response to distinguish differences among the human islet donors also supports the direct recognition of the polymorphic MHC products expressed on the human islet cells. The future of immunosuppressive therapies in transplantation and autoimmune disease depends on their ability to induce long-term, antigen-specific unresponsiveness. The capacity of CTLA4Ig to significantly prolong human islet graft survival in mice in a donor-specific manner suggests that blocking the interaction of costimulatory molecules such as CD28-B7 may provide an approach to immunosuppression.

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Immunosuppression in Vivo by a Soluble Form of the CTLA-4 T Cell Activation Molecule

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In vitro, when the B7 molecule on the surface of antigen-presenting cells binds to the T cell surface molecules CD28 and CTLA-4, a costimulatory signal for T cell activation is generated. CTLA4Ig is a soluble form of the extracellular domain of CTLA-4 and binds B7 with high avidity. CTLA4Ig treatment in vivo suppressed T cell–dependent antibody responses to sheep erythrocytes or keyhole limpet hemocyanin. Large doses of CTLA4Ig suppressed responses to a second immunization. Thus, costimulation by B7 is important for humoral immune responses in vivo, and interference with costimulation may be useful for treatment of antibody-mediated autoimmune disease.

Costimulatory signals delivered by antigen-presenting cells (APCs) have been proposed to control immune responses to transplanted tissues (*1*). Antigenic stimulation of T cells in vitro in the absence of costimulation leads to aborted T cell proliferation and the development of functional unresponsiveness or clonal anergy of T cells (*2*). Several molecules on APCs augment T cell proliferation (*3*, *4*) and regulate functional unresponsiveness in vitro (*4*). The B7 activation molecule binds CD28 (*5*) and delivers a costimulatory signal for T cell proliferation (*6*). T cell–dependent B cell differentiation requires the interaction of B7 with CD28 (*7*). CTLA-4, a T cell molecule homologous to CD28 (*8*), also binds the B7 counter-receptor (*9*). CTLA4Ig, a chimeric immunoglobulin C_γ fusion of CTLA-4, binds with high avidity (dissociation constant ~12 nM) to B7 and potently blocks T cell–dependent immune responses in vitro (*9*). CD28 probably participates in costimulation required to prevent anergy induction in T cell clones (*10*), in unresponsiveness in human mixed lymphocyte reactions (*11*), and in the costimulation of antigen-specific interleukin-2 production of human T cells (*12*). Despite data that indicate the importance of B7-CD28 interactions in the costimulation of in vitro T cell responses, the role of these interactions in regulating in vivo immune responses is unknown. Here, we show that CTLA4Ig is a potent suppressor of antibody responses in vivo.

Human CTLA4Ig [human CTLA-4 and human immunoglobulin (Ig)] binds to murine B7 and inhibits murine T cell responses in vitro (*13*). These findings led us to test the effects of human CTLA4Ig on murine immune responses in vivo. CTLA4Ig was purified to homogeneity by protein A chromatography from a serum-free culture medium of transfected mammalian cells (*14*). The chimeric monoclonal antibody (MAb) L6, which has a murine region and a human Fc region, was used as a control.

We first measured serum clearance of human CTLA4Ig in mice (Fig. 1). A plot of serum CTLA4Ig levels versus time was biphasic, giving a time of half-clearance ($t_{1/2}$) of ~4 hours and ~30 hours for the two components. Serum clearance after multiple injections of CTLA4Ig was more complex and appeared dose-related. The $t_{1/2}$ for the more slowly clearing component was increased to ~4 days after six daily intravenous injections of CTLA4Ig (200 μg per injection), and functionally active CTLA4Ig was detected in mouse serum for up to ~5 weeks after the last treatment with CTLA4Ig. No overt toxicity of CTLA4Ig was noted.

The ability of CTLA4Ig to suppress for-

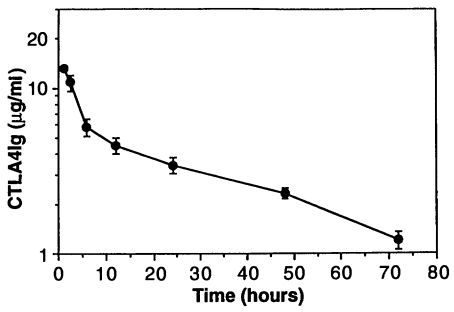

Fig. 1. Serum clearance of human CTLA4Ig in mice. BALB/c mice were each given a single intravenous injection of 50 μg of CTLA4Ig prepared from COS cells. At the indicated times, the mice were bled retro-orbitally. The binding of functional CTLA4Ig from sera to B7$^+$ CHO cells was measured by flow cytometry (*6*). CTLA4Ig concentrations were quantitated by comparison of the degree of binding with the binding of known concentrations of CTLA4Ig. Values represent mean concentrations ± SD from five mice.

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mation of plaque-forming cells (PFCs) that produce antibodies (Abs) to sheep red blood cells (SRBCs) was examined. CTLA4Ig or MAb L6 was administered daily by intravenous injection beginning immediately after administration of the SRBCs and continuing for 2 days. SRBC-specific PFCs were measured on day 14 after immunization (Fig. 2). PFC formation was suppressed in CTLA4Ig-treated mice in a dose-dependent manner. CTLA4Ig suppressed the PFC response by >50% with as little as 1 μg per injection and completely inhibited the response with 75 μg per injection. Administration of up to 100 μg per injection of chimeric MAb L6 did not significantly affect PFC formation, which indicates that suppression by CTLA4Ig was not a result of the Fc portion of the molecule.

We also examined the ability of CTLA4Ig to suppress Ab responses to a soluble protein antigen, keyhole limpet hemocyanin (KLH) (Fig. 3). Administration of CTLA4Ig after immunization suppressed Ab response to KLH. Maximal suppression was observed with a 3-day treatment with CTLA4Ig beginning immediately after immunization and continuing for 2 days thereafter (days 0 to 2). Essentially identical suppression was seen if the start of CTLA4Ig administration was delayed for up to 2 days. These data indicate that B7 is not required for up to 2 days after immunization with KLH. Thus, B7-CD28 interactions are most likely to maintain or amplify an immune response rather than initiate it (*5*). Further delay in starting CTLA4Ig treatment resulted in less effective suppression; when treatment was started on day 4 or 5, partial suppression of Ab production was observed. This may indicate that B7-CD28 interactions also function at later stages of an immune response.

The effect of CTLA4Ig on secondary Ab responses was examined. The mice were immunized with KLH, and on day 20 received a secondary immunization with KLH and treatment with CTLA4Ig or chimeric L6 (50 μg per mouse for 6 days). Serum titers of Abs to KLH rose from 2,600 ± 630 (reciprocal dilutions ± SD) on day 20 to 93,000 ± 29,000 on day 31 in chimeric L6-treated mice; in CTLA4Ig-treated mice, titers rose from 2,400 ± 100 to 23,000 ± 9,800. Thus, CTLA4Ig treatment could suppress secondary Ab responses to KLH, although less effectively than it suppressed primary responses.

Experiments were performed to elucidate the mechanism of immunosuppression by CTLA4Ig. Spleens removed on day 4 from mice immunized with SRBCs with or without chimeric L6 treatment were markedly enlarged. In contrast, spleens from mice immunized with SRBCs and then treated for 3 days with CTLA4Ig (> ~50 μg per injection) were of normal size. Cell yields of spleens from SRBC-immunized mice, with or without MAb L6 treatment, were ~1.6 times greater than of spleens from naïve and CTLA4Ig-treated mice. Thus, splenomegaly most likely resulted from antigen-induced lymphoproliferation. The relative numbers of T cells and total major histocompatibility complex (MHC) class II antigen-positive cells (B cells, monocytes, and dendritic cells) in treated and untreated mice were examined by flow cytometry (*15*). Splenocytes from naïve mice, mice treated with SRBCs plus chimeric L6, and mice treated with SRBCs plus CTLA4Ig were 45 ± 5%, 43 ± 4%, and 43 ± 7% positive for Thy-1.2, and 34 ± 5%, 38 ± 6%, and 34 ± 4% MHC class II–positive, respectively. Splenocytes isolated from mice treated with SRBCs and chimeric L6 or CTLA4Ig on day 4 had similar B7 expression (measured by CTLA4Ig binding) and also similar induction of B7 expression as shown by overnight incubation in vitro with lipopolysaccharide (*5*). Spleen B cells from SRBC- and CTLA4Ig-treated animals on day 4 did not show decreased ability to mobilize Ca^{2+} after surface Ig was cross-linked. Taken together, these results suggest that CTLA4Ig treatment did not result in gross depletions or changes in responsiveness of spleen B cells.

In vivo immunosuppression by CTLA4Ig was associated with altered T cell responses (Fig. 4). Proliferative responses were measured for spleen cells from naïve mice and from mice immunized in vivo with SRBCs and treated with chimeric L6 or CTLA4Ig. Splenocytes from SRBC-im-

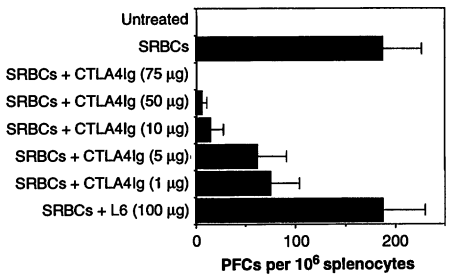

Fig. 2. CTLA4Ig suppresses in vivo induction of SRBC-specific plaque-forming cells. BDF_1 (C57BL/6 × DBA/2)F_1 mice 6 to 8 weeks of age were left untreated or immunized by intravenous injection with 5 × 10^7 SRBCs and then treated with three daily intravenous injections of the indicated amounts of CTLA4Ig or 100 μg of the chimeric MAb L6. After 14 days, spleen cells were assayed for SRBC-specific PFCs by indirect assay with the use of the method of Jerne *et al.* (*23*). Values represent mean ± SD for five mice per group. Similar results were observed when direct PFCs were measured at day 4 after injection. Suppression of PFC formation by CTLA4Ig treatment in vivo was observed in four independent experiments.

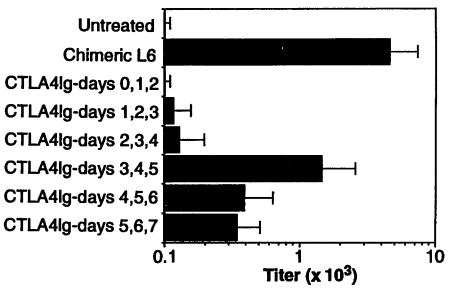

Fig. 3. CTLA4Ig suppresses primary Ab responses to KLH. BALB/c mice were left untreated or immunized on day 0 by intraperitoneal injection of 250 μg of KLH without adjuvant. The mice were then treated by intravenous (tail vein) injection of chimeric MAb L6 (50 μg per day) from days 0 to 7 or by three daily injections on the indicated days with CTLA4Ig (50 μg per injection). Abs to KLH were measured by enzyme-linked immunosorbent assay (ELISA) (*24*) on day 10. Values are expressed as mean titers (± SD) from five individual mice. Similar results were observed in another independent experiment. Suppression of Ab responses to KLH by CTLA4Ig treatment in vivo was observed in four independent experiments.

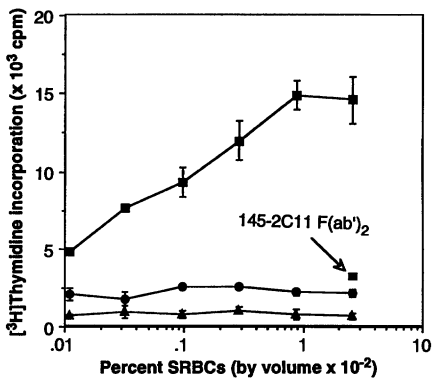

Fig. 4. Splenocytes from CTLA4Ig-treated mice show reduced antigen-specific T cell responses in vitro. BALB/c mice were left untreated (triangles) or were immunized with SRBCs (1 × 10^8 intravenously) and then treated for 3 days with CTLA4Ig (circles) or chimeric MAb L6 (squares) (50 μg per animal per day intravenously). Splenocytes were isolated on day 19 and were cultured at 2 × 10^6 cells/ml in RPMI with 10% fetal bovine serum that contained the indicated concentrations of SRBCs. Cellular proliferation was measured by addition of [^{3}H]thymidine (1 μCi/well) during the final 18 hours of a 3-day culture. Where indicated, a F(ab')$_2$ fragment of the MAb 145-2C11 (to murine CD3) (*16*) was added to a final concentration of 5 μg/ml at the beginning of the culture. Similar results were obtained in two stimulation experiments with a total of five treated and four untreated mice.

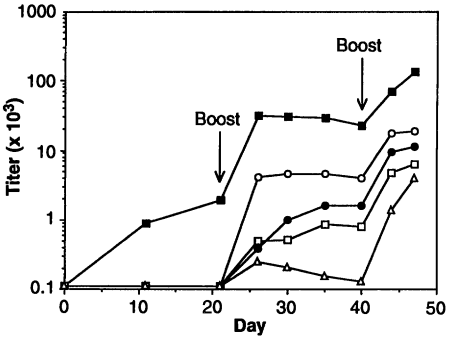

Fig. 5. Immunosuppression by CTLA4Ig is prolonged but not permanent. BALB/c mice were treated with nothing (black circles) or immunized with SRBCs plus L6 (200 μg) (black squares), CTLA4Ig (50 μg) (white circles), CTLA4Ig (100 μg) (white squares), or CTLA4Ig (200 μg) (white triangles). Treatment with L6 or CTLA4Ig was continued for 6 days. The mice were bled at the indicated times, and serum concentrations of Abs to SRBCs were measured by ELISA (*24*). All mice received additional injections of SRBCs on days 21 and 40. Values represent titers of pooled sera from five mice at each time point. Values for mice treated with SRBCs only were identical to those for mice treated with L6 plus SRBCs and have been omitted. Arrows indicate times of secondary and tertiary injections of SRBCs.

munized, chimeric L6-treated animals gave proliferative responses that were dose-dependent with respect to SRBCs; proliferation was blocked by $F(ab')_2$ fragments of a MAb to the T cell receptor complex (*16*). Thus, proliferation was T cell–dependent. Splenocytes from SRBC-immunized, CTLA4Ig-treated animals showed reduced T cell–dependent responses to SRBCs. In other experiments, splenocytes from SRBC-immunized, CTLA4Ig-treated animals showed normal responsiveness to concanavalin A. CTLA4Ig-treated animals also responded normally to allostimulation and to activation by a MAb to CD3 (*17*). These findings are consistent with previous studies (*9*, *13*) showing that CTLA4Ig blocks APC-induced T cell proliferation in vitro and suggest that inhibition of SRBC-specific immune responses in vivo resulted at least in part from reduced antigen-specific T cell function.

We determined the duration of the immune suppression that was induced by CTLA4Ig treatment after primary immunization (Fig. 5). In this experiment, treatment with CTLA4Ig or chimeric L6 was continued for 6 days after immunization. Treatment with all doses of CTLA4Ig suppressed primary SRBC IgG1 Ab responses in pooled sera by >95%. Secondary Ab responses varied according to the dose of CTLA4Ig. Mice treated with 200 μg of CTLA4Ig showed peak Ab responses that were suppressed ~80% in comparison to those of mice receiving a primary immunization and treated with chimeric L6 Mab. Similar results were obtained when IgM- and IgG2a-specific second-step conjugates were used. Mice treated with 100 μg of CTLA4Ig did not show suppressed secondary responses, and those treated with 50 μg of CTLA4Ig gave accelerated responses. Identical conclusions were reached when Ab levels in individual animals on days 25 and 30 were determined. The group treated with 50 μg of CTLA4Ig showed the greatest variation; three of five mice gave enhanced primary responses, and two mice gave titers within the primary response range. This suggests that some mice from this group exhibited immunological memory even though their primary immune responses were substantially blocked. The mice were reimmunized on day 40 with both SRBCs and KLH. SRBC responses for all groups increased with similar kinetics (Fig. 5), and KLH responses were identical (peak titers ~10,000).

Thus, treatment with large doses (200 μg) of CTLA4Ig led to prolonged immunosuppression but not permanent tolerance of SRBCs. This was also the case with mice whose primary Ab responses to KLH were inhibited by CTLA4Ig (50 μg for 3 days) (Fig. 3). We measured the amount of functional CTLA4Ig in sera from BALB/c mice (Fig. 5) by binding $B7^+$ to Chinese hamster ovary (CHO) cells. Animals treated with 50-, 100-, and 200-μg doses of CTLA4Ig had serum CTLA4Ig concentrations on day 21 of 1, 5, and 10 μg/ml, respectively. By the time the mice were reimmunized on day 40, serum CTLA4Ig levels in all groups had dropped to <2 μg/ml. These data suggest that prolonged immunosuppression was associated with the continued presence of CTLA4Ig in serum.

Recently, much attention (*10*–*12*) has been given to how CD28-B7 interactions provide the second or costimulatory signal required to maintain T cell responsiveness (*2*) in vitro. Support for the involvement of these interactions in regulation of in vivo tolerance is given by the findings of Lenschow *et al.* (*18*), who show that CTLA4Ig treatment induces long-term survival of pancreatic islet cell xenografts. There are several possible explanations why we did not induce tolerance in our studies. SRBCs and KLH are extremely potent immunogens, and inducing tolerance to them may require greater blocking of B7 or additional blocking of other costimulatory molecules (*19*). It is also possible that certain T cell populations vary in their dependence on B7 costimulation for maintained responsiveness. CTLA4Ig treatment may favor the development of interleukin-4–producing Th2 cells, which are refractory to tolerization (*20*). Finally, our results may indicate that the binding of human CTLA4Ig to murine B7 is suboptimal for induction of tolerance. Despite these possibilities, our data suggest that virtually complete suppression (>95%) of in vivo immune responses by CTLA4Ig does not necessarily lead to tolerance.

We demonstrated that CTLA4Ig is a potent immunosuppressive agent in vivo, in agreement with previous in vitro results (*9*, *13*). Our data suggest that CTLA4Ig has attractive features for an immunosuppressive drug (that is, in vivo stability, low toxicity, and high specificity). Immune regulation and tolerance induction have been achieved by administration of MAbs to T cell molecules involved in signal transduction (*21*) or combinations of MAbs that block intercellular adhesion of lymphocytes (*22*). Immunosuppression by CTLA4Ig has shown promise as an approach to manipulate immunity to transplants (*18*). Our results showing that CTLA4Ig also suppressed humoral responses suggest potential uses of CTLA4Ig in the treatment of Ab-mediated autoimmune diseases.

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14. Initially, CTLA4Ig was produced by transient transfection of COS cells as described (*9*). Unless otherwise indicated, experiments were done with CTLA4Ig produced in stably transfected CHO cells. CTLA4Ig cDNA (*9*) was cloned into the mammalian expression vector πLN (provided by A. Aruffo) and cotransfected with the pSV2dhfr selectable marker [S. Subramani *et al.*, *Mol. Cell. Biol.* **1**, 854 (1981)] into $dhfr^-$ CHO cells. CTLA4Ig-secreting transfectants were isolated and amplified by growth in increasing concentrations of methotrexate. An amplified cell line producing 3 to 10 mg/liter was isolated. These experiments were done with CTLA4Ig preparations that contained endotoxin at <~1.5 U/mg.
15. BALB/c mice were immunized with SRBCs and then treated for 3 days with CTLA4Ig or chimeric L6 as described (Fig. 2). Splenocytes were isolated on day 4, stained with biotinylated MAb to

Thy-1.2 (30-H12) or with biotinylated MAb to I-A^d (AMS-32.1) (PharMingen) followed by fluorescein isothiocyanate–conjugated streptavidin, and analyzed by flow cytometry.
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24. Microtiter wells (Immulon 2; Dynatech) were coated with KLH or a 0.5% octyl glucoside extract of SRBC ghosts. Wells were blocked for 1 hour with sample diluent (Genetic Systems, Seattle) and then washed with phosphate-buffered saline that contained 0.05% Tween 20. Sera were serially diluted in sample diluent and incubated with coated wells for 1 hour at 23°C. Wells were washed, and Ab binding was detected by addition of horseradish peroxidase–conjugated Abs to murine IgG1 (Southern Biotech, Birmingham, AL), then detected with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Genetic Systems). Absorbances at 450 nm (A_{450}) were recorded on microtiter plate reader (Genetic Systems). Titers were determined from dilution curves as the dilution required to give a value for A_{450} of five times background.
25. We thank J. Bradshaw for help with the Ca^{2+} mobilization assays; D. Hewgill for flow cytometry assistance; P. Fell for MAb L6; L. Knox, B. Grauerholz, R. Drager, L. Paul, and other members of the CTLA4Ig working group for help in preparation of CTLA4Ig; and J. Bluestone, K. E. Hellstrom, and A. Aruffo for critical reviews of the manuscript.

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Activation-Induced Ubiquitination of the T Cell Antigen Receptor

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The ζ subunit of the T cell antigen receptor (TCR) exists primarily as a disulfide-linked homodimer. This receptor subunit is important in TCR-mediated signal transduction and is a substrate for a TCR-activated protein tyrosine kinase. The ζ chain was found to undergo ubiquitination in response to receptor engagement. This posttranslational modification occurred in normal T cells and tumor lines. Both nonphosphorylated and phosphorylated ζ molecules were modified, and at least one other TCR subunit, CD3 δ, was also ubiquitinated after activation of the receptor. These findings suggest an expanded role for ubiquitination in transmembrane receptor function.

The TCR is a multicomponent transmembrane receptor that consists of clonally derived heterodimeric antigen recognition elements and a set of invariant subunits. These invariant subunits include the members of the CD3 complex (δ, ε, and γ) and the ζ subunit (*1*). The ζ subunit exists primarily as a disulfide-linked homodimer consisting of two 16-kD monomers (*2*, *3*). A subset of ζ chains undergoes multiple tyrosine phosphorylations upon receptor stimulation such that the predominant form migrates with an apparent molecular size of 21 kD in SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*4*–*7*).

To evaluate activation-dependent changes in the migration of ζ, we incubated the T cell hybridoma 2B4 (*8*) in the presence or absence of an activating monoclonal antibody to the CD3 ε subunit of the TCR, 2C11 (*9*). TCRs were immunoprecipitated, resolved on two-dimensional nonreducing-reducing SDS-PAGE (diagonal gels), and immunoblotted with antibodies directed to ζ. In unactivated cells (Fig. 1A), the ζ homodimer appeared as a prominent 16-kD species that migrated at 32 kD before reduction. After activation (Fig. 1B), the 21-kD form of phosphorylated ζ was seen directly above ζ as described (*4*, *5*). Unexpectedly, a number of additional immunoreactive species of 24, 32, and 40 kD were also observed in a variety of disulfide-linked combinations. Less prominent forms with apparent molecular sizes of approximately 27 kD under reducing conditions were also observed.

These larger immunoreactive species were further characterized on two-dimensional gels in which nonequilibrium pH gradient electrophoresis (NEPHGE) under reducing conditions was followed by SDS-PAGE in the second dimension (NEPHGE-PAGE) (Fig. 1, C and D). This demonstrated the activation-specific appearance of a ladder-like group of species above ζ, which corresponded to the 24-, 32-, and 40-kD forms seen on the diagonal gels. At each of these molecular sizes, the proteins were homogeneous with regard to isoelectric point (pI). The 21-kD phosphorylated form of ζ migrated with a more acidic pI. A 27-kD activation-specific acidic form was also seen migrating above the 21-kD form of phosphorylated ζ. On longer exposures of the autoradiograms (*10*), other spots were seen above the 27-kD species, which gave the appearance of a second, less intense ladder-like set of species that converged with the more prominent forms toward a neutral pH. This suggested the modification of ζ and, to a lesser extent, phosphorylated ζ with multimers of a neutral 8-kD protein.

Normal murine splenocytes were next analyzed. Freshly isolated splenocytes (Fig. 2A) and cells that had been incubated at 37°C for

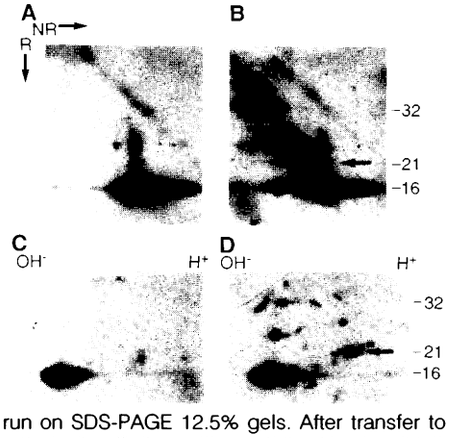

Fig. 1. Activation-induced modifications of ζ in 2B4 hybridoma cells. The 2B4 cells (10^8 cells per condition) and LK cells (an Fc receptor–bearing B cell hybridoma) (*20*) were incubated at 37°C for 45 min in the absence (**A** and **C**) or presence (**B** and **D**) of 2C11 as described (*7*). Cells were chilled to 4°C with phosphate-buffered saline and phosphatase inhibitors (*21*). Cell pellets were lysed in lysis buffer that contained Triton X-100, protease and phosphatase inhibitors (*21*), and postnuclear supernatants immunoprecipitated with a monoclonal antibody directed against the α subunit on 2B4 cells, A2B4-2 (*2*, *22*). Immunoprecipitates were separated under nonreducing conditions in SDS-PAGE tube gels (10.5%) (A and B) or run in NEPHGE tubes (*2*) with a pH range from 3 to 10 (C and D). After equilibration, all tubes were run on SDS-PAGE 12.5% gels. After transfer to nitrocellulose membranes, proteins were immunoblotted with affinity-purified anti-ζ antibodies (551) and detected by ^{125}I-labeled protein A (ICN) (*23*). The 21-kD form of phosphorylated ζ is indicated by arrows (B and D). The spot directly above the arrow in (D) is a hyperphosphorylated form of ζ. Antiserum 551 was raised to a peptide that corresponded to amino acids 151 to 164 of murine ζ (*23*) and does not recognize the η alternative splice of ζ. The diagonal gels (A and B) and the NEPHGE gels (C and D) are from separate experiments. NR, nonreducing; R, reducing. Molecular size markers are indicated to the right in kilodaltons.

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