sigma) and 12.5 U/ml recombinant human IL-2 (a gift from Cetus, San Francisco, CA). The 3.7.2 cells were expanded on 10⁴ irradiated (20 Gy) E-depleted splenocytes from DBA/2 mice. The pigeon cytotoxic c-reactive murine Th1 clone, A.E7, was expanded as previously described (45). The human cell lines HeLa (adenocarcinoma), 1309L (monocytes), Jurkat E6 (T lymphoma), and U266BL (EBV-transformed B myeloma), and the retroviral packaging cell line Phoenix-ecco (http://www.stanford.edu/group/nolan/NL-Phoenix.html) were obtained from the ATCC.

Anergy models

To induce anergy with the anti-CD3 mAb, T cell clones were cultured for 18–24 h in plates coated with 10 μg/ml 145-2C11, except for A.E7, which was anergized on plates coated with 1 μg/ml. After this induction period, cells were transferred into uncoated plates and incubated for a variable rest period. Control cells were incubated in uncoated plates for the same total amount of time or were activated according to the maintenance protocol. As a separate approach, T cell anergy was induced on chemically modified splenocytes as previously published (30). Briefly, irradiated (30 Gy) E-depleted B10.A splenocytes were treated with the chemical cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) (Sigma), washed intensively, and used to anergize A.E7 cells in the presence of 1 μM peptide Ag DASP (NH₂-KKANELLYALKQATK-COOH, a gift from Dr. Marc Jenkins, University of Minnesota, Minneapolis, MN). The APC to A.E7 ratio was 7:5:1. As a control, A.E7 cells were activated on irradiated splenocytes that had not been treated with EDCI in the presence of the DASP Ag. Twenty hours later, A.E7 cells were separated from the APC by density gradient centrifugation on Ficoll-Hypaque and rested for another day before being lysed for RNA or protein analyses.

In the third model, T cell anergy was induced on 1-A²-transfected EL-4 cells (ELAD) as described previously (46). Briefly, pGL10 cells were incubated with mitomycin C (Sigma)-treated ELAD cells in a 1:1 cell ratio in the presence of 0.1 μg/ml OVA323–339 (University of Chicago Cancer Research Center Oligopeptide Synthesis Facility). The pGL10 cells were separated from the dead APC after 20–24 h and rested for an additional 24 h. As a control, pGL10 was activated on ELAD cells that coexpressed the costimulatory ligand B7-1 (ELAD-B7) in the presence of OVA peptide. The expression of the transfected molecules on the cell surface of the APCs was verified by flow cytometry.

Finally, anergy was induced with the non-cross-linking, nonmitogenic anti-CD3-IgG3 in Th1 clones by incubating the cells for 20 h with 1 μg/ml of this mAb. The T cells were washed three times and incubated in growth medium until harvested for cell lysis or restimulation (47).

T clone proliferation assay to verify anergy induction

Cells inactivated by the various methods were evaluated in a restimulation assay on days 4–7 as follows. Rested anergic or control OVA-specific T clones (2–5 x 10⁴) were restimulated in 96-well microtiter plates with 2–5 x 10⁵ E- and T-depleted irradiated (20 Gy) splenocytes from DBA/2 mice in the presence of various amounts of OVA. A.E7 was stimulated at 2-5 x 10⁵ T cells/well with 2-5 x 10⁵ E- and T-depleted irradiated (30 Gy) splenocytes from B10.A mice in the presence of various amounts of pigeon cytocrome c (Sigma). Exogenous IL-2 at 20 U/ml was added to selected wells to examine whether the T cells were viable and responsive to growth factor. Thirty-seven kilobecquerels of [3H]-thymidine (Amersham, Arlington Heights, IL) was added to each well for the final 8–14 h of a 60- to 72-h restimulation. Samples were examined in triplicate and harvested, and the emission of beta irradiation was determined using the Packard TopCount System and the MicroScint 0 scintillation solution (Packard Instruments, Meriden, CT). The counts per minute reported is the mean of the triplicate samples. For most experiments described, the restimulation cuvette contained anergic or resting cells in the absence of 1-A²-transfected EL-4 cells and rested for 24 h before the system using ELAD transfectants, which resulted in a 3- to 10-fold reduction in proliferative activity (data not shown).

RNA isolation and Northern blot analysis

Total RNA was isolated from live cells by applying the cell lysate to a cesium chloride gradient ultracentrifugation (48, 49) or using Trizol (Life Technologies, Gaithersburg, MD) according to the manufacturer’s recommendation. An additional final ethanol precipitation step was included. Tissues were derived from a B10.A mouse and lysed in Trizol. Five micrograms of total RNA was loaded onto a 1.2% agarose/1% formaldehyde gel along with a RNA size marker (Life Technologies). Northern blotting, hybridization with 32P-labeled cDNA, and stripping of blots were performed as described previously (50). The cDNA probe for GRP1 detection was a 305-bp GRP1-specific sequence from the 3′-untranslated region obtained after a digest with BamH1 and XhoI. This probe did not cross-hybridize with mRNAs representing other GRP1 family members (data not shown). Equal loading and transfer of RNA were verified by UV illumination of the ethidium bromide-stained 28S and 18S ribosomal RNA on the blot. Some autoradiograms (Kodak X-OMAT, Eastman Kodak, Rochester, NY) were quantitatively evaluated using the Computing Densitometer 12SE and Imagequant software (Molecular Dynamics, Sunnyvale, CA). Images of ethidium bromide-stained 18S ribosomal RNA on the Northern blot were quantitated using a UMAX S-12 desktop scanner (Hsinchu, Taiwan) and 1D Image Analysis Software (version 2.0.2, Kodak).

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3 Abbreviations used in this paper: GRP1, general receptor of phosphoinositides 1; AICD, activation-induced cell death; ARF1, ADP-ribosylation factor-1; ARNO, ARF nucleotide binding site opener; DDRT-PCR, differential display of reverse transcribed RNA by PCR; ELAD, 1-A²-transfected EL-4 cells; EDCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; eGFP, enhanced green fluorescence protein.
Cloning of full-length murine GRP1

The DDRT-PCR reamplificates were cloned into the pBluescript II SK" phagemid (Stratagene, La Jolla, CA), and plasmid DNA from several independent bacterial colonies was sequenced as well as used to probe Northern blots in an effort to verify the anergy association. At the time of GRP1 cloning, there was no match in the nucleotide databases; thus, the GRP1 DDRT-PCR product was used to screen a NIH-3T3 cDNA library (AZAP II, Stratagene), and two independent plagues were isolated and converted into the phagemid state. Nested deletions of the cDNA clones were sequenced, and gene-specific nested primers were designed to obtain missing 5' sequence information by RACE from NIH-3T3 poly(A)+ RNA with the Marathon cDNA amplification kit and the long distance, high fidelity KlenTaq (Clontech, Palo Alto, CA) according to the manufacturer's recommendation. A full-length cDNA clone was assembled from the largest RACE clone obtained and was sequenced with overlapping primers. Additional RACE clones were obtained and sequenced to verify the coding sequence. The complete gene sequence including the previously unpublished 3'-untranslated region is available from GenBank under accession number AF084221.

Chromosomal localization of GRP1

Fluorescence in situ chromosomal hybridization to human PHA-stimulated peripheral blood lymphocytes arrested in metaphase was performed as described previously (52). The bioin-labeled GRP1 probe was a human full-length cDNA clone in the pcDNA I/Amp vector (Invitrogen, Carlsbad, CA). This clone was isolated and provided by Dr. Donald Stauton (ICOS, Bothel, WA). Its coding sequence is identical with the GenBank entry for ARNQ5, accession number AJ223957. Chromosomes were identified by staining with 4,6-diamidino-2-phenylindole dihydrochloride. Images were obtained using a Zeiss Axiohot microscope (New York, NY) coupled to a cooled charge-coupled device camera. Separate images of 4,6-diamidino-2-phenylindole dihydrochloride-stained chromosomes and the hybridization signal were merged using image analysis software (NU200 and Image 1.57).

For murine chromosome localization GRP1 was mapped by analysis of the progeny of the genetic cross: NFS/nu or C57BL/6J transgenic for GRP1 on a C57BL/6J background. The progeny of these crosses were typed for >1200 makers distributed on 19 autosomes and the X chromosome. Recombination was calculated according to the method of Green (54), and genes were ordered by minimizing the number of recombinants. The GRP1 probe was a 305-bp GRP1 3'-untranslated region obtained after a digest with BamHI and XbaI.

The SDS-PAGE and Western analysis

Cells were lysed in a 0.5% Triton X-100 (Sigma) containing reducing lysis buffer, and 40–70 μg of the total lysate protein, as determined with the Bio-Rad DC kit (Bio-Rad, Hercules, CA), was subjected to SDS-PAGE and subsequently transferred to polyvinylidene fluoride membrane (51). Ponceau staining was performed to control for uniform protein loading. The blots were immunostained with the rabbit anti-GRP1 antisera and developed with an anti-rabbit HRP-coupled antiserum and the ECL chemiluminescence substrate (Amersham). In some experiments, the blots were reprobed with anti-actin Abs after stripping in a reducing SDS buffer to control for uniform protein loading. Some exposures were quantitated using the Computing Densitrometer 325E and the ImageQuant Software (Molecular Dynamics).

Recombinant vaccinia virus

The GRP1, ARNO, or cytohesin-1 CDNA was cloned into the pcglTGK vaccinia virus expression vector (55). Infection with the recombinant virus directed the expression of chimeric proteins with amino-terminal Ig domains in the infected cells.

Immunofluorescence

HeLa, U937, Jurkat E6, and U266 cells were infected with recombinant vaccinia virus. Eight hours postinfection cells, were placed on poly-L-lysine-covered microscope slides for 1 h in a humidified chamber at 37°C and processed as described previously (56), except that a 1% (v/v) paraformaldehyde in PBS solution was used.

Cells were examined using a confocal laser scanning microscope (Leica TCS-NT system, Leica) attached to a Leica DM IRB inverted microscope with a PLAPO 63 × 1.32 oil immersion objective. Confocal images were collected as 512 × 512 pixel files and processed with the help of the Photoshop program (Adobe).

Adhesion assay

Jurkat E6 cells were infected with recombinant vaccinia virus. Five to six hours after infection, cells were labeled with 12 μg/ml bisbenzimide H33342 fluorochrome trihydrochloride (Calbiochem, La Jolla, CA) for 30 min at 37°C, collected by centrifugation, resuspended in HBSS, and delivered to maxisorp 96-well plates (NUNC, Roskilde, Denmark) at 1.5 × 10^5/well. Before adhesion, plates were coated with goat anti-human IgG Ab (Fcγ specific) at 0.85 μg/well for 90 min at 25°C, blocked with 1% (v/v) BSA/PBS, incubated with culture supernatants from COS cells secreting ICAM-1 receptor-globulin fusion protein (55), and subsequently used in the assay. Where indicated, cells were incubated with 40 nM PA 0.5 h before the adhesion assay. To determine whether the binding was LFA-1 specific, replicate samples were set up in the presence of the blocking anti-LFA-1 Abs MEM-30 and MEM-48 (39). Cells were then allowed to adhere for 1 h at 37°C, and unbound cells were carefully washed off three times with 300 μl of HBSS. Bound cells were assayed in 100 μl 2% (v/v) formaldehyde/PBS using a fluorescence plate reader (Cytofluor II, PerSeptive Diagnostics, Cambridge, MA). The signal of 1.5 × 10^7 cells/ well at 490 nm corresponds to 100% adhesion. Each value is the mean of two determinations conducted in triplicate.

Retroviral transduction of lymph node cells

The murine viral vector pLXIN (Clontech, Palo Alto, CA), was modified to yield pLXIE by replacing the polyomavirus resistance gene with the gene encoding enhanced green fluorescence protein (eGFP). Into the EcoRI site of this resulting vector, the 5'- untranslated region and the coding region of GRP1 were cloned to yield pLGLIE, which allowed expression of both GRP1 and eGFP from a single, bicistronic transcript in transduced cells. To produce retrovirus-containing supernatant, Phoenix-eco packaging cells were transfected with pLXIE or the control vector pLXIE using the CaPO4 transfection method (51). The supernatant was filtered through a 0.2-μm pore size, syringe filter to separate the virus from contaminating Phoenix-eco cells.

T cells from pooled inguinal, brachial, axillary, superficial cervical, and mesenteric lymph node cells of a DO11.10 mouse were activated on OVA-pulsed, irradiated (20 Gy) isogenic spleen feeder cells. The T cells were cultured normally to avoid skewing toward a particular pathway, purposefully skewed toward Th1 development using 2 ng/ml recombinant murine IL-12 (a gift from David Pesky, Hoffmann-La Roche, Nutley, NJ) and 10 μg/ml anti-IL-4 mAb, 1B11, or purposefully skewed toward Th2 development using recombinant murine IL-4 (BioSource International, Camarillo, CA) and 10 μg/ml of anti-IL-12 p40 mAb. Three days later, the activated T cells were infected with retrovirus-containing supernatant in 15 μg/ml polybrene and cultured for another 4 days in medium with IL-2. On day 7, live cells were recovered upon centrifugation on Ficoll/mitomizone, stained with sCD4-PE, and sorted for eGFP and CD4 expression using a FACStarplus (Becton Dickinson, San Jose, CA). For assessment of the population’s stability, the sorted cells were restimulated under skewing conditions and sorted again 7 days later. These cells were maintained thereafter by weekly stimulation on OVA-pulsed spleen cells of the same specificity providing the exogenous IL-2 (10 U/ml). The stability of eGFP expression over time was examined 7 days after each stimulation by flow cytometry, gating on propidium iodide-negative cells.
Proliferation assay and cytokine ELISA

To examine proliferation and cytokine production, 10,000 retrovirally transduced and sorted cells were restimulated on irradiated splenic feeder cells in the presence of OVA peptide. Cytokine production was measured 23 h later from culture supernatant by ELISA (Endogen, Woburn, MA). [3 H]TdR incorporation was analyzed on day 3.

Results

Anergic Th1 clones express a genetic program different from a responsive control: identification of GRP1

We were interested in differences in the genetic program expressed in anergic vs responsive Th1 clones, reasoning that anergic Th1 clones might express a special gene program to maintain their unresponsiveness over the weeks that anergy has been shown to persist (31). Thus, two murine Th1 clones, pGL2 and pGL10, were exposed to immobilized anti-CD3 mAb to induce anergy or were treated as indicated in Fig. 1 to yield a population that was responsive to a subsequent mitogenic stimulus. Two Th1 clones were used to minimize the chance of isolating clone-specific artifacts. The anti-CD3 protocol successfully induced functional unresponsiveness in both Th1 clones, while the control cells remained responsive to restimulation on day 7 (Fig. 1A). As an additional control, anti-CD3-treated pGL2 cells were cultured in the presence of exogenous IL-2 to prevent anergy induction. As shown in Fig. 1A (C), this treatment resulted in a partial restoration of cell function, which is in agreement with previously published results (23). The RNA from all five anergic and control samples was subjected to DDRT-PCR analysis (33). One thousand and two hundred different PCR reactions, using 12 different poly(T)-anchored primers and 20 different arbitrary primers, were analyzed. Fig. 1B depicts a typical result obtained with two different primer combinations. PCR products differing in expression level between the two anergic and three control samples were isolated, reamplified, cloned, and sequenced. Among the 64 PCR isolates with different expression in anergic vs control cells, 26 were selectively present in control cells, while 38 were selectively expressed in anergic cells. Further analyses revealed that most of the PCR products were either false positives or represented mRNAs that could not be detected using the less sensitive Northern blot analysis. One PCR product, however, detected a mRNA species in Northern blots that was not present in control cells but was induced in anergic Th1 clones (asterisk in Fig. 1B; see Figs. 2 and 3). The sequence of the PCR product that represented only the very 3′-untranslated region did not match any database entry. Its corresponding mRNA was 3.5–4 kb as judged by Northern blot analysis (data not shown). The full-length cDNA was subsequently isolated and cloned as described in Materials and Methods.

Except for one discrepant nucleotide that did not affect the predicted protein sequence, the coding sequence of the cDNA sequence obtained was identical with that of GRP1 (34). GRP1 is a...
member of a gene family that displays several functions, such as regulation of integrin avidity (55) and guanine nucleotide exchange activity on ARF family G proteins (35, 57–59). These diverse functions have led to a complicated nomenclature within the family. Other members are known as cytohesin-1 (55) and ARF nucleotide binding site opener (ARNO) (59). The deduced protein structure of all family members, including GRP1, is comprised of an amino-terminal coiled coil domain that may promote dimerization (59), followed by a catalytically active SEC7 homology domain (35) and a pleckstrin homology domain. The latter has been demonstrated to facilitate binding to phosphoinositide 3,4,5-trisphosphate and hence membrane targeting of the protein to sites of activation (34). The carboxyl terminus of the GRP1 family is rich in basic amino acids, and it has recently been shown for cytohesin-1 that this polybasic c domain cooperates with the pleckstrin homology domain (60). Analysis of GRP1 protein by in vitro translation and overexpression of GRP1 from our cloned cDNA in a murine T cell hybridoma determined that the protein was 46 kDa, which is in accordance with the predicted Mr of 46.3 kDa (data not shown). The GRP1 mRNA was expressed in various mouse tissues, notably in heart and lung, whereas it was absent from a liver sample (Fig. 1C). A similar expression pattern was observed in nonlymphoid tissues derived from a recombinase-activating gene-2-deficient mouse. These results suggest that GRP1 expression observed in the lung was due not to infiltrating lymphocytes but, most likely, to tissue-specific fibroblasts, because various fibroblast lines were found to express GRP1 at high levels constitutively (data not shown).

*FIGURE 2.* Kinetics of GRP1 mRNA and protein induction in anergized Th1 clones. A.E7 or pGL2 cells were anergized on plate-bound anti-CD3 mAb 145-2C11. Aliquots were lysed at the time points indicated (in hours) and subjected to Northern (A and B) or western (C and D) blot analyses. The ethidium bromide staining pattern of the 18S ribosomal RNA band on the blot is presented as a control for the Northern blot. The Western blot was sequentially probed with the antisera against GRP1 and actin. Arrows in C indicate the position of the 46-kDa marker. In some repeats of this experiment control cells were exposed to plates coated with the anti-H2 class I mAb 30-5-7S. No induction of GRP1 was observed under these conditions (data not shown). GRP1 expression levels shown in this experiment control cells were exposed to plates coated with the anti-H2 class I mAb 30-5-7S. No induction of GRP1 was observed under these conditions (data not shown). GRP1 expression levels shown in this experiment control cells were exposed to plates coated with the anti-H2 class I mAb 30-5-7S. No induction of GRP1 was observed under these conditions (data not shown). GRP1 expression levels shown in this experiment control cells were exposed to plates coated with the anti-H2 class I mAb 30-5-7S. No induction of GRP1 was observed under these conditions (data not shown). GRP1 expression levels shown in this experiment control cells were exposed to plates coated with the anti-H2 class I mAb 30-5-7S. No induction of GRP1 was observed under these conditions (data not shown).
The GRP1 is a gene induced in anergic T cells

We identified GRP1 with the DDRT-PCR in an anergy model based on a single time point following clonal Th1 inactivation using a mAb against CD3ε in the absence of costimulation (Fig. 1B). To determine the time course of GRP1 mRNA and protein expression, a temporal analysis of GRP1 using the anti-CD3 model of anergy was performed. The results obtained by Northern and Western blotting confirmed that GRP1 was selectively induced in anergic Th1 clones (Fig. 2). The GRP1 mRNA was barely detectable, and protein was undetectable in resting Th1 clones before stimulation, and no significant induction of mRNA or protein occurred during the first 12 h of anti-CD3 exposure. Under continued exposure to anergizing conditions, maximal expression of GRP1 mRNA was observed at ~48 h, and although the signal declined, GRP1 mRNA was still expressed at 5-fold over background levels at 7 days (Fig. 2, A and B). The GRP1 protein levels also reached a maximum around 48 h and continued to be expressed at near maximum levels (91%) at 7 days (Fig. 2, C and D). Thus, GRP1 protein was expressed at a high level concurrent with the functional state of anergy. Although the polyclonal anti-GRP1 serum used in our Western blots reacted with multiple family members, the signal detected in the T cell clones represents GRP1, because 1) no protein was detected in nonanergized Th1 clones, although mRNA for multiple family members was observed (Fig. 2 and data not shown); and 2) Northern blots hybridized with a GRP1 probe that detected four mRNA species revealed that only the 3.5- to 4-kb signal, representing GRP1, is up-regulated during anergy induction. Thus, GRP1 seems to be the only member of the family that is significantly induced upon clonal inactivation.

To verify the association between GRP1 and anergy using alternate costimulation-dependent anergy models, we analyzed GRP1 mRNA expression in A.E7 cells anergized on chemically modified APC (30). This modification is believed to leave Ag presentation by the APC intact, but to destroy its costimulation capacity. The restimulation proliferation assay confirmed that anergy was successfully induced in the cells exposed to the chemically modified APC (data not shown). The GRP1 mRNA was induced at 48 h in A.E7 cells stimulated with chemically modified APC (Fig. 3A, sample E) and remained elevated at 7 days, whereas cells stimulated with untreated, peptide-pulsed APC (sample U) did not express GRP1 mRNA above background levels (sample O) at the 7 day point. Thus, GRP1 was induced and persisted specifically in the T cells anergized by two distinct approaches.

Because the costimulation pathways affected by the chemical modification of the APC are ill defined, a third approach was employed that directly examined GRP1 expression in a B7-deficient T cell anergy model. Murine EL-4 thymoma cells transfected with I-A^d (ELAD cells) in the absence or the presence of cotransfected B7-1 (ELAD-B7) (46) were used as APC. Culturing pGL10 cells in the presence of chicken OVA peptide-loaded ELAD cells induced anergy in the Th1 clone, whereas exposure to Ag-loaded ELAD-B7 did not alter T cell responsiveness (data not shown). Once again, GRP1 mRNA and protein were strongly induced at 48 h following stimulation with ELAD cells, but were barely detectable in pGL10 cells stimulated with ELAD-B7 (Fig. 3B).

Unique induction of GRP1 in Th1 and Tc1 cells

The results described above were limited to analyses of murine Th1 clones. Th2 clones have been reported to be resistant to the induction of anergy (13). Therefore, GRP1 expression was examined in anergy-resistant Th2 clones stimulated with anti-CD3 under the anergizing conditions used for the Th1 studies. In accordace with previous reports, the Th2 clones failed to become anergized upon anti-CD3 treatment (Fig. 4B). Furthermore, GRP1 was not induced in the Th2 clones, in contrast to the Th1 clones that clearly up-regulated GRP1 protein 48 h after the CD3 stimulus (Fig. 4A). The Th2 clones did, however, express low amounts of GRP1 protein constitutively. Similar anergy studies were performed with the 3.7.2 cytolitic CD8^+ T cell clone, which secreted IFN-γ upon mitogenic activation (Tc1). These cells could be anergized with the anti-CD3 Ab (Fig. 4B). When anergic and control 3.7.2 cells were analyzed for GRP1 protein expression, a significant increase above basal levels of GRP1 expression in the anergic cells was observed (Fig. 4A). Thus, the up-regulation of GRP1 correlated with the capacity of T cells to become anergized.

T cell anergy induced by a TCR antagonist induces GRP1

It is clear from the previous studies that GRP1 was induced in T cells anergized by methods that fail to provide T cell costimulation. However, the results did not distinguish between a relationship of GRP1 with the absence of CD28 signaling vs the anergic state per se. T cell inactivation with partial TCR agonists has been shown to be a fundamentally different method to anergize T cells. Partial agonists induce anergy by altering early events in TCR signal transduction proximal to the TCR complex, even in the presence of costimulation (16). We have shown previously that a non-mitogenic anti-CD3-IgG3 Ab, incapable of being cross-linked on costimulation-competent APC, triggers a partial intracellular signal that induces anergy in Th1 clones similar to that observed with peptide-based partial TCR agonists (47, 62). As shown in Fig. 5A, the nonmitogenic anti-CD3-IgG3 Ab induced anergy in the pGL10 clone. Unresponsiveness occurred in the treated pGL10 cells even if costimulation was provided by splenic APC (Fig. 5C). Moreover, as in the costimulation-deficient anergy models, the GRP1 protein expression was up-regulated by this T cell inactivating protocol (Fig. 5, B and D). Thus, GRP1 expression was specifically associated with the T cell unresponsiveness in two independent models of anergy induction.
FIGURE 5. The GRP1 is induced in pGL10 anergized with the nonmitogenic Ab anti-CD3-IgG3. The pGL10 cells were grown in growth medium (no Ab, A and B), exposed to immobilized 145–2C11 (2C11), or inactivated in the presence of soluble anti-CD3-IgG3 (IgG3, A and B). In a different experiment, shown in C and D, pGL10 cells were incubated in growth medium in the presence of irradiated, syngeneic spleen cells with no further compounds added (no Ab) or were inactivated with soluble anti-CD3-IgG3 (IgG3) in the presence of spleen cells. The occurrence of unresponsiveness was examined in a restimulation assay (anti-CD3-IgG3 (IgG3) in the presence of spleen cells. The occurrence of unresponsiveness was examined in a restimulation assay (anti-CD3-IgG3, A and B). This feature was not shared by cytohesin-1 (Fig. 6B, c and d) or ARNO (Fig. 6B, e and f), which could both be found in the cytoplasm as well. In Jurkat cells, the cytoplasm to nucleus ratio was sufficiently large to conclude that GRP1 was largely absent from the cytoplasm and exclusively localized to the cortex (Fig. 6B).

The GRP1 induces LFA-1/ICAM-1-mediated adhesion

Human cytohesin-1 was shown to promote CD18-mediated adhesion upon overexpression in Jurkat cells (55). We wanted to know whether GRP1 could affect adhesion in a similar manner, especially after our observation that GRP1 localizes to the plasma membrane in Jurkat cells (Fig. 6B). Jurkat E6 cells, expressing Ig fusion proteins of cytohesin-1, GRP1, or ARNO, were examined in adhesion assays as described previously (55). As shown in Fig. 6D, Jurkat cells, which expressed a control protein (the isolated Ig domains), adhered minimally to ICAM-1. Upon phorbol ester treatment the adhesiveness of these cells increased 4- to 5-fold as previously described (60). The GRP1 or cytohesin-1 overexpression induced 6- and 8-fold increases in specific adhesion to ICAM-1, respectively, whereas ARNO had no significant effect. The observed adhesion to ICAM-1 was reduced to background levels if a pair of blocking anti-LFA-1 Abs was included into the assay (data not shown), indicating that the adhesion was LFA-1 specific. Thus, GRP1 and cytohesin-1 induced LFA-1-mediated lymphocyte adhesion, which corresponds well to the localization of these proteins to the plasma membrane in lymphoid cells (Fig. 6). These results were also consistent with empirical observations that homotypic adhesion as well as adhesion to tissue culture dishes increased in anergic Th1 clones (data not shown).

In addition, we observed a specific interaction of the cytoplasmic domain of CD18 with GRP1 in a yeast two-hybrid assay (data not shown), which was performed as described previously for cytohesin-1 (55), indicating that GRP1 and CD18 can interact directly.

Unique distribution of GRP1 protein to the plasma membrane in lymphoid cells

To begin to understand the intracellular site of GRP1 function, we compared the subcellular localizations of overexpressed GRP1, ARNO, and cytohesin-1 in various cell lines using confocal immunofluorescence microscopy. Recombinant vaccinia virus-derived Ig fusion proteins were used because they had previously been found to be very useful in these types of analyses for three reasons. First, the amino-terminal tagging does not interfere with known functions of cytohesin-1 (55, 56). Second, these chimeric proteins could be visualized with the same highly specific reagent, an Ab directed against the Fc portion of human IgG. Finally, as shown in Fig. 6C, similar expression levels could be achieved for each Ig fusion protein, excluding the possibility that differences in compartmentalization observed for the three Ig fusion proteins would be due to different expression levels. Analysis of the subcellular distribution revealed two important features of GRP1. First, GRP1 displayed a cell type-specific localization pattern. In nonlymphoid HeLa (Fig. 6Ac) and U937 cells (Fig. 6Af), GRP1 showed diffuse cytoplasmic appearance with only a small degree of plasma membrane staining. In fact, the fusion protein seemed to accumulate in intracellular vesicular compartments. In contrast, in the lymphoid cell lines, U266 (Fig. 6Ad) and Jurkat E6 (Fig. 6Bd), GRP1 almost exclusively localized to the plasma membrane. This GRP1-specific staining pattern in the lymphoid cells was not observed with the other family members. Cytohesin-1 was detected in the cytoplasm and at the plasma membrane in all cell lines analyzed (Fig. 6A, a, d, and g, and Fig. 6Bc), while ARNO showed mostly diffuse cytoplasmic staining (Fig. 6A, b and h, and Fig. 6Be), except for the immature macrophage cell line U937 (Fig. 6Ae) where the fusion protein had a punctuate appearance in the membrane. The second unique characteristic of GRP1 was its exclusive localization to the lymphocyte cortex (Fig. 6Bg), similar to the distribution observed with plasma membrane-associated actin (Fig. 6B, a and h).

The GRP1-transduced T cells do not maintain ectopic gene expression

Jurkat T cells are easy to transfect and have been used successfully in the past to study signal transduction and adhesion. However, the autonomous proliferation of Jurkat cells interferes with studies directed toward analyzing the role of GRP1 in cell proliferation triggered by TCR-mediated antigenic stimulation. Therefore, TCR-transgenic lymph node CD4+ T cells from DO11.10 mice were transduced with GRP1 using a retroviral expression strategy. The goal was to generate Th1 cells that overexpressed GRP1 and to access their proliferative capacity and cytokine production upon antigenic stimulation. To achieve this, the lymph node T cells were activated under Th1 skewing conditions, transduced, and sorted by FACS for expression of the coexpressed marker eGFP. Because eGFP was expressed from a bicistronic RNA, preceded by GRP1, eGFP expression correlated with GRP1 expression in the transduced T cells (Fig. 7C). The FACS-purified T cells were examined for continued expression of eGFP/GRP1 after Ag stimulation and cell expansion. A selective loss of green fluorescence was observed in the GRP1 population. Three weeks after the sort, the fraction of eGFP-positive cells among the GRP1-transduced cells was only 21% (Fig. 7A). Furthermore, the mean fluorescence intensity of eGFP in the GRP1-transduced population declined as well (Fig. 7, A and B), suggesting that the proliferating cells could only tolerate low levels of GRP1 expression. In contrast, cells transduced with a control vector containing eGFP remained >70%...
eGFP positive over the course of the experiment. The same high percentage of the cells remained eGFP positive when a retroviral construct containing GRP1 cDNA in an antisense orientation was used (data not shown). Thus, it seemed unlikely that the observed instability of the GRP1-transduced population was an artifact caused by genomic imprinting of the integrated retroviral construct. Rather, these results indicated that there existed strong negative selection pressures in T cells expressing the GRP1 transgene. The mechanism of the observed instability of the GRP1-transduced population was further addressed by examining whether ectopic GRP1 expression altered the cells’ ability to proliferate and/or produce cytokines. Control and GRP1-transduced DO11.10 T cells were sorted into eGFP− and eGFP-deficient cells and restimulated with OVA peptide. No difference in [3H]TdR incorporation was observed among the various groups, including GRP1-transduced cells (eGFP+ or eGFP−) and control transduced T cells (Fig. 7D). Similar results were observed with T cells skewed either toward the Th1 or the Th2 pathway. Th2 cells, however, incorporated more [3H]TdR, presumably because they produced significant amounts of IL-4 (data not shown). In addition, there was no difference in ectopic expression of GRP1 on IL-2 production (Fig. 7E). IL-4 in the Th2 cells (data not shown) or IFN-γ in the Th1 cells (data not shown). Increased IL-2 production was observed in all cells that did not express eGFP, indicating that the retroviral vector affected cytokine production (Fig. 7E). The lack of difference in cytokine production by GRP1-transduced cells compared with that by a vector control was confirmed by intracellular cytokine staining, gating on the eGFP-positive cells (data not shown).

Finally, we wanted to address whether GRP1-transduced cells were more sensitive to activation-induced cell death (AICD). Transduced T cells were exposed to immobilized anti-CD3 mAb, and the percentage of propidium iodide-positive (apoptotic) cells was analyzed. In general, cells expressing eGFP were protected from AICD compared with eGFP-negative cells. However, ectopic GRP1 expression did not affect cell death as examined by propidium iodide or annexin V binding capacity (data not shown). Thus, the mechanism for the reproducibly observed instability of
the GRP1-transduced T cell population cannot be attributed to decreased cytokine production, reduced proliferation, or an increased propensity to undergo AICD.

Discussion

There has been much progress in understanding the basis of peripheral tolerance. More than a decade ago, Jenkins, Schwartz, and colleagues defined a process of clonal inactivation, termed T cell anergy, that was induced as a direct consequence of costimulation blockade in vitro. In the last few years an increasing number of in vivo correlates have been defined (32, 63). In addition, numerous reports have identified molecular differences between anergic and responsive T cells (64–71). These differences include alterations in early and late signal transduction events and transcriptional regulation of IL-2 (72). However, the findings that the induction of T cell anergy is dependent on protein synthesis and is extremely long lived (31) support the hypothesis that newly synthesized negative regulatory factors control unresponsiveness. Therefore, we employed DDRT-PCR to identify such regulatory factors control unresponsiveness.

The strong correlation between anergy and GRP1 induction suggests a functional relationship. For instance, up-regulation of GRP1 in the anergic T cells might lead to an inability to respond to a mitogenic stimulus. However, overexpression of GRP1 following retroviral transduction of T cells was not sufficient to affect the cell’s ability to proliferate, produce cytokines, or undergo AICD (Fig. 7). We did observe, however, a reproducible instability of the GRP1-expressing population compared with that of the control. The expression of ectopic GFP was rapidly lost during culture. Thus, GRP1 could be important for the anergic cell’s stability. This effect may be dependent on other as yet unidentified cofactors, such as the GRP1 interaction partners ARF1 or cytohesin-1.

Unlike activated T cells, anergic cells cannot home to the marginal zone of the germinal centers to interact with activated B cells (32). It is conceivable that GRP1 binds LFA-1 (Fig. 6) and affects LFA-1-mediated adhesion, thus interfering with migration of the anergic T cell. In this scenario, CD28 is not merely a costimulatory signal, but is also a regulator of migration; the prevention of GRP1 induction (Fig. 3B) could affect LFA-1-mediated adhesion, and the ability of CD28 costimulation to abrogate expression of the T cell chemokine lymphotactin (74, 75) would interfere with the recruitment of additional T cells. Adoptive transfer experiments with T cells derived from GRP1-deficient mice will be required to further address this issue.

In summary, we have shown that GRP1 is an integrin regulatory protein and a potential marker for clonal anergy, which is synthesized de novo upon inactivation, a finding that makes GRP1 different from all previously reported molecules associated with anergy. Because GRP1-transduced cells proliferate and produce cytokines normally, but adhere spontaneously to ICAM-1, GRP1 induced in anergic cells may affect LFA-1-dependent T cell responses, including adhesion.

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