Translational Control of Interleukin 2 Messenger RNA as a Molecular Mechanism of T Cell Anergy

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Summary

T cell stimulation by triggering through the T cell receptor (TCR) in the absence of costimulatory signals or by calcium ionophore induces unresponsiveness in T cells to further stimulation, a phenomenon known as anergy. In freshly isolated T cells, calcium ionophore induces expression of interleukin (IL)-2 messenger (mRNA), but this mRNA is not translated and not loaded with ribosomes. In addition, while plate-bound anti-CD3 stimulation of resting T cells leads to IL-2 mRNA expression and IL-2 secretion, in cells pretreated with calcium ionophore before anti-CD3 stimulation, the IL-2 mRNA remains polysome unloaded and no IL-2 is produced. These observations show that IL-2 expression is controlled at the translational level, by differential ribosome loading. Furthermore, our data suggest that translational control of IL-2 mRNA may be a molecular mechanism by which anergy is attained.

Antigenic stimulation of resting T lymphocytes induces expression of more than 100 different genes (1), secretion of lymphokines, and proliferation in response to IL-2 both in an autocrine and paracrine fashion (2). Antigenic stimulation requires triggering of the TCR, as well as additional costimulatory signals (3–5), and can be mimicked by a combination of calcium ionophore and phorbol esters (6). Triggering of the TCR in the absence of costimulatory signals leads to a state of unresponsiveness to further stimulation known as anergy (7). Anergy can also be induced by calcium ionophore alone, in freshly isolated T cells and some T cell clones (8, 9). The biochemical base for anergy remains controversial (10–14). However, it is known that anergy can be reversed with either exogenous IL-2 (15, 16) or a combination of PMA and ionophore (8, 9, 17).

To study the mechanism of anergy induction, we have stimulated human peripheral blood T cells with calcium ionophore. This signal, although sufficient to induce the expression of IL-2 mRNA (18–20), did not lead to secretion of IL-2. Indeed, here we describe that upon ionophore stimulation, IL-2 mRNA is not translated and not loaded with ribosomes. Upon subsequent stimulation of these cells with plate-bound anti-CD3, the IL-2 mRNA remains unloaded and no IL-2 is produced. Furthermore, the translational blockade, like anergy, can be reversed by a combination of phorbol esters and calcium ionophore. Taken together, our data suggest that translational control of IL-2 may represent a mechanism by which anergy is attained.

Materials and Methods

Cell Purification and Culture Conditions. Fresh T cells were isolated from buffy coats by Ficoll-Hyphaque followed by Percoll gradient centrifugation (Pharmacia Biotech Inc., Piscataway, NJ). T cells were resuspended in DMEM supplemented with 10 mM Hepes, 2 mM glutamine, 5 × 10^{-5} M B-mercaptoethanol, and either 5% heat-inactivated autologous or FCS (Readysysteme, Zurzach, Switzerland). Cells were cultured for 12 h (unless otherwise indicated) at 37°C in a humidified incubator containing 5% CO₂ in the presence or absence of 10 μg/ml plate-bound anti-CD3 mAb (clone 66.1), calcium ionophore (1 μg/ml, A23187), or calcium ionophore in combination with 1 ng/ml PMA. IL-2 biological activity was measured in a bioassay using CTLL-2 cells (21), and IL-2 units were determined by comparison to a standard.

Preparation and Analysis of RNA. Total RNA was prepared using the guanidinium thiocyanate/acid phenol method and analyzed by Northern blotting as described (22, 23). Northern blots were subsequently hybridized with 32P-labeled full-length IL-2 and HLA (24) probes.

Biosynthetic Labeling and Immunoprecipitations. Purified T cells were stimulated for 11 h with calcium ionophore, either alone or in combination with PMA. After several washes in methionine-free media, cells were pulsed for 15 min with [35S]methionine in methionine-free media. Cell lysis and immunoprecipitations were done as described (25). Sequential immunoprecipitations of each lysate were performed in the following order with rabbit preimmune serum, rabbit anti–IL-2 antisera (catalogue no. 1300008, Boehringer Mannheim GmbH, Mannheim, Germany), and mouse anti-IL-2 (clone 66.1), calcium ionophore (1 μg/ml, A23187), or calcium ionophore in combination with 1 ng/ml PMA. IL-2 biological activity was measured in a bioassay using CTLL-2 cells (21), and IL-2 units were determined by comparison to a standard.

Polysome Gradients. Total cytoplasmic extracts were fractionated in 15–40% sucrose gradients (26–28). After fractionation and deproteinization, RNA samples were denatured with 4.6 M formaldehyde at 65°C, blotted onto nylon membranes using a slot blottor (Schleicher & Schuell, Inc., Keene, NH), and immobilized by UV fixation. Filters were hybridized with a human IL-2 cDNA probe. As controls, the 0.3-kb EcoRI-EcoRI fragment of mouse β₂-microglobulin (29), the 1.1-kb PstI-PstI fragment of mouse β-actin (30), and an oligonucleotide 5’-ACGGGAGGT-
IL-2 expression after calcium ionophore stimulation of human peripheral blood T cells. (A) IL-2 mRNA levels from T cells noninduced (n.I.) or induced for 12 h with (a) plate-bound anti-CD3 mAb (anti-CD3); (b) calcium ionophore (Iono); or (c) calcium ionophore in combination with PMA (PMA+Iono). The specific signals for IL-2 and HLA class I mRNAs were quantified using a phosphoimager (Molecular Dynamics, Sunnyvale, CA). The relative IL-2 mRNA levels were obtained after dividing the signal obtained for IL-2 mRNA by the signal obtained for HLA class I mRNA and giving the value of 1.0 to ionophore-stimulated cells. (B) Secreted IL-2 into the supernatants of the cells induced as described in A and measured as biological activity in a bioassay using CTLL-2 cells. (C) Immunoprecipitation of IL-2 and HLA class I molecules after biosynthetic labeling of T cells stimulated with calcium ionophore, alone or in combination with PMA. As control, rabbit preimmune sera were used. The arrowheads indicate the migration of IL-2 and HLA class I proteins.

TTCTGTCCTCCC-3' complementary to nucleotides 4205–4225 specific for human 28 S RNA were used. Hybridization and washing conditions were as described (23).

Results and Discussion

Calcium Ionophore Induces the Expression of IL-2 mRNA but not IL-2 Synthesis. It is well known that while resting T cells do not express detectable levels of IL-2 mRNA, stimulation with a combination of phorbol esters and calcium ionophore, or plate-bound anti-CD3 mAbs, induce the expression of this mRNA (Fig. 1 A), and secretion of IL-2 into the supernatant (Fig. 1 B). Furthermore, calcium ionophore by itself is able to induce expression of IL-2 mRNA (Fig. 1 A) (18–20). However, this signal alone does not lead to IL-2 secretion into the supernatant (≤0.2 U/ml; Fig. 1 B). The differences in IL-2 biological activity between supernatants from cells stimulated with calcium ionophore, alone or in combination with PMA (130–140-fold; Fig. 1 B), could not be explained by differences in IL-2 mRNA levels (6–8-fold; Fig. 1 A), nor by differences in stimulation kinetics (data not shown). In addition, T cells stimulated with plate-coated anti-CD3 mAb secrete detectable IL-2 (3.6 U/ml; Fig. 1 B), although they express IL-2 mRNA levels comparable (0.8-fold) to ionophore-stimulated cells (Fig. 1 A). Taken together, these data clearly indicate that the lack of detection of secreted IL-2 is not caused by the differences in mRNA levels, and they suggest that IL-2 mRNA can be translationally regulated in ionophore-stimulated cells.

To determine if the lack of secreted IL-2 into the supernatant of calcium ionophore-stimulated cells was caused by a lack of IL-2 mRNA translation, newly synthesized IL-2 was immunoprecipitated from lysates of cells stimulated with calcium ionophore, alone or in combination with PMA, after metabolic labeling of the cells for 15 min with [35S]methionine. As shown in Fig. 1 C, newly synthesized IL-2 was readily immunoprecipitated from lysates of cells stimulated with calcium ionophore alone. Subsequent immunoprecipitation of the lysates with anti-HLA class I antibodies showed that the lack of translation of IL-2 is specific, since we could immunoprecipitate comparable amounts of newly synthesized class I molecules under both stimulation conditions (Fig. 1 C). Taken together, these data indicate that the lack of IL-2 biological activity in calcium ionophore-stimulated cells results from a specific lack of translation of IL-2 mRNA.

The Lack of IL-2 Translation Is Linked to a Differential Ribosome Loading of IL-2 mRNA. To dissect the defect of IL-2 mRNA translation in calcium ionophore-stimulated cells, we analyzed sucrose gradients from lysates of cells stimulated with calcium ionophore alone or in combination with...
Figure 2. Polysome fractionation of cytoplasmic IL-2 mRNA. Distribution of IL-2 and β2-microglobulin mRNA in polysome gradients from T cells stimulated with calcium ionophore, alone (I) or in combination with PMA (P+I), for 12 h. Different cell numbers were used for each gradient to give similar signal strength for IL-2.
inhibited the response to anti-CD3 mAbs, but also the proliferative response in allogeneic stimulations (J.A. Garcia-Sanz, unpublished results).

To determine if the lack of IL-2 secretion in anti-CD3 mAb–stimulated T cells after pretreatment with calcium ionophore was caused by the translational inhibition of IL-2 mRNA, we analyzed the distribution of cytoplasmic mRNAs isolated from these cells in polysome gradients. Polysome gradients from cells stimulated with anti-CD3 mAb over night culture in media showed two peaks of IL-2 mRNA, one on the top fractions of the gradient, representing IL-2 mRNA with few or no ribosomes, and another at the bottom of the gradient, representing ribosome-loaded IL-2 mRNA (Fig. 4). In cells stimulated with anti-CD3 mAb after pretreatment with ionophore, however, we detected only one peak of IL-2 mRNA at the top of the gradient. The peak corresponding to ribosome-bound IL-2 mRNA was not present. As a control, we analyzed the distribution of β-actin mRNA under the same conditions. Despite the differences in β-actin mRNA steady-state levels detected in Fig. 4 A, which reflect differences in transcription rate upon stimulation (37, 38), its distribution pattern on the polysome gradients was similar (Fig. 4 B). As an additional control, we analyzed the distribution of 28 S RNA.
RNA in these gradients. The first fraction containing 28 S RNA corresponds to the fraction containing the 60 S ribosomal subunits, and the following two fractions correspond to mRNAs with one ribosome attached (39). As shown in Fig. 4, the fractions containing the peak of IL-2 mRNA after pretreatment with ionophore corresponded to the first three fractions containing 28 S RNA. This indicated that IL-2 mRNA was able to form a translation preinitiation complex, but only one ribosome was able to bind IL-2 mRNA.

To rule out the possibility that the lack of translation in anti-CD3-stimulated cells after pretreatment with calcium ionophore was caused by the toxicity of the ionophore, T cells were incubated overnight with ionophore and then stimulated for an additional 6 h with a combination of phorbol esters and ionophore. Under these conditions, anergy should be reversed and indeed, secreted IL-2 was detectable (Fig. 3 C). Furthermore, polysome gradients of these cells showed that a minor peak of free IL-2 mRNA (top of the gradient), most of IL-2 mRNA was present at the bottom of the gradient, corresponding to polysome bound mRNA. Again, the distribution of β-actin and 28 S RNAs was unchanged (Fig. 4). Thus, the translational repression of IL-2 was relieved by the same conditions that reversed T cell anergy.

Conclusions. The data presented here demonstrate that conditions which induce T cell anergy in freshly isolated human peripheral T cells are also able to induce a translational repression of IL-2 mRNA. This translational inhibition of IL-2 mRNA was maintained upon subsequent stimulation with plate-bound anti-CD3 mAb, but could be reversed by a combination of phorbol esters and ionophore, conditions that also reverse the anergic phenotype. The inhibition of IL-2 mRNA translation is demonstrated both by the lack of IL-2 synthesis upon biosynthetic labeling of the cells and by a shift in the distribution of IL-2 mRNA on polysome gradients towards the fractions containing a ribosomal subunit or a single ribosome. This shift of IL-2 mRNA upon ionophore treatment is consistent with a mechanism allowing the formation of the translation preinitiation complex and the loading of one ribosome, followed by a block of IL-2 mRNA translation. This block of IL-2 mRNA translation would be responsible for the anergic state of the cells. The reversal of both the anergic state and the specific block of IL-2 mRNA translation by phorbol esters in combination with ionophore is most likely caused by the higher levels of IL-2 mRNA expressed.

The phenomenon of T cell anergy has been described as the induction of a lack of response to further stimulation. At the molecular level, changes of phosphorylation patterns and lack of ZAP70 recruitment have been shown to play a role in the induction of anergy (10, 11). Furthermore, in some models, a lack of IL-2 transcription and a role for different DNA-binding proteins, including AP-1, NRE-α, PRE, as well as NFA-T, have been described (12–14). These data, together with functional data, suggest that anergy can be induced at different nonexclusive levels. Our data clearly indicates that translational regulation of IL-2 mRNA is a control point for T cell activation, which may be one of the mechanisms by which anergy is reached.

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