Idiotypic immunization induces immunity to mutated p53 and tumor rejection

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The p53 molecule might serve as a common tumor-associated antigen, as the tumor suppressor gene p53 is mutated and the p53 protein is often over-expressed in tumor cells. We report that effective immunity to p53 can be induced through an idiotypic network by immunization of mice with a monoclonal antibody (PAb-240) specific for mutated p53, or with a peptide derived from the complementarity determining region (CDR) 3 of the variable domain of the light chain (VL) of this antibody. The immunized mice produced IgG antibodies to p53 and mounted a cytotoxic reaction to a tumor line bearing mutated p53. The idiotypically immunized mice were resistant to challenge with the tumor cells. Thus antibodies to p53 might serve as immunogens for activating resistance to some tumors. At the basic level, these findings indicate that a network of p53 immunity may be organized naturally within the immune system.

Mutations of p53 have been detected in about 70% of colon cancers, 30–50% of breast cancers, 50% of lung adenocarcinomas and almost all small-cell carcinomas of the lung, among other tumors[1]. The prevalence of p53 mutations in cancers of different origins can be explained by the function of *p53* as a tumor-suppressor gene[2]. Thus, inactivation of the p53 protein by mutations or other factors could enable the growth of tumor cells that might otherwise be led into growth arrest or apoptosis by the action of wild-type p53 (ref. 2). Mutated p53 protein accumulates in tumor cells, and might serve as a tumor-associated antigen for immunotherapy. Indeed, attempts have been made to induce tumor immunity using mutated peptide sequences of p53 (refs. 3,4). However, p53 is a self antigen, and mechanisms of self tolerance could reduce the immunogenicity even of mutated p53.

We reasoned that immunity to p53 might be inducible using an anti-p53 antibody as an idiotypic immunogen, in place of p53 itself. In the terminology of idiotypic networks, an antibody to an antigen, (that is, antibody Ab1) (ref. 5) might function as an immunogen and elicit a second antibody (Ab2) specific to Ab1 (ref. 5). Ab2, in turn, can spontaneously induce a third Ab, (Ab3; ref. 5). Since Ab1 might bind both the antigen epitope and Ab2, Ab2 might mimic the structure of the antigenic epitope. Hence, Ab3, which is activated by Ab2, might also recognize the original antigen, and so function, like Ab1, as an antibody to the antigen. In other words, an epitope of the target antigen and an epitope of Ab2 might look alike, at least to Ab1, and Ab1 and Ab3 might act alike in recognizing the antigen. Attempts have been made to use Ab2 antibodies that mimic tumor antigens to induce Ab3 anti-tumor immunity[6]. Anti-idiotypic networks however, can be inuced by Ab1 as well as Ab2. Rather than using a classic Ab2 approach, we decided to explore the possibility of using an Ab1 anti-p53 antibody to induce active immunity to p53. BALB/c mice immunized with a BALB/c monoclonal antibody to mutated p53, PAb-240 (Ab1), developed their own IgG antibodies to mutated p53 (Ab3) (Fig. 1). In contrast, no antibodies to mutated p53 were induced by immunization with the control monoclonal antibody 9E10, specific for the onco-protein myc.

The singular specificity of an antibody molecule, its idiotype, is formed by the CDRs that create its unique antigen-binding site[7]. CDR peptides have been used in the past to induce immunity to reovirus.[8,9] To test whether idiotypic determinants of PAb-240 (Ab1) could induce anti-p53 immunity, we cloned and sequenced the variable (V) domains of the light (L) and heavy (H) chains of Pab-240, and synthesized peptides corresponding to the VL CDR3 and VH CDR3 segments. These CDR3 peptides of PAb-240 (Ab1) were used as immunogens. The VL CDR3 peptide, but not the VH CDR3 peptide, induced IgG anti-p53 antibodies (Fig. 1). Thus, the anti-p53 antibodies were likely to have been induced by activation of an idiotypic network response.

As the VL CDR3 peptide was synthesized as a linear amino acid sequence, it can have little or no structural similarity to the folded shape of the antigen-combining site of the intact PAb-240 molecule[7]. However, the CDR domains may form a reverse turn, and thus have some conformational similarity with their own linear sequence. As the bare CDR3 peptide is not an antibody to p53, the induction of anti-p53 by the VL CDR3 peptide of Ab1 cannot be attributed simply to structural complementarity. T cells recognize processed, linear peptides and not native, folded protein conformations; hence, we suspected that anti-idiotypic T cells might have a role in the p53 idiotypic network.

Effector T cells can be detected functionally by cytotoxicity to antigen-bearing target cells. We tested whether immunization with the VL CDR3 peptide of PAb-240 might lead to the lysis of the Meth A tumor cell line, a tumor of BALB/c origin bearing a p53 mutation[10]. The CDR3 peptide induced significantly ($P < 0.001$) more lysis of the Meth A cells than did immunization with an immunogenic peptide of a virus, HS VP16 (Fig. 2; ref. 11). Thus, it is likely that cytolytic T cells were activated by the idiotypic immunization. The activity of T cells in

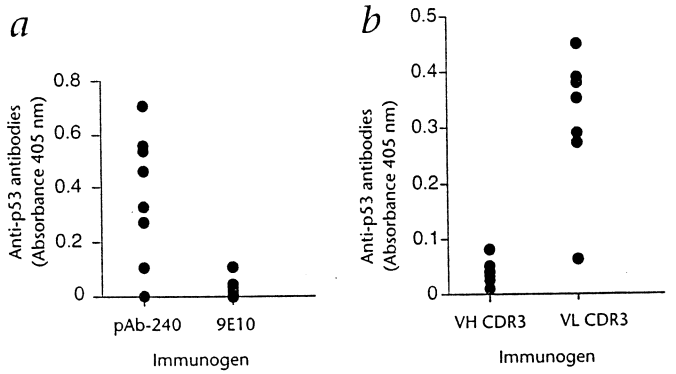

Fig. 1 ***a***, PAB-240 activates anti-p53 antibodies. BALB/c mice were immunized with PAb-240 or 9E10 monoclonal antibodies. Ten days after the boost mice were bled and the sera were tested for antibodies to mutant p53 using an standard ELISA. Results are expressed as absorbance values of individual mouse era. The difference between the groups immunized with Pab-240 or with mAb 9E10 was significant ($P < 0.001$). ***b***, Pab-240 VL CDR3 peptide activates anti-p53 antibodies. BALB/c mice were immunized with the CDR peptide and boosted 2 weeks later. Experimental animals were bled ten days later and their sera were tested for antibodies to mutant p53 as in (*a*). The difference between the two groups was significant ($P < 0.001$)

the p53 network is also indicated by the fact that the Ab3 antibodies to p53 were of the IgG isotype, an antibody type dependent on the activation of helper T cells[12].

Although we have not yet isolated or characterized the T cells that might recognize the VL CDR3 peptide, the p53 network seems to be effective. Immunization to whole PAb 240, but not to 9E10, led to resistance to challenge with Meth A tumor cells implanted *in vivo* (Fig. 3*a*). The VL CDR3 peptide, but not the VH CDR3 peptide of PAb-240, could also immunize mice against the Meth A tumor (Fig. 3*b*). The effects were long lasting; rejected tumors did not recur during a two-month period of observation.

In general, immunization to anti-p53 monoclonal antibody mutated epitopes such as those on PAb-240 protects against Meth A tumors, which express p53. We have analyzed several peptides from the CDR regions of antibodies against wild-type p53 and found them to be in effective in protection from Meth A tumors. However, certain epitopes of antibodies directed against wild-type p53 afford some protection (data not shown), though it is significantly less than that provided by immunizing with epitopes of antibodies such as PAb-240, which is directed against mutated epitopes.

On a practical level, our results indicate that active immunity to mutant p53 can be induced by an idiotypic network triggered by an anti-p53 antibody or by a peptide fragment of the antibody. The need to isolate and purify immunogenic p53 can be avoided. Moreover, it seems that the network can activate both helper and effector T cells. We are now attempting to isolate and characterize these cells.

At a basic level, it is of fundamental interest that an idiotypic network appears to be centered on p53. The ability of a CDR3 peptide of an Ab1 antibody to activate p53 immunity, tumor cell lysis and tumor inhibition indicates that the p53 network may be organized within the immune repertoire prior to immunization. Had the VL CDR3 peptide served merely as a primary immunogen, the response should have been limited to the peptide alone, as would be expected for any other peptide immunization. The linear VL CDR3 peptide of PAb-240 by itself should be unrelated to p53, both structurally and chemically. The VL CDR3 peptide may have already been associated with p53 through a pre-existing lymphocyte network that could account for the induction of p53 immunity[13]. The CDR peptide may have induced an antibody (AB2) that resembles p53 in some way. In either case, there is something unique about the p53 network, because AB3 antbodies do not arise after every AB1 immunization. As yet, we do not know how the p53 network organizes itself and how it might include mutated p53. It has been suggested that at least some mutated p53 molecules may assume a conformation that is also assumed by wild-type p53 when it is in a physiologically inactive state[14]. In other words, mutated p53 may not be structurally foreign to the immune system. Study of the p53 idiotypic network should help clarify the possible role of natural p53 immunity in immunosurveillance.

Methods

Mice. Inbred mouse strain BALB/c was obtained from Olac (Oxon, UK). Female mice were used 8–10 weeks of age.

Monoclonal antibodies. The anti-p53 mouse monoclonal antibody Pab-240 (IgG1/κ), specific for a mutated conformation of the p53 molecule[15] was raised in ascites fluid and purified by protein A affinity chromatography followed by dialysis in PBS. The anti-myc oncoprotein antibody 9E10 (IgG1/κ) (ref. 16) was used as a control antibody.

Cloning and sequencing of antibody V regions. Total RNA from anti-p53 monoclonal antibody hybridoma secreting cells was isolated using the TRI Reagent (Molecular Research Center, Cincinnati, Ohio), according to the manufacturer's instructions. Briefly, 1–2 × 10^6 cells were precipitated, washed and homogenized in 1 ml reagent. After homogenization, RNA was extracted with phenol/chloroform and precipitated with ethanol. Half of the RNA was then used for cDNA preparation. For preparation of cDNA, poly $(dT)_{15}$ primer (Boehringer) was used. First-strand cDNA was synthesized using reverse transcriptase (USB, Cleveland, Ohio). The cDNA was subjected to PCR amplification using forward primers located in the constant region or in the J region, and backward primers located in the V region[17], were used for amplification using *Taq* DNA polymerase (Promega). PCR products were cloned in the plasmid pGEM (Promega) and sequencing was carried out using automated sequencing (Applied Biosystems), and analyzed using Applied Biosystems and GCG package programs. To avoid misincorporation of Taq polymerase, DNA amplification was performed twice, from two RNA preparations. Sequencing was carried out using primers specific to both the 3′ and 5′ ends. The sequences from all reactions were found to be identical for each PCR product.

Synthetic Peptides. The CDR3-based peptides of the VL chain, YYCQHIRELTRSEGGPS, and VH chain YYCARLLRYFAMDYWGQGTT (CDRs are underlined) from PAb-240 were prepared with an automated synthesizer (Applied Biosystem model 430A) using the manufacturer's protocols for *t* butyloxycarbonyl coupling. Peptide purity was evaluated by analytical

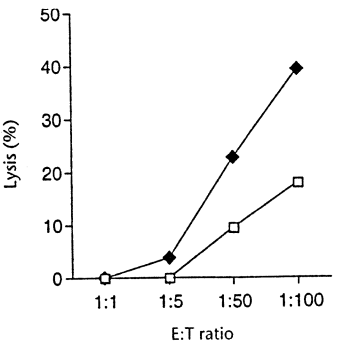

Fig. 2 Lysis of Meth A tumor cells by spleen cells of mice immunized to the VL CDR3 peptide. BALB/c mice were immunized as in Fig. 1*b* with the VL CDR3 peptide of PAb-240 (◆) or with an immunogenic peptide of virion protein 16 or herpes simples type 2 (□). Results are expressed as percentage of specific lysis. The difference between the groups at Effector:Target (E:T) ratios 1:50 and 1:100 are significant ($P < 0.001$).

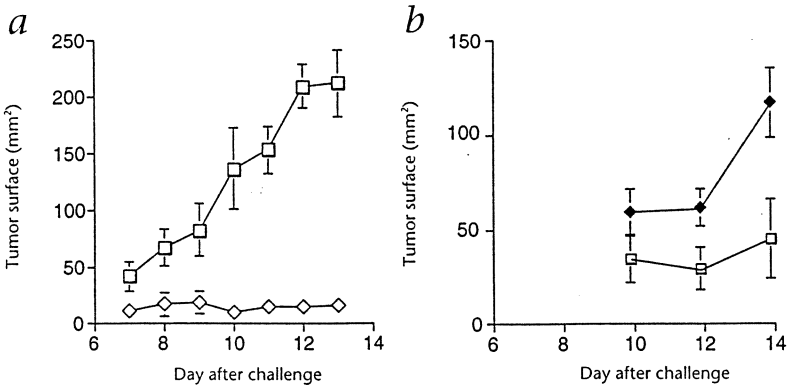

Fig. 3 ***a***, PAb-240 immunization induces resistance to tumor challenge. Groups of five mice were immunized as in Fig. 1*a* with PAb-240 (open diamonds) or with control monoclonal antibody 9E10 (open squares). Ten days after the boost, the mice were challenged by intradermal inoculation of 10^5 Meth-A tumor cells. Tumor size was estimated as a surface areas on various days after challenge by multiplying the tumor mass measured in its two broadest dimensions. ($P < 0.001$). Results are expressed as mean tumor surface +/− s.e.m. ***b***, PAb-240 VL CDR3 peptide immunization induces resistance to tumor challenge. Groups of five mice were immunized with the VL CDR3 (open squares) or the VH CDR3 peptides (filled diamonds) of PAb-240 as in Fig. 1*b*. The mice were then challenged by inoculation of Meth-A tumor cells as ***a***. The differences in tumor size between the groups were significant at days 12 and 14. ($P < 0.001$).

reverse phase HPLC and mass spectrometry analysis. The peptide DMT-PADALDDRDLEM from virion protein 16 of herpes simplex virus type 2 was used as a control.

Tumor cells. The Meth A tumor is a transplantable 3-methylcholantrene-induced sarcoma of BALB/c origin that has three mutations in the p53 coding sequence[18]. The cells were grown in RPMI media containing 10% heat-inactivated fetal calf serum (FCS), and passaged through BALB/c mice to select for tumorogenicity.

Immunization. Mice were immunized intradermally in the hind footpads with 20 µg of monoclonal antibody in a 0.1 ml emulsion 1:1 in Complete Freund's Adjuvant (CFA; Difco Laboratories, Detroit, Michigan). A booster injection with the same amount of the monoclonal antibody in PBS was given two weeks later by the same route. For immunization with the CDR peptides mice were injected subcutaneously in the dorsal flank with 0.1 mg of the peptide in a 0.1 ml emulsion 1:1 in Incomplete Freund's Adjuvant (IFA; Difco laboratories). Mice were bled 10 days after the boost, and the sera were tested for specific antibodies.

Antibody detection by ELISA. Flat bottom maxi-sorb plates (Nunc) were coated with 50 µl per well of mutant p53 at a concentration of 5 µg/ml. Recombinant proteins were prepared as described[14]. After incubation with antigen, the plates were washed and blocked over night with 5% fetal calf serum (BioLab, Jerusalem, Israel) in PBS. Test sera diluted 1:320 were then added for 90 min, followed by incubation for 75 min with 50 µl per well of Alkaline phosphatase-conjugated goat anti-mouse IgG, Fc fragment (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania). The plates were washed, incubated with the substrate p-Nitrophenyl Phosphate Disodium (Sigma) and read using an ELISA reader at 405 nm.

Cytotoxicity assay. Cytolysis was measured in a DNA fragmentation assay by [^{3}H]thymidine release as described[19]. In brief, spleen cells from BALB/c mice were taken ten days after the second immunization with 0.1 mg of the CDR peptide in IFA. Cells were cultured at a concentration of 10×10^6 cell per ml, in 50 ml of enriched media RPMI-1640 containing 2 mM glutamine, 1 mM sodium pyruvate, non-essential amino acids, 100 U/ml of penicillin, 0.25 µg/ml of fungizon (Life Technologies), 5×10^{-5} M 2-mercaptoethanol (Fluka), 10mM HEPES buffer (Sigma) supplemented with 10% fetal calf serum (Life Technologies) and in the presence of 10 µg/ml of the peptide. After five days of incubation, cells were harvested and various numbers of effector cells were added to 1×10^4 target cells that were (3H)thymidine-labeled with a final volume of 0.2 ml of enriched media per well in 96-well U-bottom plates. After four hours of incubation, samples were harvested and radioactivity counted. The percentage of specific cytotoxicity for each experimental point was calculated using the average values of the triplicates from experimental (E) and spontaneous release (S) wells as follows : % cytotoxicity = {(S-E)/S} × 100.

Meth A tumor cell challenge. Immunization of BALB/c mice was performed as described above. Ten days after the boost, the mice were injected intradermally in the right-lower abdominal quadrant, with 1×10^6 Meth A cells. Tumor growth was observed for eight weeks; the tumors were measured by their maximum and minimum diameters using a caliper. Disappearance of the tumor mass constituded rejection.

Acknowledgments

I.R. Cohen is the incumbent of the Mauerberger Chair of Immunology and the Director of the Robert Koch-Minerva Center for Research in Autoimmune Diseases. The work was supported by grants from the Minerva Foundation and from the National Institutes of Health.

RECEIVED 6 FEBRUARY; ACCEPTED 1 MAY, 1998

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Idiotype vaccines: Forgotten but not gone

New findings show that idiotype induced immunity has potential for both prophylactic and therapeutic vaccination (pages 705–709 and 710–712).

CONSTANTIN A. BONA

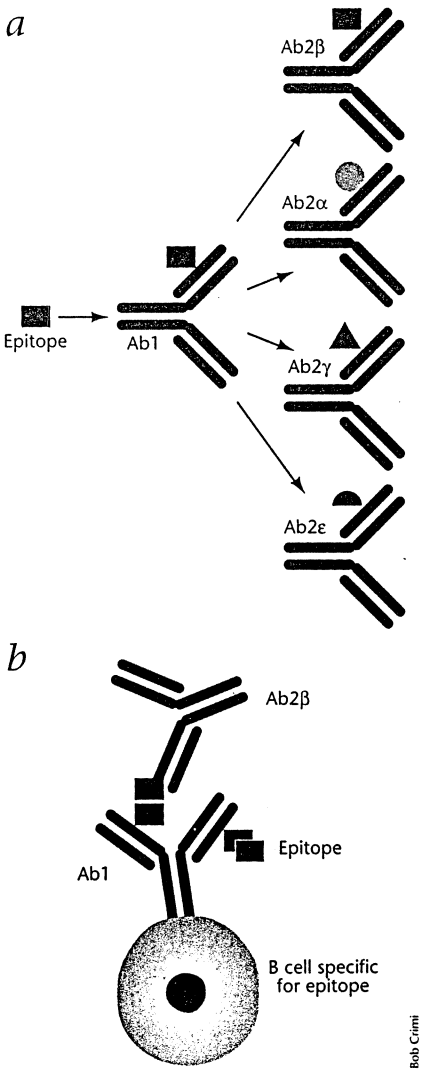
IDIOTYPES ARE ANTIGENIC determinants and phenotypic markers of *V*-region genes that encode the specificity of antibodies (and T-cell receptors)[1]. The diversity of antigen receptors on lymphocytes is reflected in the diversity of idiotypes. This means that statistically the antigen dictionary of both self and foreign antigens should be reflected in the idiotype dictionary of each individual. This assumption has lead to the concept that antigens have an internal image[2] and that idiotypes can mimic foreign and self antigens because they are topochemical copies or positive imprints of antigenic epitopes. This concept has stirred real interest because it suggests that internal image immunoglobulins (that is, anti-idiotypic antibodies that bind to the idiotype of the antigen binding site) instead of antigens could be used to make vaccines[3]. In contradistinction to the lock and key interaction between antigens and their antibody receptors, the interaction between idiotypes and antibodies is based on a three-dimensional mimicry—a degenerate lock and key. Thus, the antigen and the anti-idiotypic epitope, which differ greatly in their gross chemical structure, are recognized as similar by the same antibody (see figure).

Two papers in this issue of *Nature Medicine* show that immunity induced by idiotypic antibodies protects neonatal mice against streptococcal infection[4] and adult mice against tumor challenge[5]. In their study, Magliani *et al.*[4] genetically engineered an anti-idiotype antibody fragment that mimics the capsular polysaccharide antigen of group B streptococcus bacteria. The construct elicited an active protective humoral response in adult mice and a passive protective immunity in newborns. The Ruiz paper[5] demonstrates that a monoclonal antibody to mutant p53 (or a peptide to the light chain of the antibody) initiates both humoral and cellular immunity, which is specific for tumor cells expressing mutant p53, through an idiotypic network. Idiotypically immunized mice were resistant to challenge with a tumor expressing mutant p53 and spleen cells from these mice lysed the tumor cells *in vitro*.

It was Rubinstein and colleagues[6] who first showed that an anti-idiotypic antibody can mimic a bacterial polysaccharide, indicating that such an antibody can bind to the antigen binding site of polysaccharide-specific immunoglobulin. The importance of the Magliani work is three-fold: (1) mimicry can be achieved with a single variable chain fragment (2) such constructs can induce not only an antibody response but also immune memory (3) the animals produce sufficient IgG antibodies to confer passive immunity to newborns.

Numerous studies have shown that internal image immunoglobulins mimic tumor-associated antigens, such as GD2, gp37, gp52, SV40 tumor large antigen, and those expressed by melanomas and cancers of the colon and ovary[7]. The tumor suppressor protein p53 is mutated and overexpressed in many different tumors. As Ruiz *et al.* argue in their paper, the mutated p53 protein is an excellent target for the new idiotypic approach to immunotherapy. They clearly show that an antibody specific for mutant p53 elicits immunity against this protein. However, their most important observation is the induction of cellular immunity with a linear peptide corresponding to the CDR3 (third complementarity determining region) of the light chain of the anti-p53 antibody. This observation is particularly important because idiopeptides, usually produced by processing of endogenous proteins, are generally poor immunogens. T cells (in particular $CD8^+$ T cells) are the best effector cells in antitumor immunity so the finding that an idiopeptide elicited a cytotoxic response is encouraging. Research has shown that antitumor immunity can be induced by internal image immunoglobulins mimicking tumor-associated antigens[8]. The knowledge gained from such studies has led to clinical trials aimed at evaluating the efficacy of immunotherapy using anti-idiotype antibodies against tumor-associated antigens of colon and ovarian cancer and melanoma. The immunity elicited by antibodies against mutant p53 could prove beneficial for developing vaccines against a large group of tumors.

There is a legion of experimental data



The heterogeneity of anti-idiotypic antibodies including internal image antibodies that mimic foreign or self antigen epitopes. ***a***, An epitope of a given antigen may induce the synthesis of an antibody (Ab1) specific for that antigen. This in turn induces the synthesis of a heterogeneous population of anti-idiotype antibodies: Ab2β, an internal image antibody that expresses an idiotype mimicking the antigenic epitope; Ab2α, an antibody that is not blocked by antigen and that may express regulatory idiotopes; Ab2γ, an antibody that is blocked by antigen; Ab2ε, an antibody that recognizes the antigenic epitope as well as the idiotype of Ab1. ***b***, Both the idiotype of the Ab2β internal image antibody and the antigenic epitope stimulate B cells bearing an immunoglobulin receptor (Ab1), which is specific for the epitope that originally initiated antibody production.

from preclincial trials demonstrating that internal image antibodies mimicking viral, bacterial and parasitic antigens induce specific protective immunity against infectious agents[7]. So, why have idiotype-based vaccines not been adopted?

There are several explanations for the failure of idiotype vaccines to penetrate the field of human vaccinology. First, there are technical problems related to production of human anti-idiotype monoclonal antibodies. This is an important limitation because murine antibodies by virtue of their xenogeneic origin elicit a strong antibody response in humans, which neutralizes the mouse antibodies and diminishes their half-life through rapid clearance. Second, in contrast to live attenuated or killed vaccines, which induce strong and long lasting immune memory, the idiotype vaccines do not. The induction of a strong memory is a prerequisite for a good vaccine. Third, whereas IgG antibodies are the predominant class of antibody after immunization with current vaccines, IgM antibodies predominate in the humoral response following immunization with idiotype vaccines. Nevertheless, the strong immunity elicited by idiotypic vaccines in the Magliani and Ruiz studies offers a refreshing ray of hope that internal image antibodies can be exploited for vaccination.

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