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Biochemistry

A 20-kDa protein associated with the murine T-cell antigen receptor is phosphorylated in response to activation by antigen or concanavalin A

(T-cell activation/phosphorylation/receptor-associated protein/anti-clonotypic antibody/photoactivatable affinity reagent)

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ABSTRACT **Antigen or concanavalin A activation of a murine T-cell hybrid specific for pigeon cytochrome *c* and restricted to the E^k_α:E^k_β immune response-associated (Ia) molecule resulted in phosphorylation of a 20-kDa protein that was specifically coprecipitated by a monoclonal antibody binding the T-cell antigen receptor. There was no evidence for phosphorylation of the antigen receptor itself. The phosphorylation of the 20-kDa polypeptide was dependent on the concentration of antigen or lectin used to activate the T-cell hybrid and reached a maximum 40 min after the addition of antigen. Moreover, the phosphorylation induced by antigen in the presence of Ia molecule-bearing B cells was specifically blocked by the addition of appropriate anti-Ia molecule monoclonal antibodies. The 20-kDa protein was also radioiodinated with a hydrophobic photoactivatable labeling reagent. The amount of iodinated 20-kDa protein immunoprecipitable with the anti-receptor antibody did not increase with T-cell activation, indicating that the phosphorylation occurred on a molecule that was constitutively associated with the antigen receptor. Concanavalin A also induced phosphorylation of a 20-kDa polypeptide in a second antigen-specific major histocompatibility complex-restricted T-cell hybrid. Again, the phosphorylated polypeptide was precipitated only by a monoclonal antibody specific for the antigen receptor on this hybrid. Thus, the antigen or concanavalin A-induced activation of T-cell hybrids results in the rapid phosphorylation of a 20-kDa protein that is associated with the T-cell antigen receptor.**

The activation of most T lymphocytes requires the interaction of a clonotypic antigen receptor on the T-cell membrane with a foreign antigen and a gene product of the major histocompatibility complex (MHC) expressed on the surface of antigen-presenting cells such as macrophages or B cells. In the past 2 years, the antigen receptors on these so-called MHC-restricted T cells have been extensively characterized. Monoclonal antibodies that bind the receptors have been used to isolate these molecules from cloned murine (1–6) and human (7–11) T cells. The receptors have a disulfide-linked heterodimeric structure consisting of α and β chains each of $\approx$40 kDa. cDNA clones encoding the murine (12, 13) and human (14) β chain and the murine α chain (15, 16) have been identified. Sequence information indicates that these chains have both variable and constant domains and display considerable amino acid homology to immunoglobulin molecules.

The biochemical events that follow the interaction of the T-cell receptor with antigen and a product of the MHC are poorly understood. However, recent work has demonstrated that the activation of protein kinases accompanies ligand binding to and the activation of a variety of receptors including those for insulin (17), insulin-like growth factors (18), epidermal growth factor (19), and platelet-derived growth factor (20). Therefore, we initiated studies to investigate whether antigen- or lectin-induced T-cell activation was associated with specific phosphorylation events. In these experiments, we used two cloned, MHC-restricted T-cell hybrids and the monoclonal antibodies directed against the respective antigen-specific receptors on these T cells. These studies failed to demonstrate any phosphorylation of the antigen receptor itself; instead, we observed a T-cell activation-dependent phosphorylation of a 20-kDa protein that appears to be closely associated with this receptor.

MATERIALS AND METHODS

Cells. 2B4, the pigeon cytochrome *c*-specific E^k_α:E^k_β immune response-associated (Ia) molecule-restricted T-cell hybrid grows continuously in culture in a 2:1 mixture of RPMI 1640 medium to Eagle's Hanks' amino acids medium supplemented with 10% fetal calf serum. In response to antigen and in the presence of an antigen-presenting cell bearing the E^k_α:E^k_β Ia molecule, the T-cell hybrid secretes interleukin 2 (IL-2), which can be measured by a sensitive bioassay (3). A keyhole limpet hemocyanin-specific A^k_α:A^k_β Ia molecule-restricted T-cell hybrid, SKK-9.11 (21), as well as the A^k_α:A^k_β- and E^k_α:E^k_β-bearing B-cell tumor, LK 35.2 (22), which was used as the antigen-presenting cell in these experiments, were kindly provided by P. Marrack and J. Kappler (National Jewish Hospital, Denver, CO). Both T-cell hybrids secrete IL-2 when stimulated with concanavalin A in the absence of any presenting cells.

Antigens and Lectins. Pigeon cytochrome *c* was obtained from Sigma. The preparation of the COOH-terminal fragment 81–104 by CNBr cleavage has been described (3). A peptide corresponding to an analogue of the COOH-terminal residues of tobacco hornworm moth cytochrome *c* having a glutamic acid as its NH_2 terminus (moth 93–103, 93E) was prepared by solid-phase synthesis by Lee Maloy (National Institutes of Health) and was purified by reversed-phase, high-pressure liquid chromatography. Concanavalin A was obtained from Pharmacia.

Monoclonal Antibodies. The clonotypic antibody A2B4-2 specifically binds to and inhibits antigen-induced IL-2 release from only the 2B4 hybrid. In addition, this antibody immunoprecipitates the heterodimeric antigen receptor from this cell (3). The monoclonal antibody KJ16-133, described

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Abbreviations: Ia, immune response-associated; MHC, major histocompatibility complex; ^{125}I-TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]-iodophenyl)diazirine; IL-2, interleukin 2.

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by Haskins *et al.* (21), binds to the T-cell antigen receptor on the SKK-9.11 cell but not to the clonotypic receptor on 2B4. Culture supernatant fluid containing this antibody was provided by J. Kappler and P. Marrack. An anti-Thy1 monoclonal antibody, G7, in the form of culture supernatant fluid was provided by K. Gunter (National Institutes of Health) (23). Cells producing the anti-Ia monoclonal antibodies 10.2.16 (anti-A^k_α:A^k_β; γ_{2b}) (24), 17.3.3 (anti-E^k_α:E^k_β; γ_{2a}), and 14.4.4 (anti-E^k_α; γ_{2a}) (25) were provided by D. Sachs (National Institutes of Health).

T-Cell Labeling and Stimulation. The phosphorylation experiments were performed with 2B4 and SKK-9.11 T cells incubated with [^{32}P]orthophosphate to label their intracellular ATP pools. For this incubation, the cells were harvested from tissue culture flasks (5–7 × 10^5 cells per ml), washed 3 times in phosphate-free RPMI 1640 medium at 4°C, and resuspended in this medium with 10% dialyzed fetal calf serum (medium A). The B cells were similarly harvested, washed, and resuspended. T cells (1–2 × 10^9) were then incubated with 10 mCi of [^{32}P]orthophosphate (ICN; carrier free, 285 Ci/mg; 1 Ci = 37 GBq) in 50 ml of medium A in a 150-cm^2 flask at 37°C for 2 hr. For activation, the ^{32}P-loaded T cells and the washed B cells, each resuspended at a concentration of 5 × 10^7 cells per ml, were mixed 1:1 in 15- or 50-ml conical tubes. Antigen, lectin, or anti-Ia antibodies were added to the B cells prior to the addition of ^{32}P-loaded T cells. The tubes were then incubated in a shaking water bath at 37°C.

Surface iodination of the T cells was performed using the lactoperoxidase-catalyzed reaction as described (3). Intrinsic membrane proteins were iodinated with 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (^{125}I-TID), a photoactivatable hydrophobic labeling reagent (26). Prior to this labeling, 2 × 10^8 2B4 cells in 4 ml of medium were either activated or not with 10 μg of concanavalin A per ml. After 1 hr in a 37°C shaking water bath, the two preparations were washed 3 times and resuspended in 5 ml of Hanks' basic salt solution with 15 mM glutathione at pH 7.6 and 4°C. The cells were transferred to glass tubes and 625 μCi of ^{125}I-TID (Amersham; 5 mCi/ml) was added to each tube. The cells were incubated on ice for 5 min and then exposed in an ice water bath to a 200 W mercury lamp at 15 cm for 15 min. The cells were washed 3 times with Hanks' solution prior to lysis.

Cell Lysis, Immunoprecipitation, and NaDodSO$_4$/Polyacrylamide Gel Analysis. After activation and/or radiolabeling, cells were washed 3 times with ice-cold phosphate-buffered saline containing the following phosphatase inhibitors: 0.4 mM sodium vanadate/0.4 mM EDTA/10 mM sodium fluoride/10 mM sodium pyrophosphate. The cell pellets were then solubilized with a lysis solution containing 0.6% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate/300 mM NaCl/50 mM Tris·HCl, pH 7.6/0.1 mM phenylmethylsulfonyl fluoride/leupeptin at 100 μg/ml, and the above phosphatase inhibitors and were allowed to stand on ice for 30 min. The lysates were centrifuged at 11,000 × *g* for 15 min to remove nuclei and debris. Supernatant fluid containing the KJ16.133 monoclonal antibody was adsorbed to Sepharose beads covalently conjugated with the MAR 18.5 anti-rat κ-chain monoclonal antibody (27). The other monoclonal antibodies, in the form of culture supernatant fluids, were preadsorbed to protein-A Sepharose (Pharmacia) beads for 2 hr at 4°C. The beads were washed once in lysis buffer. The antibody–Sepharose complexes were incubated on ice with the postnuclear supernatant fluids for 45–60 min. The resins were then washed 3 times with a buffer containing 0.1% Triton X-100/300 mM NaCl/50 mM Tris·HCl, pH 7.6/phenylmethylsulfonyl fluoride/leupeptin/phosphatase inhibitors, and the associated ^{32}P-labeled proteins were eluted from the beads by boiling for 5 min in 1% NaDodSO$_4$/50 mM Tris·HCl, pH 6.8/20% (vol/vol) glycerol/0.1% phenol red/5% 2-mercaptoethanol. Samples were electrophoresed in 16.5% polyacrylamide gels cross-linked with Acrylaide and attached to Gel-Bond (FMC, Rockland, ME) using the buffer system of Laemmli (28). After drying, gels were exposed to Kodak x-ray film at −70°C for 20–40 hr, using intensifying screens (DuPont Lightning Plus). Autoradiographs were analyzed using a Hoefer scanning densitometer. The extent of phosphorylation was determined by measuring the areas under the densitometric peaks and is expressed in arbitrary units.

RESULTS

The T-cell antigen receptor can be radioiodinated on the surface of 2B4 cells and specifically immunoprecipitated using the monoclonal antibody A2B4-2. When such immunoprecipitates were analyzed, it was determined that the antigen receptor on the 2B4 cell exists in two forms. The 42- to 44-kDa α chain of the heterodimer is associated either with a β chain of apparent molecular size 42–44 kDa (β) or with one of 46–48 kDa (β′). The upper band observed on the NaDodSO$_4$/polyacrylamide gel run under reducing conditions (Fig. 1, lane A) represents only the 46- to 48-kDa β′ chain of the receptor, while the lower band contains both the α and β chains of the receptor (29).

To address the question of whether phosphorylation of the antigen receptor accompanied T-cell activation, T cells were loaded with [^{32}P]orthophosphate and then activated using either an appropriate antigen and the Ia-bearing B-cell tumor LK 35.2, or the lectin concanavalin A in the absence of B cells. In no instance was an activation-associated phosphorylation of the clonotypic receptor observed when ^{32}P-labeled cell lysates were immunoprecipitated using the A2B4-2 monoclonal antibody and the immunoprecipitates analyzed on 10% polyacrylamide gels (data not shown). However, under both activation regimens, polypeptides of lower molecular size appeared to be phosphorylated and specifically immunoprecipitated. To improve the resolution of these

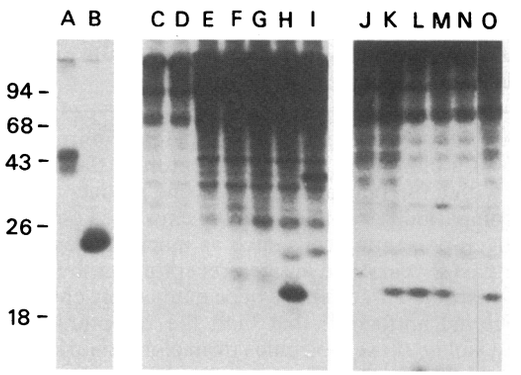

FIG. 1. NaDodSO$_4$/polyacrylamide gel electrophoresis analysis of radioiodinated and phosphorylated T-cell polypeptides. Lysates from surface ^{125}I-labeled 2B4 cells were immunoprecipitated with the A2B4-2 anti-receptor monoclonal antibody (lane A) or with G7, an anti-Thy1 monoclonal antibody (lane B). In a separate experiment, the 2B4 T cells were labeled with [^{32}P]orthophosphate and incubated for 60 min at 37°C alone (lane C), with 100 μM antigen (moth 93–103, 93E) (lane D), with the B-cell tumor LK 35.2 (lanes E, F, and J) with B cells plus antigen (lanes G–I, and K), with concanavalin A [20 μg/ml (lane L), 10 μg/ml (lane M), 5 μg/ml (lane N)] or with the LK 35.2 B cells plus 10 μg of concanavalin A per ml (lane O). The cells were washed, lysed, and immunoprecipitated with the monoclonal antibodies A2B4-2 (lanes C, D, F, H, and J–O) or G7 (lane I), or with control supernatant fluids from SP2/0 cells without antibody (lanes E and G). Gel electrophoresis and autoradiography were performed as described. Lanes C–I and J–O represent two separate experiments. Numbers on left are kDa.

polypeptides, 16.5% polyacrylamide gels were used (Fig. 1). Many ^{32}P-labeled polypeptides were seen in the immunoprecipitates, but the presence of nearly all of these bands was independent of activation requirements, and they were also present when the anti-receptor antibody was omitted. In contrast, a ^{32}P-labeled polypeptide of 20–22 kDa (pp20) appeared to be present only when the A2B4-2 antibody was used (Fig. 1, lanes H and K). This polypeptide was seen only when the strict requirements for antigen activation of the T cells were met. No ^{32}P-labeled pp20 was seen when B cells were omitted and T cells alone were incubated in the absence (lane C) or presence (lane D) of appropriate antigen. Similarly, when T cells and B cells were co-incubated without antigen, no ^{32}P-labeled pp20 was present in A2B4-2 immunoprecipitates (lanes F and J).

In addition to requiring the appropriate combination of components for antigen-mediated activation, the monoclonal antibody specific for the clonotypic receptor (A2B4-2) was essential for immunoprecipitation of ^{32}P-labeled pp20. Replacement of A2B4-2 with supernatant fluids of the nonsecreting parent myeloma cell SP2/0 (lanes E and G) or with the anti-Thy1 monoclonal G7 (lane I) resulted in no ^{32}P-labeled pp20 on the gel. A second ^{32}P-labeled polypeptide of 25 kDa (pp25) was also specifically precipitated by the anti-receptor monoclonal antibody A2B4-2 (lane H). However, the phosphorylation of pp25 did not appear to share the strict requirements for activation seen with ^{32}P-labeled pp20. The increase in intensity in pp25 seen in Fig. 1 (compare lanes H and F) was not seen in all experiments. It is noteworthy that the 2B4 cells bear the surface molecule Thy1, which can be radioiodinated and precipitated using G7 (lane B) and that two phosphoproteins were specifically precipitated by G7 (lane I). One of these polypeptides comigrated with the surface-labeled Thy1 and the other appeared to be ≈43 kDa. However, the phosphorylation of these ^{32}P-labeled polypeptides also did not correlate with T-cell activation (data not shown).

When T cells were activated with the lectin concanavalin A, ^{32}P-labeled pp20 was immunoprecipitated by the A2B4-2 antibody. The amount of ^{32}P-labeled pp20 was dependent on the concentration of concanavalin A used (lanes L–N) and, in this regard, resembled lectin-mediated stimulation of IL-2 release from the 2B4 cells (data not shown). In contrast to activation by antigen, lectin did not require B cells. Likewise, the amount of ^{32}P-labeled pp20 present in A2B4-2 immunoprecipitates was independent of the inclusion of the B cells (compare lanes M and O).

An important characteristic of the activation of the 2B4 cells is that it is MHC-restricted, as well as being specific for pigeon cytochrome *c*. Antigen activation of 2B4 is blocked by monoclonal antibodies against the E^k_α:E^k_β Ia surface molecules, but not by antibodies against A^k_α:A^k_β Ia molecules. The latter Ia molecules are also expressed on the surface of the antigen-presenting B cell used here but are not involved in the activation of this particular T cell. The addition of ascites containing an anti-E^k_α:E^k_β antibody (17.3.3) completely inhibited the appearance of ^{32}P-labeled pp20 in A2B4-2 immunoprecipitates, even when the ascites was added at a 1:2500 dilution (Fig. 2, lanes C–E). A second anti-E^k_α antibody (14.4.4) also inhibited, although it displayed an apparently lower titer of inhibitory activity (lanes F–H). In contrast, little or no inhibition was seen when an anti-A^k_α:A^k_β monoclonal antibody (10.2.16) was included (lanes I and J). This monoclonal antibody had no greater effect than addition of an ascites induced by the SP2/0 myeloma cell, which contained no anti-Ia antibody (lanes K–M). Thus, the apparent induction of phosphorylation of ^{32}P-labeled pp20 required recognition by the T cell of E^k_α:E^k_β Ia molecules in addition to pigeon cytochrome *c*.

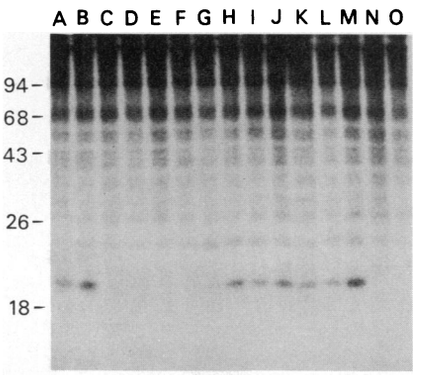

FIG. 2. Inhibition of pp20 phosphorylation by anti-E^k_α:E^k_β but not anti-A^k_α:A^k_β monoclonal antibodies. [^{32}P]Orthophosphate-labeled 2B4 cells were incubated for 60 min at 37°C with LK 35.2 B cells, 100 μM pigeon cytochrome *c* fragment 81–104, and dilutions of ascites containing anti-Ia antibodies as follows: no anti-Ia ascites (lanes A and B); anti-E^k_α:E^k_β 17.3.3 at 1:100 (lane C), 1:500 (lane D), or 1:2500 (lane E); anti-E^k_α 14.4.4 at 1:100 (lane F), 1:500 (lane G), or 1:2500 (lane H); anti-A^k_α:A^k_β 10.2.16 at 1:100 (lane I) or 1:500 (lane J); control SP2/0 ascites at 1:100 (lane K), 1:500 (lane L), or 1:2500 (lane M). In lanes N and O, T cells and B cells were similarly incubated without antigen or ascites. Immunoprecipitation, gel electrophoresis, and autoradiography were performed as described. Numbers on left are kDa.

It was not clear from these data whether the activation-induced appearance of ^{32}P-labeled pp20 reflected phosphorylation of a polypeptide constitutively associated with the T-cell antigen receptor, or whether activation led to an association of the receptor with an intrinsically phosphorylated 20-kDa protein. The hydrophobic labeling reagent ^{125}I-TID partitions into the lipid bilayer and upon photoactivation generates a reactive carbene that selectively labels intrinsic membrane structures (26). Nonstimulated and concanavalin A-activated 2B4 cells were incubated with this reagent, membrane proteins were photolabeled, and immunoprecipitates were prepared with the A2B4-2 monoclonal antibody. Iodinated peptides that comigrated with pp20, pp25, and with the lower molecular size band of the surface-iodinated antigen receptor were specifically immunoprecipitated (Fig. 3, lane B). Moreover, immunoprecipitates from cells that were activated with lectin prior to photolabeling (lane D) had the same amount of the 20-kDa ^{125}I-labeled protein as the nonstimulated cells (lane B). Assuming this 20-kDa protein is the same as pp20, based on its size and precipitation by the anti-receptor antibody, the results presented in Fig. 3 make it unlikely that the appearance of pp20 reflects an increased association of this protein with the receptor during T-cell stimulation. Instead, the phosphorylation reactions observed in Figs. 1 and 2 appear to take place on molecules already associated with the antigen receptor.

The appearance of ^{32}P-labeled pp20 in A2B4-2 immunoprecipitates is characterized by the kinetics shown in Fig. 4*A*. In this experiment, T cells loaded with [^{32}P]orthophosphate were incubated with B cells and the synthetic peptide antigen moth 93–103, 93E at 100 μM. The amount of ^{32}P-labeled pp20 increased from negligible amounts at time 0 to a maximum at ≈40 min. The antigen-induced phosphorylation of pp20, assayed after 1 hr of incubation, was dependent on antigen concentration (Fig. 4*B*). The COOH-terminal CNBr fragment of pigeon cytochrome *c* (residues 81–104) and the synthetic peptide (moth 93–103, 93E) appeared to have similar potency in leading to the appearance of ^{32}P-labeled pp20 in A2B4-2 immunoprecipitates. These two polypeptides also display similar potency in the stimulation of antigen-mediated secretion of IL-2 by 2B4 cells (data not shown).

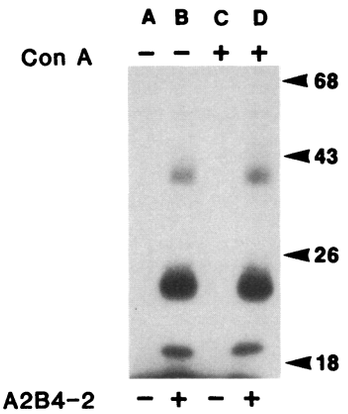

FIG. 3. Amount of ^{125}I-TID-labeled antigen receptor-associated polypeptides does not change with T-cell activation. Concanavalin A activated (+) or nonactivated (−) 2B4 cells were labeled with ^{125}I-TID. Lysates were immunoprecipitated with A2B4-2 antibody (+) or with control SP2/0 supernatant fluids without antibody (−). Gel electrophoresis and autoradiography were performed as described. Numbers on right are kDa.

The preceding experiments were performed solely with the 2B4 hybrid and the A2B4-2 anti-receptor monoclonal antibody. To determine whether this phosphorylation could take place in other T cells, the keyhole limpet hemocyanin-specific T-cell hybrid SKK-9.11 and a monoclonal antibody binding its antigen receptor were used (21). In the experiment shown in Fig. 5, SKK-9.11 (lanes A–D) and 2B4 (lanes E–H) were labeled with [^{32}P]orthophosphate. To bypass any potential problems stemming from differences in antigen processing, lectin was used to activate both cells. One-half of each preparation was stimulated with concanavalin A, and immunoprecipitates were prepared with A2B4-2, the antibody that bound only 2B4 cells, and with KJ16-133, the antibody that bound only SKK-9.11 cells. The ^{32}P-labeled pp20 protein was specifically precipitated from concanavalin A-activated SKK-9.11 by the KJ16-133 antibody and from activated 2B4 by the A2B4-2 monoclonal reagent, but not

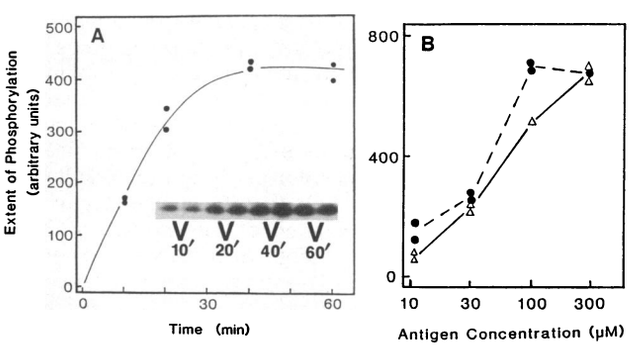

FIG. 4. (*A*) Kinetics of phosphorylation of the 20-kDa protein associated with the antigen receptor. The 2B4 T cells labeled with [^{32}P]orthophosphate, LK 35.2 B cells, and 100 μM antigen (moth 93–103, 93E) were mixed and incubated at 37°C for the indicated times. (*B*) Phosphorylation of pp20 as a function of antigen concentration. [^{32}P]Orthophosphate-labeled 2B4 cells were incubated for 60 min at 37°C with LK 35.2 B cells and the indicated concentrations of either pigeon cytochrome *c* fragment 81–104 (△) or a synthetic peptide analogue of tobacco hornworm moth cytochrome *c*, moth 93–103, 93E (●). After activation in these separate experiments, the cells were washed, lysed, and immunoprecipitated with the monoclonal antibody A2B4-2. NaDodSO$_4$/polyacrylamide gel electrophoresis and autoradiography were performed, and the extent of phosphorylation of the pp20 species was analyzed by densitometry. Values are expressed in arbitrary units that reflect integrated peak areas.

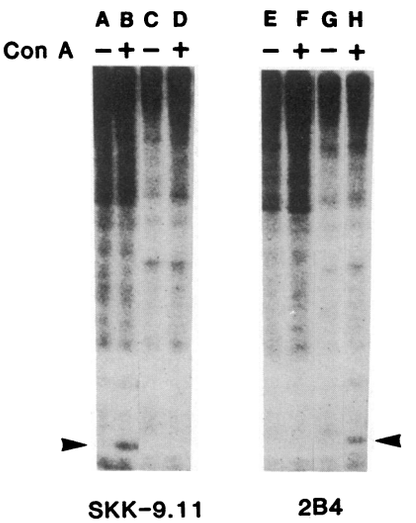

FIG. 5. Phosphorylated pp20 can be specifically immunoprecipitated by anti-receptor antibodies from two lectin-activated T-cell hybrids. [^{32}P]Orthophosphate-labeled SKK-9.11 cells (lanes A–D) or 2B4 cells (lanes E–H) were activated with 15 μg of concanavalin A per ml as indicated (+). Immunoprecipitates from cell lysates were prepared with the KJ16-133 monoclonal antibody absorbed to MAR 18.5-Sepharose (lanes A, B, E, and F) or with A2B4-2 adsorbed to protein-A Sepharose (lanes C, D, G, and H). Position of phosphorylated pp20 is indicated by arrows.

vice versa. Thus, ^{32}P-labeled pp20 was specifically coprecipitated only when the clonotypic T-cell antigen receptors were precipitated by their respective antibodies. This result indicates that the pp20 was precipitated because of its association with the antigen receptors and not because of some fortuitous cross-reaction of the anti-receptor antibodies with an unrelated 20-kDa cellular protein.

DISCUSSION

We have shown that, under conditions of T-cell activation, there is a rapid phosphorylation of a 20-kDa polypeptide that is immunoprecipitated by monoclonal antibodies specific for the T-cell antigen receptor. This phosphorylation occurs as a consequence of either a MHC-restricted antigen-specific induction event or by the binding of the lectin concanavalin A to the T cells in the absence of B cells. These findings suggest that the two activation mechanisms operate, at least in part, through a common post-binding pathway. It is important to emphasize that the increase in phosphorylation of this one protein is highly specific. Although others have noted a rapid increase in the phosphorylation of a 65-kDa protein with T-cell activation (30), in our studies there was no discernable difference in the level or distribution of any cellular phosphoproteins when equal aliquots of total ^{32}P-labeled lysate from either unactivated, antigen-activated, or concanavalin A-stimulated cells were compared on one-dimensional polyacrylamide gels (data not shown). Other phosphoproteins specifically precipitated in these experiments, as for example, Thy1 or the pp25 that was specifically coprecipitated with the A2B4-2 monoclonal antibody, also did not increase in amount of ^{32}P incorporation. Only one identified protein, pp20, appeared to increase markedly in its level of phosphorylation with T-cell activation.

The monoclonal antibody A2B4-2 could immunoprecipitate the 20-kDa or 25-kDa ^{125}I surface-labeled proteins, but the labeling efficiency varied greatly from preparation to preparation. In contrast, the hydrophobic photoactivatable reagent ^{125}I-TID labeled the 20- and the 25-kDa receptor-associated proteins quite well. The amount of iodinated

20-kDa protein co-immunoprecipitated with the antigen receptor did not change upon T-cell activation. This suggests that the induction of ^{32}P-labeled pp20 reported here represents enhanced phosphorylation of pp20 and not activation-induced association of the phosphoprotein with the receptor. We cannot distinguish, however, between activation of a phosphokinase upon T-cell stimulation or an alteration in the 20-kDa protein that renders it a substrate for a constitutively active kinase.

Two important conclusions can be drawn from the experiments using the T-cell clone SKK-9.11. First, pp20 can be immunoprecipitated from activated SKK-9.11 and 2B4 cells only by using the appropriate anti-receptor antibodies. This demonstrates that pp20 is immunoprecipitated by virtue of its association with the antigen receptor. It is not a phosphoprotein fortuitously immunoprecipitated by the A2B4-2 antibody because of immunologic cross-reactivity of pp20 with the 2B4 antigen receptor. Second, pp20 is found in clones other than 2B4, and it is phosphorylated in response to activation in the two different T-cell lines tested. This suggests that the pp20 phosphorylation reported here may be a general phenomenon occurring upon activation of all T cells.

The system described here is in many ways analogous to other systems in which binding to receptor activates a protein kinase. Considerable analysis of receptors for insulin (17), insulin-like growth factor (18), epidermal growth factor (19), and platelet-derived growth factor (20) indicates that binding of ligand to the respective receptors is associated with the stimulation of a phosphokinase activity. All of these receptors are themselves protein kinases and, specifically, tyrosine kinases. We do not know the nature of the kinase that is responsible for the T-cell phosphorylation events reported here. The question of whether it is the receptor or part of the receptor complex needs to be addressed. Despite these uncertainties, these findings suggest that the T-cell antigen receptor can be added to a growing list of receptors in which the binding of ligand leads to activation of phosphokinase activity.

It is tempting to speculate that the site of phosphorylation is one of the elements of the as yet undefined murine T3 complex. Antibodies to the human 20-kDa T3 glycoprotein can coprecipitate several other proteins, including a 25-kDa glycoprotein and a nonglycosylated 20-kDa protein, in addition to the clonotypic receptor (31–33). In the mouse, some evidence has been presented that a 20- to 22-kDa and a 25- to 27-kDa protein can be chemically cross-linked to the clonotypic structure and coprecipitated as a complex (34, 35). In the present report, evidence is presented that two proteins of these sizes labeled with either ^{32}P or by hydrophobic photoaffinity reagents are coprecipitated by the A2B4-2 antibody, which preferentially binds a mouse clonotypic structure.

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