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Altered peptide ligands induce quantitatively but not qualitatively different intracellular signals in primary thymocytes

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ABSTRACT **Interaction of the T cell receptor (TCR) with peptide/major histocompatibility complexes (MHC) in the thymus is of critical importance for developing thymocytes. In a previous study, we described an antagonist peptide that inhibited negative selection of transgenic thymocytes induced by an agonist peptide. In this study we show that this antagonist peptide can induce positive selection of $CD8^+$ thymocytes more efficiently than the agonist or the weak agonist peptides, whereas the opposite is true for their ability to cause negative selection. The intracellular signals induced in thymocytes by such peptides after TCR ligation was examined in $CD4^+8^+$ double-positive thymocytes from F5/β_2m^o/Rag-1^o transgenic mice. TCR ligation with either the agonist, weak agonist, or antagonist peptide variants resulted in hyperphosphorylation of CD3ζ, CD3ε, ZAP-70, Syk, Vav, SLP-76, and pp36–38. The extent of phosphorylation of these intracellular proteins correlated with the efficiency with which the peptide analogs induced apoptosis of immature thymocytes. Unexpectedly, there was no correlation between the upstream TCR signaling pathways analyzed and the capacity of the different peptides to induce positive selection.**

Central to intrathymic development of T cells are interactions between the T cell receptor (TCR) and peptide/major histocompatibility complexes (MHC) on thymic epithelium. It is generally accepted that low-affinity recognition of peptide results in survival (positive selection), whereas high-affinity interactions, or no interaction, result in thymocyte death (negative selection or neglect, respectively). Recently, using peptide analogs generated by mutating the antigenic peptide in amino acid positions predicted to interact with the TCR, it was shown that thymocytes can be either positively or negatively selected in the presence of antagonist or agonist peptides (1–6). In addition, it has been shown that positive selection also can be inhibited by some variant ligands (7).

To address the relationship between signals induced by peptide analogs and signals that lead to positive or negative selection, we used mice transgenic for the F5 TCR utilizing the Vα4 and Vβ11 chains. This TCR recognizes a nonamer peptide of the influenza nucleoprotein, NP68 (ASNENMDAM), in association with MHC class I H-2D^b (8). We have described previously an antagonist peptide, NP34 (ASNENMETM) for this receptor (9). In the present study we have used, in addition, a peptide analog that is a weak agonist for this receptor, NP4Q (ASNQNMDAM) (O.W., unpublished data) as well as a control peptide GAG [from the GAG protein of the SF2 strain of HIV (390–398): SQVTNPANI] (10). This peptide appears to have similar affinity for H-2D^b and has the ability to stabilize MHC molecules on the surface of RMAS cells with the same efficiency as nucleoprotein peptides (9).

Fetal thymic organ cultures (FTOCs) were tested to assess the ability of the agonist and variant peptides to promote either positive selection or death. In addition, we analyzed the intracellular signals induced by TCR ligation in $CD4^+8^+$ double-positive thymocytes from F5/β_2m^o/Rag-1^o mice after their exposure to MHC molecules presenting NP68, NP34, or NP4Q peptides. F5/β_2m^o/Rag-1^o mice lack mature single-positive cells, thus analysis of their thymus excludes signaling by mature T cells and allows examination of $CD4^+8^+$ immature thymocyte cells, which have not undergone any positive- or negative-selection interactions. Thus, we were able to examine the phosphorylation state of key signaling molecules that have been shown to affect T cell function and thymocyte development (11–20).

The results reported here indicate that in the F5 TCR system the antagonist peptide is more efficient at inducing positive selection of $CD8^+$ T cells, whereas the agonist peptide is more efficient at promoting cell death of immature thymocytes. We have extended these studies and directly analyzed the intracellular signals induced in thymocytes by these peptides. The data establish that the antagonist peptide stimulates qualitatively similar signals that differ only quantitatively to those induced by the agonist peptide.

MATERIALS AND METHODS

F5 TCR Transgenic Mice. Mice transgenic for the $\alpha\beta$ TCR, from the F5 cytotoxic T cell clone, were generated in our laboratory as reported (21). These mice were crossed onto β_2m^o (22) and Rag-1^o (23) backgrounds and are referred to as F5/β_2m^o/Rag-1^o mice. TAP1-deficient mice were obtained from S. Tonegawa (24).

Peptides. Four nonamer peptides were used: NP68 [from the nucleoprotein of influenza virus A/NT/60/68 (NP366–374)], NP34 [from the nucleoprotein of influenza virus A/PR/8/34 (NP366–374)], NP4Q, and a control peptide GAG [from the GAG protein of the SF2 strain of HIV (390–398): SQVTNPANI] (10). The latter peptide stabilizes MHC molecules in RMAS cells with the same efficiency as the NP peptides (9). Peptides were synthesized on an Applied Biosystems 430A peptide synthesizer.

Cell Culture and Media. The thymic epithelial cell line YO1 (H-2^b) was generated as described previously (9) and was maintained in RPMI 1640 medium (Life Technologies, Paisley, Scotland) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics (RP10 me-

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Abbreviations: MHC, major histocompatibility complex; TCR, T cell receptor; FTOC, fetal thymic organ culture; PTK, protein tyrosine kinase.
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dium), in the presence of 100 units/ml interferon γ (Genzyme) at 33°C in 5% CO_2.

FTOC. For negative selection experiments, fetal thymic lobes were isolated from day 15 F5/Rag-1^o embryos and transferred onto Nucleopore polycarbonate filters (Costar). The thymic lobes were cultured at 37°C, 5% CO_2 in RP10 medium. After 4 days of culture the filters were transferred to medium containing different concentrations of NP68, NP4Q, NP34, and GAG peptides and cultured for 12 hr. Thymocytes then were harvested for analysis by gently disrupting the thymic lobes manually in 1.5 ml Eppendorf tubes and analyzed by flow cytometry. For positive-selection experiments, the FTOCs were set up by using thymic lobes from day 16 F5/Tap1^o embryos. Thymic lobes were cultured as above, with the indicated concentrations of peptide being added from the beginning of the culture and medium containing peptide being changed every 24 hr. After 10 days of culture, thymocytes were harvested and analyzed by flow cytometry.

Flow Cytometry. For flow cytometric analysis the following mAbs and second-layer reagents were used: fluorescein isothiocyanate-conjugated YTS169.4 [anti-CD8α (25)], phycoerythrin-conjugated CD4 (Boehringer Mannheim), biotin-conjugated KT11 [anti-Vβ11 (26)], and streptavidin-RED 670 (Life Technologies). For analysis of DNA content, thymocytes were stained with 5 μg/ml 7-Aminoactinomycin D (7AAD) (Sigma) in PBS containing 2% FCS, 0.1% sodium azide, and 0.3% Saponin (Sigma). Stained cells were analyzed on a FACScan flow cytometer (Becton Dickinson) by using CELLQUEST software (Becton Dickinson).

Stimulations, Precipitations, and Immunoblotting of Thymocytes. Thymocytes (1×10^8) were centrifuged briefly onto a monolayer of YO1 cells that were preincubated with various peptides for 2 hr at 37°C. After 5 min (unless stated otherwise) thymocytes were removed, rapidly sedimented, and lysed in a 1% BRIJ 96 (Sigma)-containing lysis buffer [150 mM NaCl/50 mM Tris, pH 7.5/5 mM EDTA/10 mM NaF/10 mM disodiumpyrophosphate (Sigma)], and protease inhibitors (chymostatin, leupeptin, antipain, and pepstatin A) (Sigma) and sodium orthovanadate (Sigma) for 1 hr at 4°C. Immunoprecipitations were carried out overnight with either protein A Sepharose (Pharmacia) or glutathione Sepharose 4B (Pharmacia).

Immunoprecipitates were resolved by SDS/PAGE (Pharmacia) under reducing conditions and transferred onto polyvinylidene difluoride membranes (Millipore) by Western blotting. The blots were probed with antiphosphotyrosine mAb (4G10) followed by horseradish peroxidase-conjugated goat anti-mouse Ig (Southern Biotechnology Associates) and developed by chemiluminescence (Pierce and Warriner, Chester, U.K.). After stripping, total amounts of protein immunoprecipitated were detected by using relevant antibodies.

Antibodies, Fusion Proteins, and Anti-Sera. The antibodies used for immunoprecipitation and immunoblotting were as follows: rabbit anti-ZAP-70 polyclonal sera was generously provided by N. S. C. van Oers (University of Texas Southwestern Medical Center, Dallas) (27); anti-ZAP-70 mAb was kindly provided by J. Tite (Glaxo–Wellcome); anti-Syk polyclonal sera were kindly provided by M. Turner (National Institute of Medical Research, London) (28); and anti-SLP-76 polyclonal antibody was a kind gift of O. Acuto (Institut Pasteur, Paris). Rabbit anti-Vav antiserum, Vav-1, was raised against a synthetic peptide corresponding to residues 575–590 of human proto-Vav (29). 4G10 mAb was from Brian Drucker (Oregon Health Sciences University, Portland). M. D. Waterfield (Ludwig Institute, U.K.) kindly provided us with the GST-Grb-2 fusion protein. The following secondary antibodies were used in immunoblotting: horseradish peroxidase-conjugated goat anti-mouse Ig (Southern Biotechnology Associates) and peroxidase-conjugated Protein A (Amersham).

RESULTS

Antagonist and Agonist, but Not Weak Agonist, Peptides Induce Positive Selection of Thymocytes. FTOCs from MHC class I-deficient mice, transgenic for different TCRs, have been used previously to demonstrate thymocyte positive selection by individual peptides (30, 31). Experiments using particular TCR transgenic mice demonstrated that only antagonist peptides could positively select thymocytes, whereas other studies showed that low levels of agonist peptides were also capable of doing so (1, 3, 4, 6, 32). To establish whether or not NP68, NP4Q, and NP34 could induce positive selection of F5 thymocytes, fetal thymic lobes were isolated from day 16 F5/TAP1^o embryos and cultured *in vitro* in the presence of different concentrations of the various peptides. Thymic lobes cultured for 10 days with medium alone or 10 μM GAG produced low numbers of Vβ11^{hi} CD8$^+$ thymocytes (Fig. 1*A*). Culture in the presence of 100 nM–10 μM NP68 induced a more than 2-fold increase in the number of mature Vβ11^{hi} CD8$^+$ thymocytes produced. However, 10 μM NP34 induced a 4- to 5-fold increase, over background, in the numbers of mature F5 thymocytes produced by these FTOCs. When the functional capacities of mature thymocytes from various cultures were examined, only those selected by 10 μM NP34 were shown to proliferate in response to antigenic peptide (NP68) (data not shown). From these data we conclude that the antagonist peptide NP34 was considerably more efficient than the antigenic peptide at positively selecting F5 thymocytes, although NP68 appeared to positively select F5 thymocytes over a broader range of concentrations than NP34. Interestingly, the weak agonist NP4Q did not positively select F5 thymocytes at any of the concentrations tested (Fig. 1*A*).

Agonist, Weak Agonist, and Antagonist Peptides Induce Negative Selection of Thymocytes with Different Efficiencies. As mentioned earlier NP68 induces negative selection and apoptosis of thymocytes *in vivo*. To assess the relative efficiency with which NP68 and the variant peptides could induce apoptosis of immature thymocytes, fetal thymic lobes were isolated from day15 F5/Rag^o embryos and cultured *in vitro* for 4 days. At this stage the thymic lobes contained around 60% double-positive thymocytes and less than 1% mature CD8$^+$ thymocytes. The FTOCs then were transferred to medium containing different concentrations of the various peptides and apoptosis measured after 12 hr when using 7AAD staining.

The basal level of cell death was not increased significantly when thymocytes were incubated with the control peptide GAG as compared with thymocytes cultured in medium alone (Fig. 1*B*). As expected, and in agreement with the *in vivo* data (21), NP68 induced apoptosis of double-positive thymocytes efficiently and in a dose-dependent manner (detectable at 10 nM) (Fig. 1*B*). In contrast, concentrations as high as 10 μM of NP4Q and 100 μM of NP34 were required to induce detectable levels of apoptosis.

CD3ζ, CD3ε, ZAP-70, and Syk Proteins in Thymocytes Are All Tyrosine Phosphorylated in Response to Agonist, Weak Agonist, and Antagonist Peptides. One of the earliest events after TCR stimulation is the activation of protein tyrosine kinases (PTKs) that phosphorylate tyrosine residues within a number of membrane-associated and adapter molecules (33). In thymocytes, TCR ligation with CD3 antibodies induces Lck-dependent hyperphosphorylation of CD3ζ and associated ZAP-70 PTK, as well as CD3ε and Syk PTK (27, 34). To investigate whether TCR ligation with agonist and peptide variants resulted in phosphorylation of these intracellular proteins, thymocytes from F5/β_2m^o/Rag-1^o mice were exposed to thymic epithelial cells presenting NP68, NP4Q, or NP34 peptides and the phosphorylation state of CD3ζ, CD3ε, ZAP-70, and Syk was assessed by Western blotting.

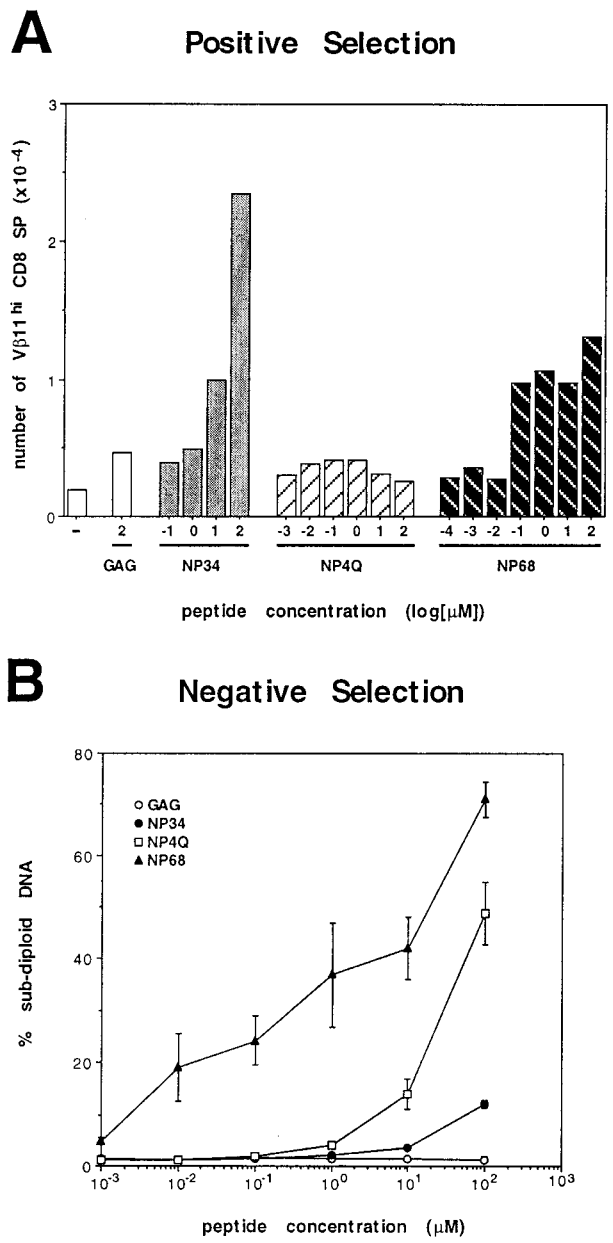

FIG. 1. Positive selection and apoptosis of double-positive thymocytes in response to variant and agonist peptides. (*A*) Fetal thymic lobes from day 16 F5/TAP1° embryos were cultured for 10 days with the indicated concentrations of the various peptides. Thymocytes were harvested and stained with anti-CD4, anti-CD8, and anti-Vβ11 mAbs. The bar chart shows the absolute number of Vβ11^{hi} CD8$^+$ thymocytes produced in each case. Each bar represents the average number from a pool of four individual FTOCs. Positive selection using NP68 and NP34 was seen in three and seven independent experiments, respectively. NP4Q failed to cause any significant positive selection in two independent experiments. (*B*) Fetal thymic lobes from F5/Rag-1° day 15 embryos were cultured for 4 days in medium alone and then transferred to medium containing different concentrations of NP68, NP34, NP4Q, or GAG peptides. Thymocytes were harvested after 12 hr and stained with anti-CD4 and anti-CD8 mAbs and with 7AAD. The graph depicts the percentage of double-positive thymocytes containing subdiploid DNA. The mean and standard deviations of three fetal thymic lobes are shown for each point.

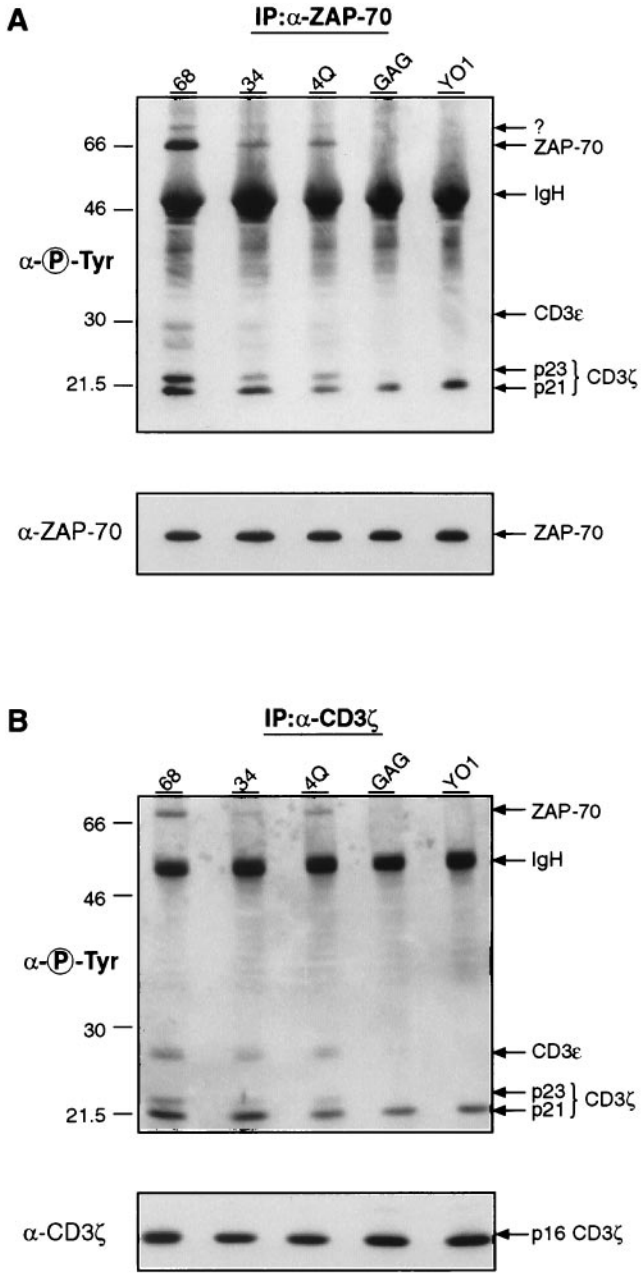

FIG. 2. ZAP-70, CD3ζ, and CD3ε molecules are tyrosine phosphorylated after TCR stimulation with agonist and variant peptides. Thymocytes (1×10^8) from F5/β_2m°/Rag-1° mice were stimulated for 5 min with YO1 cells preloaded with 10 μM of either NP68, NP34, NP4Q, or the control peptide GAG or YO1 cells alone as described in experimental procedures. ZAP-70 and associated proteins were analyzed by immunoprecipitating with an anti-ZAP-70 antibody (*A*) or with an anti-CD3ζ antibody (*B*). Tyrosine-phosphorylated proteins were detected by using an anti-phosphotyrosine antibody, 4G10 (α-P-Tyr), and are indicated by the arrows. The migration of molecular mass markers is indicated on the left. Data are representative of five experiments.

The constitutively phosphorylated p21-isoform of CD3ζ was found to be associated with unphosphorylated ZAP-70 in double-positive thymocytes from F5/β_2m°/Rag-1° mice (Fig. 2*A*), consistent with published data (27, 35). Ligation of the F5 TCR on double-positive thymocytes with either NP68, NP4Q, or NP34 resulted in hyperphosphorylation of ZAP-70-associated CD3ζ generating the p23-isoform (Fig. 2*A*). Although both peptide variants induced hyperphosphorylation of ZAP-70-associated CD3ζ, the levels were lower than those after stimulation with NP68, but significantly higher than background stimulation induced by the control peptide or with YO1 cells alone. The aforementioned changes in CD3ζ were also seen after direct immunoprecipitation of this molecule (Fig. 2*B*). The CD3ε chain also became tyrosine phosphory-

lated in response to NP68 and to a lesser extent in response to NP4Q and NP34 (Fig. 2*B*).

Ligation of the F5 TCR on double-positive thymocytes with either NP68 or NP4Q induced phosphorylation of both ZAP-70 and Syk PTKs (Figs. 2 and 3*A*). Interestingly, phosphorylation of both these PTKs also occurred after stimulation with NP34, the antagonist peptide. However, stimulation with the agonist peptide NP68 induced more phosphorylation of ZAP-70 and Syk than the weak agonist, which, in turn, induced more phosphorylation than the antagonist peptide (Figs. 2 and 3*A*). Western blotting confirmed that similar amounts of ZAP-70 and Syk had been precipitated after each stimulation (Figs. 2 and 3*A*). Stimulation of thymocytes with YO1 cells alone or preloaded with GAG did not result in tyrosine phosphorylation of any of these PTKs. We conclude that stimulation of the F5/β_2m^o/Rag-1^o thymocytes with an antagonist peptide induces a qualitatively similar response to that induced by the agonist peptide. However, TCR engagement

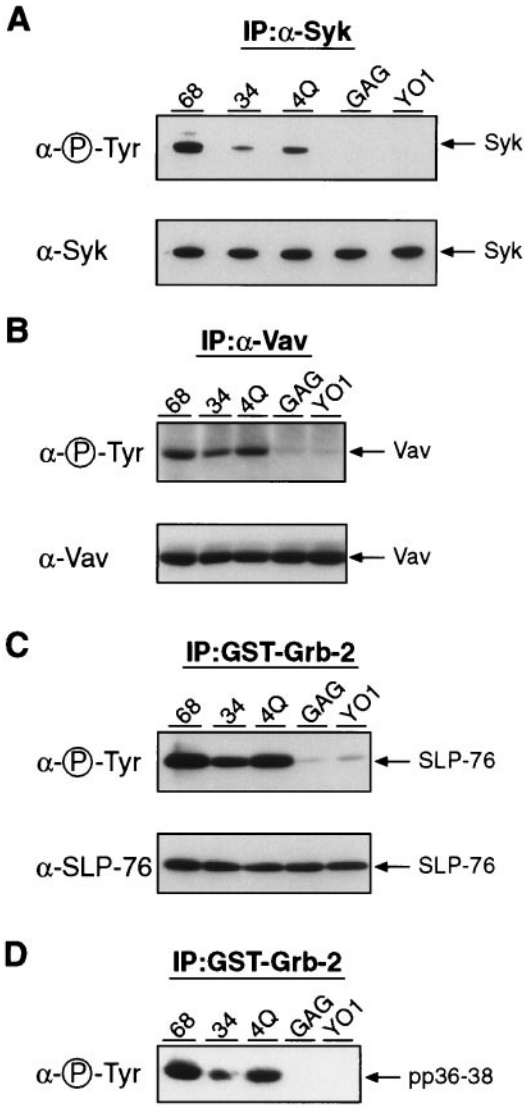

FIG. 3. Syk, Vav, SLP-76, and pp36–38 are tyrosine phosphorylated after thymocyte stimulation with agonist and variant peptides. Thymocytes (1×10^8) from F5/β_2m^o/Rag-1^o mice were stimulated for 5 min with YO1 cells preloaded with 10 μM of either NP68, NP34, NP4Q, a control peptide GAG, or with unpulsed YO1 cells. Syk was immunoprecipitated with an anti-Syk antibody (*A*) whereas Vav was immunoprecipitated with an anti-Vav-1 antibody (*B*). Both SLP-76 (*C*) and pp36–38 (*D*) were immunoprecipitated with a GST-Grb2 fusion protein. Data are representative of four experiments.

with the antagonist ligand resulted in quantitatively lower phosphorylation of CD3ζ, CD3ε, ZAP-70, and Syk as compared with that induced by the agonist or weak agonist peptides.

Vav, SLP-76, and pp36–38 Proteins Are Tyrosine Phosphorylated in Response to Agonist, Weak Agonist, and Antagonist Peptides. Activation of cytoplasmic PTKs by the TCR induces a complex cascade of protein tyrosine phosphorylation and interactions between signaling proteins that are crucial for activating downstream signaling pathways (33, 36). Because TCR ligation with the agonist, partial agonist, or antagonist peptides resulted in tyrosine phosphorylation of ZAP-70 and Syk, we were interested in assessing whether these peptides could induce phosphorylation of downstream targets of these PTKs, such as Vav, SLP-76, and pp36–38 (37–43). Tyrosine phosphorylation of these molecules was observed after stimulation of thymocytes with NP68, but not with the control peptide GAG or unpulsed YO1 cells (Fig. 3 *B–D*). Interestingly, TCR stimulation with either the weak agonist NP4Q or antagonist NP34 also resulted in their phosphorylation. Once again the intensity of tyrosine phosphorylation differed, with the antigenic peptide inducing more phosphorylation of Vav, SLP-76, and pp36–38 than the weak agonist peptide NP4Q, which, in turn, stimulated more phosphorylation than the antagonist peptide NP34 (Fig. 3 *B–D*).

It was possible that TCR ligation with the cognate and antagonistic peptide could induce differential tyrosine phosphorylation of substrates other than the ones described above and therefore undetected in these assays. To address this question total cell lysates from stimulated thymocytes were Western blotted and stained with an anti-phosphotyrosine-specific antibody. Using this assay we found that TCR ligation with either the agonist, weak agonist, or the antagonist resulted in tyrosine phosphorylation of the same substrates (data not shown). We conclude that stimulation of double-positive thymocytes with either cognate or variant peptides resulted in qualitatively similar, but quantitatively different, phosphorylation of PTKs and adapter molecules.

Agonist, Antagonist, and Partial Agonist Peptides Induce Signaling Events with Similar Kinetics. Although NP34 and NP4Q induce similar, but quantitatively lower, TCR proximal signals to those induced by NP68 5 min after exposure to antigen, it was possible that the kinetics of the response to variant peptides differed from those of the agonist. To examine this possibility, a time course experiment was carried out. Thymocytes from F5/β_2m^o/Rag-1^o mice were stimulated over a period of 30 min with the various peptides, and the levels of tyrosine-phosphorylated CD3ζ and ZAP-70 were analyzed as described previously. Stimulation of thymocytes with NP68, NP4Q, and NP34, but not GAG, resulted in hyperphosphorylation of CD3ζ and ZAP-70. Tyrosine phosphorylation of these proteins by all peptides occurred rapidly, reaching maximal levels within 30 sec, and was maintained over the 30-min period of the experiment (Fig. 4*A*). Again the maximal extent of phosphorylation reflected the antigenicity of the peptide used. A similar pattern in the kinetics of phosphorylation of SLP-76 and pp36–38 also was observed (data not shown). Thus, we conclude that there is no difference in the tyrosine phosphorylation kinetics of TCR proximal signaling molecules after stimulation with variant peptides over this time course.

The Proximal TCR Signals Induced by Variant Peptides Are Similar to Those Induced by Low Concentrations of Agonist Peptide. After the observations described above it was of interest to analyze whether in primary immature thymocytes the signals induced by NP34 and NP4Q could be reproduced by low concentrations of the agonist peptide. To this end, we examined the state of phosphorylation of CD3ζ and ZAP-70 after exposure to varying doses of agonist peptide (10 pM–10 mM). As shown previously (Fig. 2), CD3ζ and ZAP-70 both were tyrosine phosphorylated after thymocyte stimulation

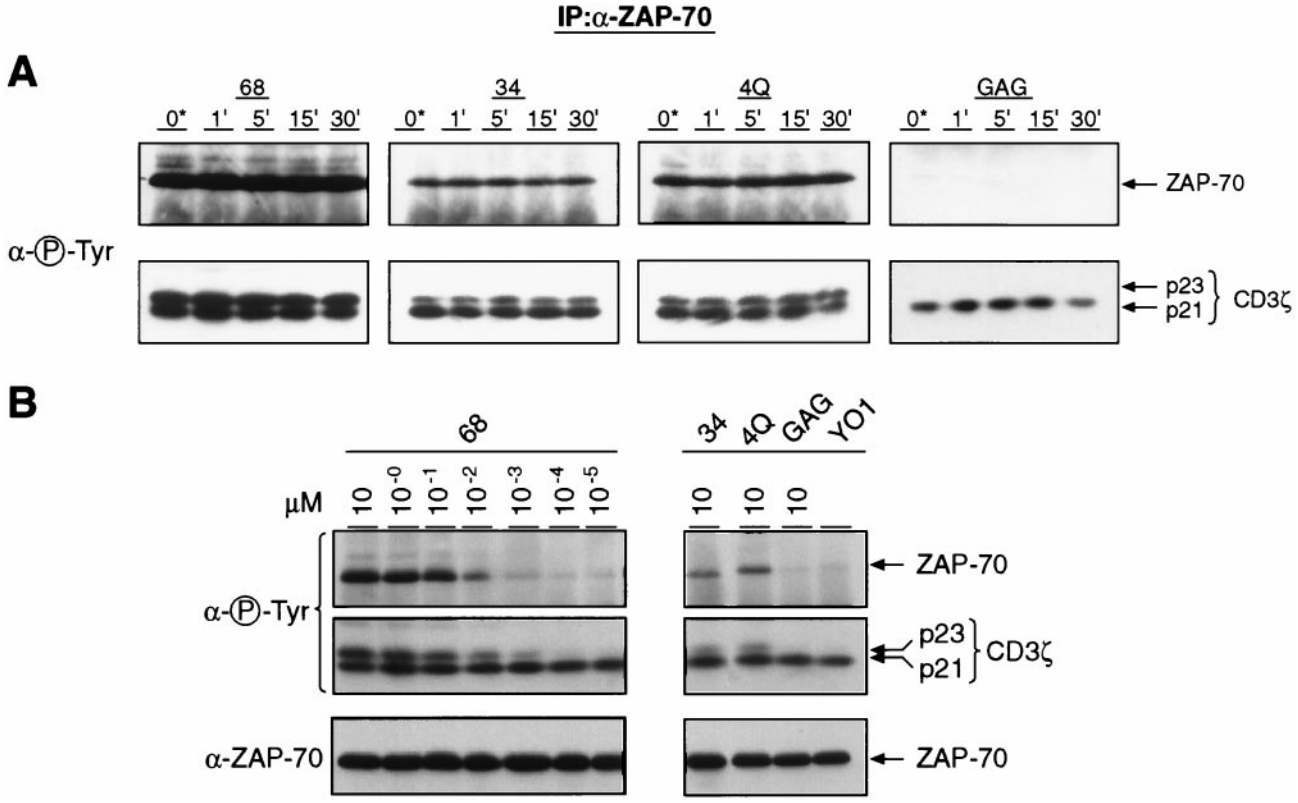

FIG. 4. The peptide variants induce similar kinetics of intracellular signaling, and low concentrations of antigenic peptide induce equivalent signals to high concentrations of weak agonist and antagonist peptides. (*A*) Thymocytes (1×10^8) from F5/β_2m°/Rag-1° mice were stimulated over a period of 30 min with YO1 cells preloaded with 10 μM of either NP68, NP34, NP4Q, or GAG, or YO1 cells alone. 0*, time taken to centrifuge cells onto the monolayer of APCs (30 sec). (*B*) Thymocytes stimulated for 5 min with YO1 cells preloaded with various concentrations of NP68, 10 μM to 10 pM, or 10 μM NP34, NP4Q, or GAG or YO1 cells alone. ZAP-70 and associated proteins were analyzed by immunoprecipitating with an anti-ZAP-70 antibody. Data are representative of four independent experiments.

with 10 μM of NP68, NP34, and NP4Q, but not with the control peptide GAG (Fig. 4*B*). Decreasing the concentration of NP68 resulted in reduced levels of phosphorylated CD3ζ and ZAP-70 (this was also the case for CD3ε, SLP-76, and Vav pp36–38; data not shown) (Fig. 4*B*). Stimulation with 1–10 nM of NP68 induced an intensity of phosphorylation similar to that seen with 10 μM of NP34. In contrast, up to 10–100 nM of NP68 was needed to obtain levels of phosphorylation similar to those seen with 10 μM of NP4Q.

DISCUSSION

Positive selection of $CD8^+$ T cells by weak agonist and antagonist peptides has been described in different transgenic TCR systems (1, 3, 4, 6, 32). In the F5 transgenic model, peptide NP34 was shown to be an antagonist for the F5 TCR (9). In the present study, we show that immature thymocytes developing in the presence of high concentrations of this antagonist peptide are positively selected efficiently, in agreement with previous studies (1).

Positive selection of $CD8^+$ T cells expressing the F5 TCR also was found when immature thymocytes developed in the presence of the agonist peptide, although the mature thymocytes produced were not responsive to the antigenic peptide, in agreement with previous studies (32, 44). Agonist peptides were shown to be efficient in positive selection in several studies (3, 4, 6), indicating that positively selecting peptides are not necessarily antagonists (3). Interestingly, in this system we were unable to positively select $CD8^+$ cells in the presence of the weak agonist NP4Q.

Negative selection of immature T cells in FTOCs by agonist peptides has been well documented (1, 4, 32). It has been shown previously that thymocytes expressing the F5 TCR are deleted if the NP68 peptide is expressed as a self-antigen (8) or after acute injection of this peptide (21, 45). We extended these observations to show that this peptide is also capable of causing profound deletion of immature thymocytes at both high and low concentrations in FTOCs. As predicted by the avidity/affinity model, high concentrations of both variant peptides also induced low but detectable cell death above background. In summary, high concentrations of antagonist peptide resulted in efficient positive selection, whereas high concentrations of agonist peptide induced efficient cell death. Conversely, low concentrations of agonist peptide also can induce positive selection. It is important to note that although NP4Q falls between NP68 and NP34 in its efficiency at inducing negative selection, it fails to positively select F5 thymocytes at any concentration tested.

It has been proposed that the strength of signal in a cell determines the fate of the cell. Strong signals induce death whereas weak signals induce survival (4, 6). We report here that the tyrosine phosphorylation of key PTKs and adapter molecules in the TCR proximal signaling cascade after ligation with the agonist peptide also is seen after stimulation with the peptide variants. Although the signals induced by peptide variants are qualitatively similar to that of the agonist peptide they are quantitatively different. Ligation of the F5 TCR with the antagonist peptide results in a lower signal than that of the weak agonist, which, in turn, is lower than the agonist peptide. Thus, although the initial TCR signal appears to be qualitatively similar, the outcome of interaction with an agonist and an antagonist differs. Notably concentrations of NP68 that produce levels of signaling similar to those induced by NP34 cause profound negative selection (cf. Figs. 1*B* and 4*B*). We

propose that during low-affinity interactions, TCR ligation with an antagonist peptide, the signal transduced does not reach threshold levels necessary to trigger expression of key genes, which could lead to the death of the cell (46). It is at this level that quantitative differences result in qualitatively different outcomes, for example, life versus death. Given that the data in this paper argue that it is the signaling strength that determines the biological outcome of an interaction between TCR and peptide/MHC complexes, it is surprising that NP4Q (weak agonist) cannot positively select F5 T cells. To explain this, additional parameters may have to be considered, such as the ability of different ligands to cause specific clustering of surface molecules along with their cytoplasmic partners (47).

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