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Activation of T Cells by Cross-Linking Qa-2, the *Ped* Gene Product, Requires Fyn

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Abstract

Problem—Qa-2, the product of the *Ped* (preimplantation development) gene, regulates the rate of cell division of preimplantation mouse embryos by an unknown mechanism. Due to the limited availability of preimplantation embryos, T cells were used as a model system to assess the possible roles of Fyn and Lck, and two downstream effectors, PI-3 kinase and Akt, in Qa-2 induced cell proliferation.

Method of study—Resting T cells were stimulated to proliferate by treating with mouse anti-Qa-2 antibody, cross-linking with anti-mouse immunoglobulin, and adding PMA. The effects of kinase inhibitors on this proliferation were studied. Co-immunoprecipitates of T-cell lysates were analyzed for possible associations between Qa-2 and Fyn or Lck. Fyn knockout mice (Fyn^{-/-}) were used to determine whether Fyn is required for T-cell activation induced by cross-linking Qa-2.

Results—An inhibitor of Src family kinases and inhibitors of PI-3 kinase and Akt suppressed proliferation of resting T cells induced by cross-linking Qa-2. Fyn, but not Lck, co-immunoprecipitated with Qa-2. Fyn^{-/-} T cells failed to proliferate in response to Qa-2 cross-linking.

Conclusion—Fyn, PI-3 kinase, and Akt are required for the activation of T cells by cross-linking Qa-2.

Keywords

Akt; Fyn; *Ped* gene; phosphatidylinositol-3 kinase (PI-3 kinase); Qa-2; signaling; T cells

Introduction

Qa-2 protein has at least two functions. It regulates the rate of cell division in preimplantation mouse embryos and also mediates proliferation of resting T cells after cross-linking Qa-2. Qa-2 is the product of the mouse preimplantation development (*Ped*) gene, which confers a multifaceted reproductive advantage to Qa-2-positive mice.^{1–3} Embryos from strains of mice that express Qa-2 protein cleave at a faster rate than those from Qa-2-negative strains. The mechanism by which Qa-2 protein regulates division rate in mouse embryos is not known.

Mouse Qa-2 is the functional homolog of human HLA-G,^{4,5} a molecule of considerable interest because of its involvement in human reproduction.⁶ Both Qa-2 and HLA-G are class Ib (non-classical) major histocompatibility complex (MHC) proteins. Qa-2 is structurally very

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similar to class Ia (classical) MHC proteins,⁷ but unlike the class Ia proteins, Qa-2 is nonpolymorphic.⁸ Additionally, while class Ia molecules are trans-membrane proteins, Qa-2 has a glycosylphosphatidylinositol (GPI) linkage to the cell.⁹ As expected, the GPI linkage of Qa-2 protein facilitates the location of the protein to cholesterol- and glycoprotein enriched membrane microdomains (lipid rafts),^{5,10} compatible with a role for Qa-2 in cell signaling.

During development, Qa-2 is expressed by zygotes and preimplantation embryos.¹¹ In adult tissues, Qa-2 is widely distributed, but generally at low expression levels.¹² T cells express Qa-2 more abundantly than other cell types¹³ and have therefore been used for many studies that have attempted to define a role for Qa-2 protein in the immune response. Qa-2 has been implicated in NK cell inhibition,¹⁴ can serve as a restriction element for anti-tumor cytotoxic T lymphocytes,¹⁵ and is required for the selection of intraepithelial CD8 $\alpha\alpha$ /TCR $\alpha\beta$ T cells,¹⁶ which appear to be regulatory cells involved in maintaining intestinal integrity.¹⁷

The involvement of Qa-2 in regulating cell division in embryos and T cells, and the localization of Qa-2 to lipid rafts, suggest that Qa-2 is a signaling molecule. Studying signaling events directly in pre-implantation embryos is problematic due to the difficulty in collecting sufficient numbers of embryos and the fact that many signaling pathways involved in cell division are constitutively active in embryos due to their rapid rate of cell division. Fortunately, resting T cells can be induced *in vitro* to undergo cell division by cross-linking their surface Qa-2 protein.^{18,19} This induction of proliferation requires three components: (i) anti-Qa-2 primary antibody (IgG); (ii) anti-IgG secondary antibody; and (iii) phorbol myristate acetate (PMA) as a second signal. We have used this system as a model to study signaling via Qa-2, focusing on ascertaining the roles in Qa-2-mediated activation of two Src family kinases (Fyn and Lck)²⁰ and two potential downstream components, phosphatidylinositol-3 (PI-3) kinase and Akt.²¹

Materials and methods

Mice

Qa-2-positive C57BL/6J mice were bred in North-eastern University's animal care facility (accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care), from stock derived from The Jackson Laboratory (Bar Harbor, ME, USA). These mice were used for the experiments described below unless otherwise stated. *Fyn*^{-/-} mice²² (strain 129-*Fyn*^{tm1Sor/J}) and their corresponding *Fyn*^{+/+} control strain (129S1/SvImJ), were also obtained from The Jackson Laboratory. All mice were female and were 8–14 weeks old. All use and care of the mice followed the NIH guidelines.

Antibodies and Secondary Reagents

The following antibodies and secondary reagents were used: anti-Qa-2, clone 69H1-9-9 (eBioscience, San Diego, CA, USA); anti-Qa-2-biotin, (clone 1-1-2; BD PharMingen, San Diego, CA, USA); rabbit anti-mouse IgG, F(ab)₂ fragment (ICN/Capell, Aurora, OH, USA); anti-CD3-FITC (Biolegend, San Diego, CA, USA); anti-Lck, clone 3G10 (Sigma, St Louis, MO, USA); anti-Lck-biotin, prepared from clone 3G10 using NHS-biotin (Pierce Chemical, Rockford, IL, USA); streptavidin-PE/Alexafluor 647 (Invitrogen, Carlsbad, CA, USA); anti-CD16/CD32 (BD PharMingen); mouse IgG2a (isotype control, BD PharMingen); anti-mouse IgG-horseradish peroxidase (HRP; ECL kit; Amersham, Piscataway, NJ, USA); anti biotin-HRP (Cell Signaling Technology, Beverly, MA, USA).

Lymphocytes and Culture Conditions

Single cell suspensions were prepared by dispersing splenocytes in Dulbecco's Modification of Eagle's Medium (DMEM; GIBCO/Invitrogen, Grand Island, NY, USA). The cell

suspension was centrifuged over Ficoll/Hypaque (Histopaque 1083, Sigma). T cells were enriched from the resulting mononuclear cells using negative depletion column kits (R&D Systems, Minneapolis, MN, USA). The cells were suspended in culture medium consisting of DMEM supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 1 µg/mL gentamicin, and 50 µM 2-mercaptoethanol (all from Sigma). The cell preparations consistently contained $90 \pm 1\%$ CD3⁺ T cells and $<1\%$ CD19⁺ B cells, as determined by immunostaining/flow cytometry (data not shown).

T cells were stimulated via cross-linking Qa-2 in a two-step process. Cells (2×10^6 /mL) were incubated for 30 min at room temperature in culture medium containing 1 µg/mL anti-Qa-2 primary anti-body. For cross-linking, an equal volume of goat anti-mouse IgG secondary antibody in culture medium was added to the cells, to a final concentration of 50 µg/mL. Cells were cultured without removal of excess primary and secondary antibodies. A second signal was provided in the form of PMA (Sigma) at 5 ng/mL. As a positive control, activation was induced by stimulation with ionomycin (0.25 µg/mL) and PMA (5 ng/mL). PMA and ionomycin had been diluted into culture medium from stock solutions dissolved in dimethyl sulfoxide (DMSO).

Cells were incubated in triplicate cultures in 0.2 mL volumes at 10^6 cells/mL in round-bottom, 96-well culture plates at 37°C in a humidified, 7% CO₂ incubator.

For assays in which kinase inhibitors were used, the cells were exposed to inhibitor for 15 min at room temperature before adding primary antibody. The concentration of inhibitors used was in all cases at or below concentrations at which these inhibitors have been reported to be specific for the indicated enzyme. The stated inhibitor concentration was maintained through all steps of the stimulation protocol and during the culture period. The following inhibitors were used, diluted into culture medium from 10 mM stocks in DMSO: PP2, Src family kinase inhibitor²³ (Calbiochem, San Diego, CA, USA); PP3, inactive analog of PP2 (Calbiochem); wortmannin, PI-3 kinase inhibitor²⁴ (Sigma); API-2, Akt inhibitor²⁵ (Developmental Therapeutics Programs, National Cancer Institute; NIH, Bethesda, MD, USA, compound no. NSC154020 of the NCI Structural Diversity Set); LY294002, a PI-3 kinase inhibitor structurally unrelated to wortmannin,²⁶ was diluted from a 50 mM stock in DMSO (Cell Signaling Technology).

Assay of Cell Proliferation

Cell proliferation was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which correlates well with proliferation.²⁷ After 44–48 hr of culture, 20 µL of MTT [Sigma; 2 mg/mL in phosphate buffered saline (PBS), 0.01 M sodium phosphate, pH 7.4] were added to cultures. The cultures were incubated for a further 24 hr, 50 µL PBS added to each well, the plates centrifuged at $500 \times g$ for 5 min, and 200 µL of supernatant removed. Precipitate was solubilized by vigorous pipetting in 150 µL isopropanol made 0.1% in HCl, and the absorbance at 565 nm for each well was read with a Synergy microplate reader (Bio-Tek, Winooski, VT, USA), subtracting blank values (medium alone plus MTT reagents) from all readings. Absorbance was reported as the mean of triplicate cultures $\pm$ S.D. Absorbance values were compared by Student's *t*-test. For cultures in which the responses of cells from Fyn^{-/-} and control mice were compared, mean absorbance values for appropriate unstimulated cell controls were subtracted from mean experimental absorbance values, and the percentage of the response of Fyn^{-/-} cells was calculated *versus* that of the wild-type cells.

Immunostaining/Flow Cytometry

Splenic lymphocytes were suspended at 10^6 cells/50 μ L in PBS with 1% bovine serum albumin (BSA; Sigma) and 0.1% sodium azide (Sigma) at 4°C. The cells were treated for 15 min with anti-CD16/32 (20 μ g/mL) to block Fc receptors. The cells were stained with anti-CD3-FITC and anti-Qa-2-bio-tin (10 μ g/mL each; 30 min), followed by washing and addition of streptavidin-phycoerythrin/Alexafluor 647 (1.25 μ g/mL; 30 min). Data were collected using a BD FACScan flow cytometer, using Cell-Quest software (BD Immunocytometry, San Jose, CA, USA) and appropriate compensation.

Co-immunoprecipitation of Qa-2 Associated Proteins

Proteins complexed with Qa-2 in resting T cells were assessed using a modification of the solid phase capture method of Lysechko and Ostergaard.²⁸ Petri plates (60 mm; not coated for tissue culture) were incubated overnight at 4°C with anti-Qa-2 or isotype control IgG in PBS at 10 μ g/mL. After being washed with PBS three times, the plates were blocked with 2% BSA at 37°C for 45 min. T cells were added (10^7 cells in 1 mL of serum-free DMEM) and the plates incubated for 20 min at 37°C. Unbound cells were drained from the plate, and the remaining cells lysed for 10 min on ice with 0.5 mL 1% NP-40 containing protease inhibitors (Halt cocktail; Pierce Chemical) and phosphatase inhibitors (Cocktail 2, Sigma). The plates were then extensively washed with cold lysis buffer to remove cellular debris, drained, and floated in a 55°C water bath. SDS-PAGE reducing sample buffer (Pierce Chemical) was added immediately (100 μ L/plate). Initially, the buffer wetted the surface of the plate and then was harvested as it rapidly beaded after protein was eluted from the plate surface. The eluted material from each plate was used for two electrophoresis lanes unless otherwise stated.

Eluted proteins were separated on 4–20% gradient gels (Pierce Chemical), and blotted onto nitrocellulose. Blots were blocked for 1 hr in PBS/0.1% TWEEN 20 containing 5% BSA. After three additional washes in PBS/TWEEN, the membranes were incubated at 4°C overnight with antibody in 5% BSA/PBS/TWEEN. After further washing, the blots were incubated with anti-mouse-HRP or anti-biotin-HRP and developed and visualized by chemiluminescence (ECL kit, Amersham) and CL-XPosure film (Pierce Chemical).

Results

Effect of Kinase Inhibitors on the Proliferative Response to Qa-2 Cross-Linking

In the first set of experiments, the effect of kinase inhibitors on the proliferative response to Qa-2 cross-linking was tested. In the experiments depicted in Fig. 1a–d, splenic T cells gave a highly significant response to Qa-2 cross-linking in the presence of PMA, compared with control cells treated with secondary antibody and PMA only ($P < 0.001$). Treatment of the T cells with the general Src family kinase inhibitor, PP2, resulted in dose-dependent inhibition of the proliferative response, with significant inhibition at the lowest dose tested, 0.1 μ M ($P < 0.005$; Fig. 1a). The inactive analog of PP2, PP3, had no effect on proliferation ($P > 0.05$), even at the concentration equivalent to the PP2 concentration that gave complete inhibition, 5 μ M. (Complete inhibition is defined as MTT absorbance values equal to or less than those of control cultures treated with PMA and secondary antibody only). This result indicates that the activity of one or more Src family kinases is required for signaling in this system.

To determine whether PI-3 kinase is involved in signaling via Qa-2, T cells were pre-treated with the irreversible PI-3 kinase inhibitor, wortmannin. Wortmannin also inhibited the proliferative response to Qa-2 cross-linking when used at a concentration of 25 nM (Fig. 1b, $P < 0.001$). Near-complete inhibition was achieved at a concentration of 50 nM wortmannin, and full inhibition was observed at a concentration of 100 nM.

In confirmation of these results, a second PI-3 kinase inhibitor, LY294002, was also used and found to significantly inhibit stimulation via Qa-2 cross-linking (Fig. 1c, $P < 0.01$ at $1.25 \mu\text{M}$). At $5 \mu\text{M}$, inhibition of the response to cross-linking Qa-2 was nearly complete, with full inhibition observed at $10 \mu\text{M}$ LY294002. Taken together, the results using the two kinase inhibitors indicate that PI3-kinase is involved in signaling via Qa-2.

Additionally, T-cell cultures were treated with API-2, which has been shown to inhibit Akt specifically at concentrations up to $10 \mu\text{M}$.²⁵ Significant inhibition of the response to Qa-2 cross-linking in the presence of PMA was observed at a concentration of $0.025 \mu\text{M}$ ($P < 0.005$), and complete inhibition was observed at $1 \mu\text{M}$ (Fig. 1d), indicating that this kinase, a major downstream effector of PI-3 kinase, is also required for signaling via Qa-2.

The DMSO vehicle in which these various inhibitors were dissolved had no effect on cell proliferation at the highest concentration used in these studies (1:1000 dilution of DMSO; data not shown).

Co-immunoprecipitation of Src Family Kinases with Qa-2

We hypothesized that the most likely candidates for the Src family kinases required for Qa-2 signaling were p59Fyn and p56Lck, in that they are the membrane-proximal kinases involved in signaling via the T-cell receptor (TCR).²⁰ To determine whether either of these molecules is associated with Qa-2 in resting T cells, a modification of the solid phase complex capture technique of Lysechko and Ostergaard²⁸ was used, in which antibody adsorbed to a plastic surface was allowed to bind to Qa-2 on intact cells. The cells were then lysed and captured protein was eluted and analyzed by Western blotting. By using this technique, we hoped to avoid disrupting potential protein–protein interactions while avoiding the artifacts that might result when lipid raft proteins are co-immunoprecipitated from standard detergent preparations.²⁹

When T cells were added to the anti-Qa-2 coated plates, cells were clearly observed adhering to the plates. In contrast, no cells adhered to control plates coated with non-specific IgG of the same isotype. Eluates of these control plates were analyzed by Western blotting, using one-half of the sample obtained from each plate per electrophoresis lane. Neither Qa-2, Fyn, nor Lck was detected in the control samples, although IgG heavy chain was detected on the blots in which anti-mouse IgG-HRP was used as a secondary reagent (Fig. 2, lanes 1 and 4; no IgG band was expected or observed in the control lane, lane 7, developed with anti-biotin-HRP).

When the material eluted from anti-Qa-2 coated plates was analyzed, in addition to the expected IgG band, a very strong signal corresponding to Qa-2 was observed, providing a positive control of the effectiveness of capture (lane 3). It should be noted that a smaller sample of eluate was loaded into lane 3, corresponding to the eluate from 2×10^6 cells, compared with 5×10^6 cell-equivalents of eluate loaded into other lanes, and that even using this smaller sample, only a 20 s exposure was required to develop the blot. Fyn was also readily detected in the immunoprecipitated sample (lane 6; 1 min exposure), but no Lck was observed (lane 9; 30 min exposure). We conclude that Fyn is associated with Qa-2 in resting T cells, but that Lck, if present, is present in amounts below the level of detection in our system. Identical results were obtained in a duplicate experiment and in an experiment using conventional co-immunoprecipitation, in which the precipitation was initiated by adding anti-Qa-2 to a T-cell lysate (data not shown).

Response of Fyn^{-/-} Mice to Cross-linking of Qa-2

To establish whether Fyn is required for the response of resting T cells to Qa-2 cross-linking, cells from Fyn knockout mice²² were analyzed. Initially, we determined whether the T cells

of the Fyn^{-/-} mice expressed levels of Qa-2 equivalent to those of cells from Fyn^{+/+} (wild-type) control animals. Splenic lymphocytes from two of each type of mouse were pooled, immunostained, and analyzed by flow cytometry. As was found by the originators of this strain,²² we found the phenotype of splenic T cells of the Fyn^{-/-} mice to be normal with respect to the expression of CD3 (upper quadrants, Fig. 3). Also, the percentages of cells expressing both CD3 and Qa-2 (upper right quadrant, Fig. 3) were comparable. More importantly, T cells from both Fyn^{-/-} and the wild-type control mice expressed high levels of Qa-2, a characteristic required for good stimulation of cells via Qa-2 cross-linking.³⁰ The median channel fluorescence for Qa-2 expression by CD3⁺ cells (upper quadrants, Fig. 3) was the same, 2743 for wild-type cells, and 2751 for Fyn^{-/-} cells.

Cells from wild-type mice gave a robust proliferative response to Qa-2 cross-linking (Fig. 4a). As expected, this response required both cross-linking secondary antibody and PMA as a second signal. In contrast, the cells from Fyn^{-/-} mice were nearly unresponsive to Qa-2 cross-linking; their response was significantly lower than that of wild-type cells (Fig. 4a,b; $P < 0.001$). Nonetheless, the Fyn^{-/-} cells were capable of giving a strong proliferative response to a stimulus which by-passes membrane-proximal components of T-cell stimulation, ionomycin plus PMA (Fig. 4b). Thus, the Qa-2-mediated signaling pathway requires Fyn for the proliferative response to Qa-2 cross-linking.

Discussion

In this paper, we show that the signaling mechanism by which cross-linking Qa-2 induces resting T cells to proliferate depends on Fyn, PI-3 kinase, and Akt. The membrane-proximal molecules that are involved in T-cell activation downstream of the TCR are the Src family kinases, Lck and Fyn, molecules that are very similar in structure.²⁰ Although it was initially thought that Fyn was functionally redundant to Lck during TCR-mediated activation, different, but sometimes interdependent roles have now been ascribed to the two kinases.³¹ In our experiments, the proliferative response of T cells to Qa-2 cross-linking in the presence of PMA was totally suppressed using the highly specific Src family kinase inhibitor, PP2. Additionally, the response was nearly absent among T cells from Fyn knockout mice, despite those cells expressing high levels of Qa-2.

Using a modified co-immunoprecipitation technique,²⁸ we detected Fyn, but not Lck, in complex with Qa-2. This result is consistent with other reports of the association of Src family kinases with GPI-linked proteins.^{32,33} In conventional immuno-precipitation, antibody is added to cell lysates. The modification we used, in which intact cells were bound to solid phase precipitating antibody before lysis,²⁸ allows a more specific capture of lipid raft proteins (H. Ostergaard, personal communication). In contrast, extraction into lipid raft fractions prior to immunoprecipitation can permit coalescence of microdomains, possibly inducing artifactual associations among membrane proteins.^{29,34,35}

While our results with this technique and with the Fyn knockout mice point to Fyn as being of prime importance in signaling via Qa-2, as yet we cannot rule out a potential, cooperative role for Lck. For example, Lck may be drawn into a complex with Qa-2 only after activation has been initiated. The cells we studied were not activated; binding to the plate-bound anti-Qa-2 antibody that we used is insufficient to activate cells (SR De Fazio, unpublished data). However, recent data of Lovatt et al.³⁶ suggest that Lck is probably not involved in our system. These workers found that activation of Fyn alone fails to stimulate the production of diacylglycerol (DAG), whereas activated Lck efficiently promotes the formation of DAG. Activation of Fyn but not Lck following Qa-2 cross-linking is thus consistent with the requirement for a second signal provided by PMA, a DAG analog.

There are many potential downstream consequences of Fyn activation that are yet to be explored in our system. However, a review of the literature on T-cell costimulation suggested to us that PI-3 kinase²¹ might be involved in Qa-2 signaling. First, Qa-2 can serve as a co-stimulatory molecule *in vitro*, in that co-cross-linking CD3 and Qa-2 can markedly augment the response to anti-CD3.^{30,37} Second, PI-3 kinase activation is involved in the costimulation of T cells via CD28.²¹ Hypothesizing that the costimulatory ability of Qa-2 might proceed via this same pathway, we found that the proliferative response to Qa-2 cross-linking was indeed abrogated by two PI-3 kinase inhibitors. Moreover, the src-homology domains of Fyn are known to bind to the regulatory p85 domain of PI-3 kinase, with resulting activation.³⁸

A major downstream effector of PI-3 kinase is Akt (also known as phosphokinase B). Akt is an integrator of cell signals whose activity promotes several processes useful both in T-cell activation and embryo development, such as progression through the cell cycle and inhibition of apoptosis.²¹ Thus our finding that Qa-2 cross-linking requires the activity of PI-3 kinase and Akt is consistent with processes that would promote both the activation of T cells and embryo growth.

The appropriateness of T-cell activation as a model for the mechanism by which Qa-2 influences embryo development is supported by three considerations. The first of these is the marked conservation of signaling pathways that exists among cells. Second, the three kinases which we have implicated in the T-cell model of Qa-2 signaling, Fyn, PI-3 kinase, and Akt, are expressed and active in preimplantation embryos and required for normal development.^{39–43} Third, recent data obtained using congenic mouse strains that differ only in the *Ped* gene, which encodes Qa-2 protein, are consistent with Qa-2 exerting its developmental effects via the PI-3-kinase/Akt signaling pathway. Purnell et al.⁴⁴ found that while a smaller proportion of B6.K1 (Qa-2 negative) embryos developed *in vitro* to the blastocyst stage than did B6.K2 (Qa-2 positive) embryos, the B6.K1 embryos produced more than twice as much platelet-activating factor (PAF) per embryo. PAF is an autocrine trophic factor, and signals from its receptor on preimplantation embryos are transduced via PI-3 kinase.⁴⁰ We speculate that the excess secretion of PAF by B6.K1 embryos represents compensation for inadequate signaling via the PI-3 kinase/Akt pathway in the absence of Qa-2.

In summary we present Fig. 5, a model of our current understanding of the way in which Qa-2 signaling brings about cell division. Qa-2 is hypothesized to first interact with its natural ligand, as yet unknown, which is substituted in our model system by anti-Qa-2 and cross-linking antibody. The signal initiated by ligand binding is then transduced across the cell membrane. Because the GPI linkage of Qa-2 is inserted only into the outer leaflet of the membrane, this transduction is probably mediated by an associated transmembrane protein yet to be identified. Lipid modification of Fyn allows it to localize to the cytoplasmic surface of raft microdomains⁴⁵ along with Qa-2. Fyn is then activated and in turn activates PI-3 kinase.³⁸ A lipid product of PI-3 kinase activity, phosphatidylinositol 3,4,5-trisphosphate (PIP₃), is formed on the inner surface of the cell membrane, allowing recruitment to the membrane of Akt and its subsequent activation. The downstream effectors of Akt are known to promote cell division, cell survival, glucose uptake, and protein translation,^{21,46} clearly effects that would support both T-cell activation and embryo cleavage.

We have identified steps important in understanding how Qa-2 can function as a signaling molecule. However, our data represent only the beginning of the elucidation of the steps by which Qa-2 mediates regulation of cell division. The results reported in this paper suggest that the future extension of these studies to embryos should take into account the key roles that Fyn, PI-3 kinase, and Akt play in signaling via Qa-2.

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Fig. 1.

Effect of kinase inhibitors on the stimulation of C57BL/6/J resting T cells with anti-Qa-2 monoclonal antibody, cross-linking secondary anti-body, and phorbol myristate acetate (PMA). Control cultures were unstimulated (treated with PMA and secondary antibody only). Effect of: (a) the SFK inhibitor, PP2, and its inactive analog, PP3; (b) the PI-3 kinase inhibitor, wortmannin; (c) the PI-3 kinase, inhibitor LY294002; (d) the Akt inhibitor, API-2. Asterisks indicate statistical comparison of responses of stimulated cells treated with inhibitor to stimulated cells cultured without inhibitor: $**P < 0.01$. The experiments (a) and (b) were performed independently, while (c) and (d) were performed with the same cell preparation and thus share control values. All experiments are representative of two to four replicates.

Fig. 2.

Co-immunoprecipitation with plate-immobilized antibody to Qa-2 of Src family kinases from untreated splenic T cells. Eluates from this procedure were subjected to Western blotting using antibodies as indicated: Lanes 1–3, anti-Qa-2 plus anti-mouse IgG–HRP; Lanes 4–6, anti-Fyn plus anti-mouse IgG–HRP; Lanes 7–9, anti-Lck-biotin plus anti-biotin–HRP. Lanes marked 'cont' = eluate from plates coated with isotype control IgG as a negative control; '+' = lysate from 5×10^5 untreated cells as a positive control; 'IP' = complexes precipitated with anti-Qa-2 (eluate equivalent to 2×10^6 cells, lane 3; 5×10^6 cells, lanes 6 and 9).

Fig. 3. Expression of Qa-2 by T cells from Fyn knockout mice. Splenic lymphocytes from (a) Fyn ^{+/+} and (b) Fyn ^{-/-} mice were immunostained with anti-CD3-FITC and anti-Qa-2-biotin plus streptavidin-AlexaFluor 647/PE and analyzed by flow cytometry. The percentages of Qa-2-positive T cells (upper right quadrants) are indicated. This experiment is representative of duplicate experiments in which each sample represents the pool of two spleens.

Fig. 4.

(a) The proliferative response of T cells from Fyn knockout mice and Fyn+/+ wild-type mice to cross-linking of anti-Qa-2 in the presence of phorbol myristate acetate (PMA). Controls are included to demonstrate that the full combination of primary antibody, secondary antibody, and PMA is required for a response of wild-type cells. (b) Comparison of the responses of the Fyn knockout and wild-type cells to Qa-2 cross-linking plus PMA and to ionomycin plus PMA in an independent experiment. Cells were pooled from two spleens from each type of mouse in both experiments. Asterisks indicate statistical comparison of responses of Fyn-/- cells to Fyn+/+ (wild-type cells): *** $P < 0.001$. Data are representative of duplicate experiments.

Fig. 5.
Model of Qa-2 regulation of cell division (see Discussion).