Tyrosine Phosphorylation and Association with Phospholipase Cγ-1 of the GAP-associated 62-kD Protein after CD2 Stimulation of Jurkat T Cell

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Summary

Numerous substrates are tyrosine phosphorylated upon CD2 stimulation of human Jurkat T cells using a mitogenic pair of CD2 monoclonal antibodies, including the phospholipase C (PLC)γ-1–p35/36 complex. Most of these substrates are identically tyrosine phosphorylated after CD3 ligation, suggesting that both stimuli share the same biochemical pathway. We show, however, in this report that a 63-kD protein is specifically phosphorylated on tyrosine residues after ligation of the CD2 molecule. The tyrosine phosphorylation of p63 can be induced independently of other substrates when using a single CD2 mAb recognizing the D66 epitope of the molecule. Importantly, this CD2-induced tyrosine phosphorylation of p63 can also occur in the absence of the CD3ζ chain membrane expression, and is also distinct from the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$. We demonstrate, moreover, that p63 is physically linked with PLCγ-1 and p35/36 upon CD2 stimulation. Finally, we also show that a 62-kD protein coimmunoprecipitating with the p21ras GTPase activating protein (GAP) is heavily tyrosine phosphorylated only after CD2 stimulation. This ultimately suggests that p63 may represent in fact the 62-kD protein that associates with GAP after tyrosine phosphorylation. Taken together, these results demonstrate the occurrence in Jurkat cells of a tyrosine kinase pathway specifically coupled to the CD2 molecule. They also suggest a function of the p62-GAP–associated protein as a link between PLCγ-1 and p21ras activation pathways after CD2 activation.

The function of CD3 as an integral part of the antigen-specific receptor of T lymphocytes is well established (1). However, the real function of CD2 is still a debatable question. It is actually considered as a CD3-TCR alternative activation pathway promoting intercellular adhesion as well as T cell activation (2). Although not specific (CD2 ligands, CD58 and CD59, are ubiquitous molecules) (3), CD2 recognition has been reported to positively regulate CD3-TCR–mediated activation signals (4) suggesting that it may be a costimulatory signal for T cells during the antigen-specific recognition process. This assumption, about a close link between these two T cell activation pathways, was further confirmed by the finding that CD2-mediated T cell activation by specific antibodies required a functional CD3-TCR complex at the T cell surface (5). Particularly involved in this last phenomenon seems to be the CD3ζ chain which is known to be a key element responsible for cell signal transduction through the antigen-specific receptor of T lymphocytes (6–8). From all these reasons, it was postulated that CD2 and CD3 shared a common activation pathway.

This hypothesis was strengthened by the discovery that both CD3 and CD2 molecules apparently triggered the same cascade of early biochemical events, and especially the phospholipase C (PLC)[1] activation pathway (9, 10). Indeed, it has been demonstrated that the earliest event after CD3 or CD2 ligation with specific mAbs consisted in the rapid phosphorylation on tyrosine residues of numerous substrates. Among them is a 145-kD substrate, identified as PLCγ-1, one of the main isoforms of PLC in human T lymphocytes whose activity is increased by tyrosine phosphorylation (11, 12). Activated PLCγ-1 would be then responsible for the observed accumulation of the second messengers inositol(1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 and DAG are, respectively, involved in intracellular free calcium elevation and protein kinase C activation, two metabolic events critical for the appearance of downstream biological effects after CD3 or CD2 stimulation, i.e., IL-2 production and T cell

[1] *Abbreviations used in this paper:* IP_3, inositol(1,4,5)-trisphosphate; PLC, phospholipase C; PTK, protein tyrosine kinase.

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proliferation (13, 14). It was concluded, therefore, that CD3 and CD2 were coupled to the same as yet unidentified non-receptor tyrosine kinase, responsible for PLCγ-1 tyrosine phosphorylation. Several protein tyrosine kinases (PTKs) have been proposed for this coupling. This includes members of the src family of membrane-associated PTK such as $p56^{lck}$, found to be noncovalently associated with the CD4 or CD8 cytoplasmic domain (15) and activated after CD3 or CD2 cell stimulation (16–18), as well as the TCR ζ chain–associated kinase $p59^{fyn}$ (19). The potential role of another ζ-associated tyrosine phosphorylated protein with PTK activity, ZAP-70, was also envisaged (20). It has been suggested that both types of kinases (src family PTKs and ZAP-70) may interact to mediate TCR signaling.

Numerous epitopes distinguished by various sets of mAbs are present on the CD2 molecule of human T cells. Some of them are already highly expressed on resting T cells. Others are only weakly detected by the corresponding mAbs partly because of their masking with sialic acid residues on resting T cells (21). Meanwhile, a full activation via the CD2 molecule in vitro, leading to an activation of the PLC pathway, as well as late functional events, has been demonstrated to require the simultaneous ligation of these two kinds of epitopes (22). The involvement of CD2 conformational changes induced by the simultaneous binding of mAbs has been put forward. In this report, we demonstrate that stimulation of the CD2 molecule by a mitogenic pair of CD2-specific mAbs, as well as by a single CD2 mAb recognizing the cryptic epitope of the CD2 molecule expressed on Jurkat T cells, is able to specifically induce the tyrosine phosphorylation of a 63-kD protein. Importantly, we present evidence that p63 tyrosine phosphorylation appears even in the absence of CD3ζ chain membrane expression. Our results also revealed that tyrosine phosphorylation of p63 can be induced independently of the phosphorylation of PLCγ-1, suggesting that both proteins are phosphorylated by distinct tyrosine kinases. In addition, they also suggest that a complex containing PLCγ-1, its associated 35/36-kD protein and p63 can be formed upon CD2 stimulation. Ultimately, using coimmunoprecipitation experiments, we show that p63 may represent in fact the previously described p62 GAP–associated protein.

Materials and Methods

Cells. $CD2^+CD3^+$ Jurkat cells, clone E-6.1, were grown in RPMI 1640 medium (Flow Laboratories, Irvine, UK) supplemented with 10% FCS, penicillin (50 U/ml), streptomycin (50 μg/ml), 2 mM L-glutamine, and 1 mM sodium pyruvate, at a density of 0.5×10^6 cells/ml. $CD2^+CD3^-$ 31.13 Jurkat cell clone (23), kindly given by Dr. A. Alcover (Institut Pasteur, Paris, France), was cultured in the same conditions. A $CD2^-CD3^-$ Jurkat cell clone was obtained by limiting dilution of 31.13 cells followed by immunofluorescence analysis to select $CD2^-$ cells. For immunofluorescence studies, an indirect staining procedure with FITC-conjugated affinipure goat anti-mouse antibodies (Immunotech International, Marseille, France) was used. Fluorescence analysis was performed on a FACScan® cell analyzer (Becton Dickinson & Co., Mountain View, CA).

Monoclonal Antibodies. mAb UCHT1 (IgG1), specific for CD3ε chain, was obtained from Dr. P. C. L. Beverley (Imperial Cancer Research, London, UK). CD2 mAbs X11 (IgG1), D66 (IgG2b), O275 (IgG1), G144 (IgM), and GT2 (IgG1) were previously described (21, 22, 24–26). mAbs X11 and O275 recognize the previously described T11.1 epitope of the CD2 molecule (2). mAbs D66 and G144 are specific for a cryptic epitope on resting T cells because of its partial covering with sialic acid residues and which is completely unmasked after T cell activation. For all stimulation experiments, these mAbs were used at 1:500 dilution of an ascitic fluid. The 1C5H5 mAb against the TCR α chain of a human T cell clone was produced in our laboratory (27) and used as a control. Anti-PLCγ-1 mAb, antiphosphotyrosine mAb (4G10), and rabbit antiserum against COOH-terminal residues of $p56^{lck}$ and rabbit antiserum recognizing $p59^{fyn}$ were purchased from UBI Inc. (Lake Placid, NY). Anti-GAP mAb was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell Stimulation and Tyrosine Phosphorylation Analysis. Cells (5×10^6) were washed in RPMI containing 10 mM Hepes, pH 7.2, equilibrated for 10 min at 37°C and then incubated for various periods of time at 37°C in medium alone or in the presence of different stimulating agents. Activation was stopped by brief centrifugation, and cells were immediately lysed for 40 min in lysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 140 mM NaCl, 1% NP-40, 50 U/ml aprotinin, 1 mM sodium orthovanadate, and 1 mM PMSF) at 4°C. Nuclei and cellular debris were removed by centrifugation. Postnuclear supernatants were then diluted in Laemmli sample buffer (500 mM Tris-HCl, pH 6.8, 10% SDS, 10% glycerol, 5% 2-ME, and 10% bromophenol blue) and boiled for 5 min before SDS-PAGE analysis. Proteins were electrophoretically transferred for 1 h at 65 V to nitrocellulose sheet (Schleicher & Schuell, Dassel, Germany), and blots were hybridized with anti-PLCγ-1 (0.1 μg/ml) or antiphosphotyrosine mAb (0.2 μg/ml) before addition of goat anti–mouse antiserum labeled with peroxidase. Reaction was revealed with an enhanced chemiluminescence system (ECL; Amersham, Paris, France) according to the supplier's instructions.

Immunoprecipitation Experiments. For immunoprecipitation experiments, cell lysates of 10^7 stimulated cells were prepared as above except that a lysis buffer containing 1% digitonin instead of NP-40 and lacking EDTA was used. Cellular debris was removed by centrifugation, and lysates were precleared for 1 h with 20 μl of packed protein A–Sepharose beads (Sigma Chemical Co., St. Louis, MO). Precleared lysates were then incubated overnight with 2 μg of anti-PLCγ-1 mAb or 1 μg of anti-GAP mAb or rabbit antiserum against $p56^{lck}$ or $p59^{fyn}$ tyrosine kinases. Immunoprecipitates were then recovered by incubation with 20 μl of protein A–Sepharose beads for 60 min at 4°C, and washed three times in lysis buffer. The proteins were then eluted and dissolved by boiling for 5 min in Laemmli sample buffer, and subsequently resolved by SDS-PAGE. Western blot analysis was then performed using antiphosphotyrosine mAb 4G10.

Inositol Phosphate Production Measurement. Jurkat cells (10^6/ml) were labeled overnight with 5 μCi/ml myo-[2-^{3}H]inositol (ICN Flow, Orsay, France) in inositol-free medium containing 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin. After two washes in RPMI containing 10 mM Hepes, pH 7.2, cells were incubated for 20 min with 10 mM LiCl at 37°C. Aliquots of 10^6 cells were then stimulated with the indicated mAbs for the indicated period of time at 37°C. Reaction was stopped by successive addition of 50 μl NaOH 0.1 N containing 0.1% Triton X-100, 50 μl HCl, 0.1 N and 0.2 ml H_2O. The extraction procedure was continued in borosilicate glass tubes by the addition of 1.5 ml chloroform/methanol (1:2). The organic

and aqueous phases were partitioned by the addition of 0.5 ml H_2O and 0.5 ml chloroform, and the tubes centrifuged 10 min at 400 *g*. 1 ml of the aqueous phase was then applied simultaneously with 4 ml of a 5-mM cold myo-inositol on 1 ml Dowex AG 1-X8 anion-exchange resin in the formate form (Bio-Rad Laboratories, Richmond, CA), equilibrated with 0.1 M formic acid. The columns were then washed with 10 ml 0.1 M formic acid and the IP eluted with 5 ml 0.1 M formic acid plus 1.2 M ammonium formate. The eluted radioactivity was quantified by liquid scintillation counting in aqueous scintillant (ACS II; Amersham) and the results expressed in dpm (24).

Results

Stimulation of Jurkat T Cells through the CD3–TCR Complex or the CD2 Molecule Induces a Different Pattern of Protein Tyrosine Phosphorylation. CD3-TCR and CD2 activation pathways are thought to be closely linked. First, they have been shown to trigger the same cascade of biochemical events including activation of early PTKs, inositol phosphate production, and increases in intracellular free calcium concentration. Second, it is now generally admitted that the CD2-induced T cell activation needs functional CD3–TCR complex expressed at the cell surface (8, 10). However, as depicted in Fig. 1, the patterns of proteins tyrosine phosphorylated after CD3 or CD2 ligation with the CD3 mAb UCHT1 (Fig. 1, *top*), or with the CD2 mitogenic pair of mAbs X11+D66 (Fig. 1, *bottom*), were not identical. Indeed, apart from the 145-, 120-, 116-, 100-, 80-, 75-, and 35/36-kD tyrosine phosphorylated proteins induced by both stimuli, we could notice a 66-kD doublet strongly tyrosine phosphorylated over the background level only after CD3 stimulation. Conversely, a 63-kD tyrosine phosphorylated protein was only observed after CD2 ligation. This suggested that different PTKs could be activated depending on whether Jurkat cells were stimulated through CD3-TCR or CD2 molecules. One should notice here that the 145-kD band observed after CD3 or CD2 stimulation corresponded to tyrosine phosphorylated PLCγ-1 since a single 145-kD protein was revealed by anti-PLCγ-1 mAb in antiphosphotyrosine immunoprecipitates of CD3 or CD2-activated Jurkat cells (data not shown).

Delayed kinetics of tyrosine phosphorylation have been previously evidenced after the activation of PBL with CD2 mAbs as compared with CD3 stimulation (28). The time course experiments shown in Fig. 1 demonstrate that the maximum response always occurred later after CD2 stimulation with the mAb pair X11+D66. Usually it was found to peak between 3 and 5 min after the addition of the CD2 pair. In the same assay conditions, tyrosine phosphorylations after CD3 stimulation peaked very early (between 15 and 30 s). It is also clear from Fig. 1 that for a given stimulus, all proteins, including p66 and p63, shared the same tyrosine phosphorylation kinetic.

Tyrosine Phosphorylation of p63 Is Induced upon Stimulation of the D66 Epitope on the CD2 Molecule. As previously mentioned, various epitopes distinguished by different mAbs are present on the CD2 molecule of human T cells. Owing to the apparent specificity of p63 tyrosine phosphorylation after CD2 stimulation, we tested the effects of other pairs of CD2

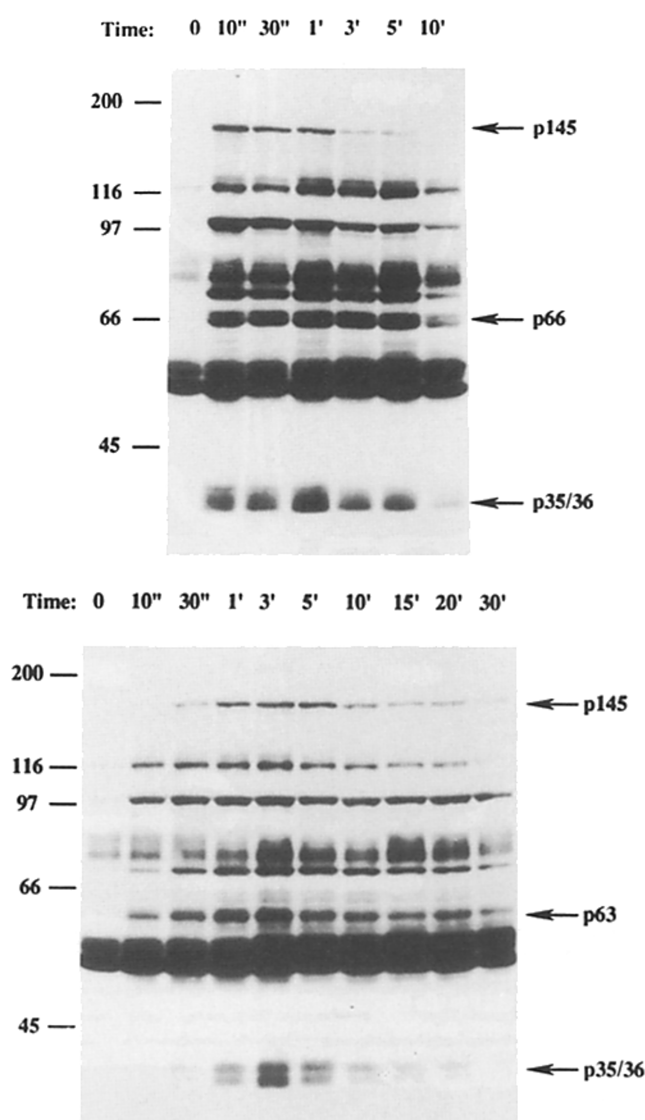

Figure 1. Different patterns of tyrosine phosphorylated proteins are induced after CD3 or CD2 stimulation. Jurkat cells (5 × 10^{6}) were stimulated with the CD3 mAb UCHT1 (*top*) or with the CD2 mAbs X11+D66 (*bottom*) for the indicated times. Total cell extracts were resolved on a 8% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with antiphosphotyrosine mAb 4G10. Positions of molecular size markers are indicated. Exposition times were 10 s for each membrane. Parallel blotting using anti-PLCγ-1 mAb revealed no change in the amount of PLCγ-1 for each stimulation time, indicating that the same quantity of cellular extract was loaded for each sample (not shown).

mAbs. mAb O275 recognizes the same epitope as X11 and mAb G144 the same as D66 (25, 26). mAb GT2 recognizes a third epitope, as evidenced by the lack of competitive binding with the mAbs named above (22). Cytofluorometry analysis of Jurkat cells after labeling with each of these CD2 mAbs as compared with CD3 mAb UCHT1 is presented in Fig. 2. High binding of mAbs X11 and O275, intermediate binding of GT2, and low binding of mAbs D66 or G144 could be evidenced.

Fig. 3 *A* shows that different couples of CD2 mAbs

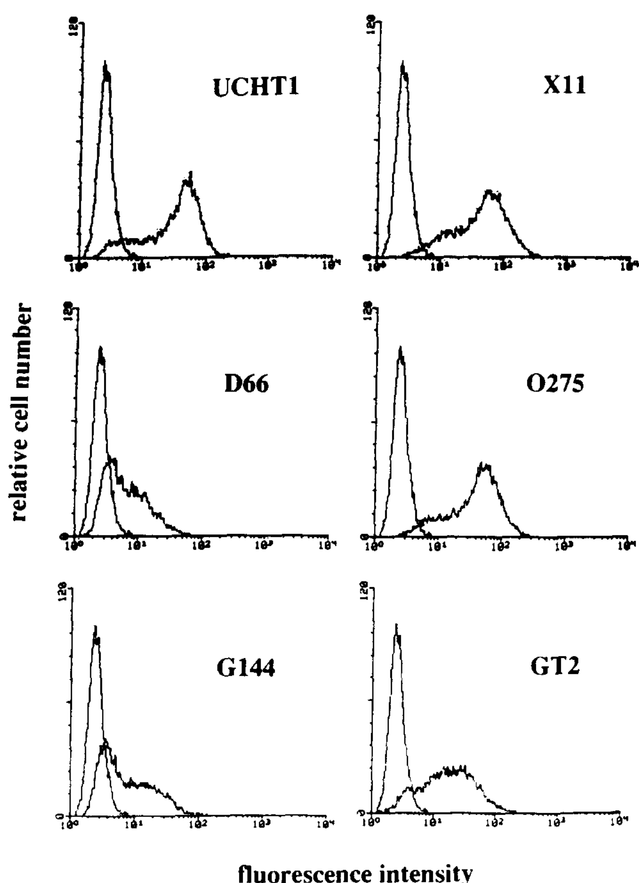

Figure 2. Immunofluorescence analysis of Jurkat T cells with different CD2 mAbs. Cells were stained for 30 min with the indicated CD2 mAb (*X11, D66, O275, G144, GT2*) or with the CD3 mAb UCHT1. An irrelevant mAb, used as the negative control, is also shown in each panel. All mAbs were used as a 1:400 dilution of an ascitic fluid. Bound mAbs were revealed using goat anti-mouse FITC conjugate and the fluorescence analyzed on a FACScan® cell analyzer.

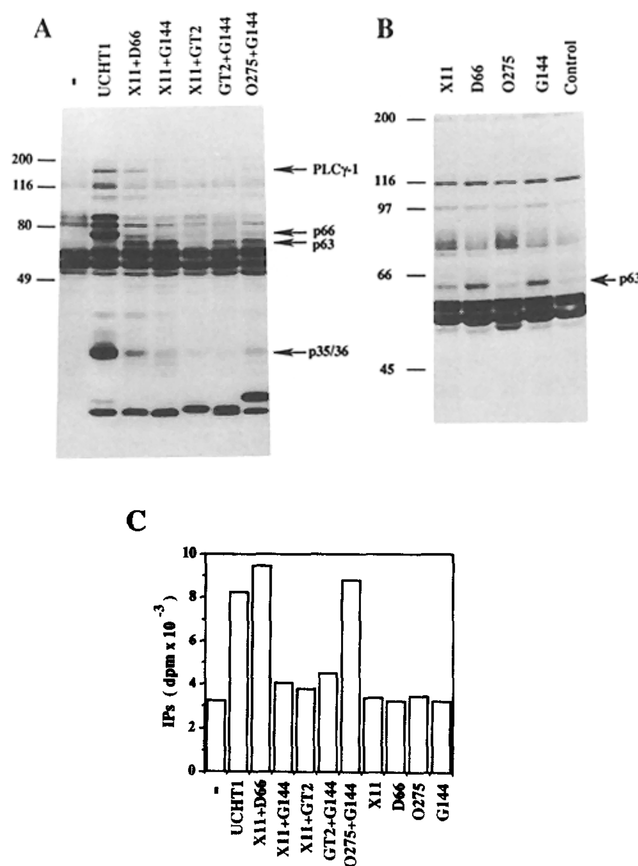

Figure 3. p63 tyrosine phosphorylation is induced upon stimulation of the D66 epitope on the CD2 molecule. (*A*) Effect of various CD2 mAbs pairs on tyrosine phosphorylation pattern in Jurkat T cells. Jurkat cells were unstimulated (−), or stimulated with the CD3 mAb UCHT1 for 1 min, or various CD2 mAbs pairs for 5 min: X11+D66, X11+G144, X11+GT2, GT2+G144, or O275+G144. After cells lysis, proteins were separated by SDS-PAGE (12% polyacrylamide gel) and visualized by immunoblotting with antiphosphotyrosine mAb 4G10. (*B*) Induction of p63 tyrosine phosphorylation by a single CD2 mAb. Control ascitic fluid (mAb 1C5H5), single CD2 mAb X11, D66, O275, or G144 were used for stimulation experiments of Jurkat T cells (5 × 10^6/sample). After lysis, postnuclear supernatants were loaded on a 8% polyacrylamide gel, and separated proteins were revealed by Western blotting using antiphosphotyrosine mAb 4G10. (*C*) IP production after CD2 stimulation. Jurkat cells (10^6/ml) were labeled with myo-[2-^{3}H]inositol and divided in aliquots (10^6 cells/sample) which were then stimulated with UCHT1 for 1 min, or CD2 mAbs pairs X11+D66, X11+G144, X11+GT2, GT2+G144, or O275+G144 for 5 min, or single CD2 mAb X11, D66, O275, or G144 for 5 min, or were left unstimulated (−). IPs were measured as described in Materials and Methods. Results are expressed in dpm as the mean of three separate experiments.

(X11+D66, X11+G144, GT2+G144, and O275+G144) were able to induce the tyrosine phosphorylation of p63 protein. None of them could, however, phosphorylate the 66-kD doublet observed after CD3 stimulation. Interestingly, we can see from Fig. 3 *A* that p63-induced phosphorylation apparently correlated with the presence in the studied combination of mAb D66 or G144 to CD2 receptor. The same results were obtained when using purified CD3 and CD2 mAb instead of ascitic fluid (data not shown). We therefore analyzed the effect of single CD2 mAb on protein tyrosine phosphorylation in Jurkat cells. We were surprised to discover that D66 and G144 could induce p63 tyrosine phosphorylation when used alone (Fig. 3 *B*). The fact that neither the single CD2 mAb X11, nor O275, nor control ascite fluid, were able to induce the p63 phosphorylation demonstrated its specific induction by stimulating the D66 epitope on the CD2 molecule. It seemed, therefore, that stimulating D66 epitope could activate a PTK acting on p63.

Importantly, these results also pointed out that p63 tyrosine phosphorylation occurred independently of PLCγ-1 phosphorylation. Indeed, mAb pairs X11+G144 and GT2+G144 could tyrosine phosphorylate p63 without inducing any detectable tyrosine phosphorylation of the 145-kD band corresponding to PLCγ-1 (Fig. 3 *A*). In the same way, D66 or G144 mAbs used alone triggered p63 but not PLCγ-1 tyrosine phosphorylation (Fig. 3 *B*). This latter result was confirmed by the fact that neither X11+G144 and GT2+G144 pairs, nor mAbs D66 or G144 used alone, could trigger inositol phosphate production (Fig. 3 *C*), as compared with the pairs that were able to trigger the phosphorylation of PLCγ-1 (see Fig. 3 *A*). Accordingly, in parallel experiments, we found

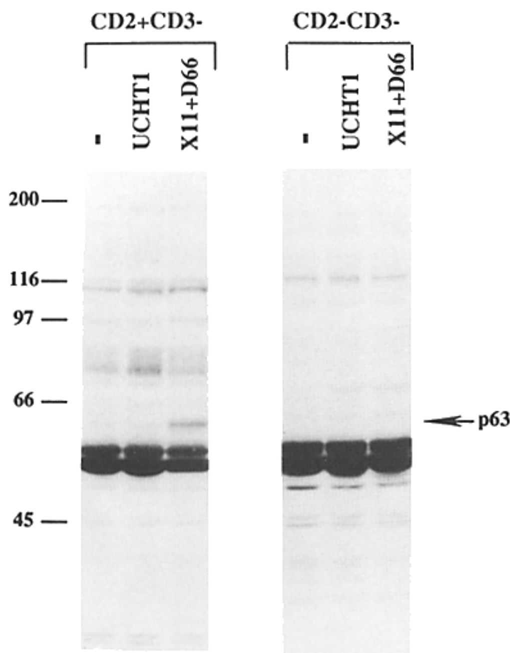

Figure 4. Tyrosine phosphorylation of p63 occurs independently of membrane CD3–TCR expression. 31.13 cells (phenotype $CD2^+CD3^-$) were stimulated with the CD3 mAb UCHT1 for 1 min, or the mAb pair X11+D66 for 5 min, or were left unstimulated (−). After lysis, total cellular extracts were run on a 8% SDS-PAGE. Proteins were then revealed by immunoblotting using antiphosphotyrosine mAb 4G10. As a negative control, the same experiments were performed in a variant cell line derived from 31.13 by limiting dilution, and lacking cell surface CD2.

a sustained intracellular free calcium increase only with those combinations leading to tyrosine phosphorylation of PLCγ-1 (data not shown).

p63 Tyrosine Phosphorylation Occurred Independently of the CD3–TCR Complex. Complete activation process of Jurkat T cells through the CD2 pathway, including early protein tyrosine phosphorylation, followed by inositol phosphate production, increased in intracellular free calcium concentration and lately IL-2 synthesis, is known to require functional CD3–TCR complex at the cell surface. Particularly required for that process seems to be the CD3ζ chain which is thought to be responsible, at least in part, for signal transduction mediated after the stimulation of the CD3–TCR molecular complex (6, 7). Whether or not tyrosine phosphorylation of p63 was dependent on CD3-TCR expression seemed, therefore, an important question to answer. For that purpose, we used a previously described Jurkat cell variant, termed 31.13, lacking the TCR β chain gene and that fails to express any membrane CD3–TCR complex or CD3ζ chain (8, 23). As shown in Fig. 4 (*left*), stimulation of this cell variant with the CD2 mAb pair X11+D66 also induced the tyrosine phosphorylation of p63, although this induction was usually weaker as in the parental cell line. Importantly, it was not accompanied by the tyrosine phosphorylation of the other substrates observed in the wild type of Jurkat cells (see Fig. 1, *bottom*). In the same cell line, the CD3 mAb UCHT1 did not stimulate any phosphorylation, a finding which was consistent with the absence of cell-surface CD3. As a control, we have also used in this experiment a cell clone, selected from the 31.13 variant, and lacking CD2 surface expression (data not shown). No tyrosine phosphorylation of p63 could be observed in this clone after stimulation with the CD2 mAb pair X11+D66 (Fig. 4, *right*). Altogether these findings demonstrated that p63 tyrosine phosphorylation only depended on the CD2 pathway and did not require CD3-TCR or ζ chain expression. These results underlined again the independence of the p63 tyrosine phosphorylation pathway since no PLCγ-1 phosphorylation could be observed after CD2 stimulation of the 31.13 variant.

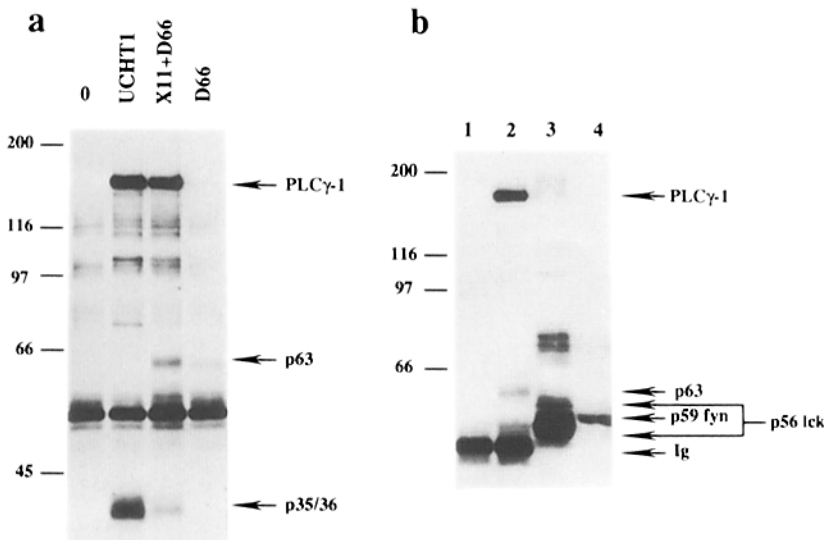

Figure 5. p63 coimmunoprecipitates with PLCγ-1 and is distinct from a tyrosine phosphorylated form of $p56^{lck}$ or $p59^{fyn}$ kinases. (*a*) Jurkat cells (10^7 for each point) were unstimulated (−), or activated with the CD3 mAb UCHT1 for 1 min, the CD2 mAb pair X11+D66 or the mAb D66 alone for 5 min. Cells were then lysed in a lysis buffer containing 1% digitonin and precleared lysates were immunoprecipitated with anti-PLCγ-1 mAb. Immune complexes were resolved by SDS-PAGE on a 8% acrylamide gel, and Western blot analysis using antiphosphotyrosine mAb 4G10 was performed. Positions of coimmunoprecipitating PLCγ-1, p35/36, and p63 are indicated. No bands except mAb H chains could be detected in immunoprecipitates performed with an irrelevant mAb (not shown). (*b*) In a separate experiment, Jurkat cells were left unstimulated (lane *1*) or were activated using the CD2 mAb pair X11+D66 (lanes *2*, *3*, and *4*), and treated as above. Lysates were then immunoprecipitated with anti-PLCγ-1 mAb (lanes *1* and *2*) or rabbit antiserum recognizing the COOH-terminal residues of $p56^{lck}$ (lane *3*) or rabbit antiserum specific for $p59^{fyn}$ (lane *4*). Immunoprecipitates were resolved by SDS-PAGE and revealed using antiphosphotyrosine mAb 4G10. Positions of tyrosine phosphorylated bands of $p56^{lck}$, $p59^{fyn}$, and p63 are indicated.

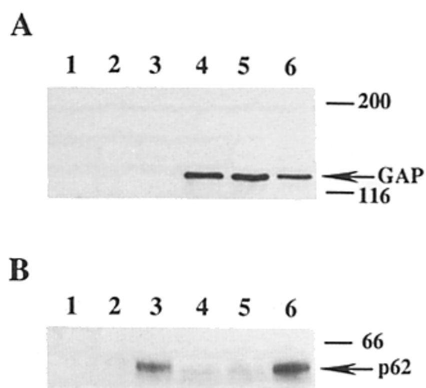

Figure 6. A 62-kD tyrosine phosphorylated protein coimmunoprecipitates with GAP specifically after CD2 stimulation of Jurkat T cells. Jurkat cells (10^7 cells for each point) were left unstimulated (lanes *1* and *4*), or were activated with the CD3 mAb UCHT1 for 1 min (lanes *2* and *5*), or the CD2 mAb pair X11+D66 for 5 min (lanes *3* and *6*). Cells were lysed in a lysis buffer containing 1% digitonin and the lysates immunoprecipitated with anti-PLCγ-1 mAb (lanes *1–3*) or anti-GAP mAb (lanes *4–6*). Immunoprecipitates were then resolved on a 8% acrylamide gel and transferred onto nitrocellulose. The membrane was then divided into two parts. (*A*) Corresponds to the upper part of the blot revealed with anti-GAP mAb. (*B*) The lower part of the blot revealed with antiphosphotyrosine mAb 4G10. After ECL reaction, exposure of the films was identical for A and B. Positions of GAP and p62 are indicated.

p63 Coimmunoprecipitates with PLCγ-1 and Is Distinct from a Tyrosine Phosphorylated Form of p56^{lck} or p56^{fyn} Kinases. We have shown that p63 was specifically tyrosine phosphorylated after appropriate CD2 stimulation. That it may represent a link in the corresponding biochemical cascade was therefore possible. Thus, a possible important role for p63 to play in the CD2 transduction pathway would be to associate with PLCγ-1 after receptor stimulation. We have therefore examined tyrosine phosphorylated proteins that coimmunoprecipitated with PLCγ-1 after CD2 stimulation of Jurkat T cells. For that purpose, cells were incubated with X11+D66 for 5 min before lysis and immunoprecipitation with anti-PLCγ-1 mAb, and then probed with antiphosphotyrosine mAb. As shown in Fig. 5 *a*, the 63-kD tyrosine phosphorylated protein was found to coprecipitate with PLCγ-1 after CD2 ligation using the mitogenic pair X11+D66. As previously reported (4), p35/36 also coimmunoprecipitated with PLCγ-1 upon these conditions of CD2 stimulation. The other proteins present in the anti-PLCγ-1 immunoprecipitate were identical in unstimulated or in CD2-stimulated Jurkat cells. These results thus suggested that p63 formed a complex with PLCγ-1 and p35/36 after CD2 stimulation, even though these proteins were likely phosphorylated by distinct PTKs. Interestingly, we found that after Jurkat cell stimulation with D66 mAb alone, p63 also could be immunoprecipitated with PLCγ-1 (Fig. 5 *a*) although, as we described above (see Fig. 3 *B*), PLCγ-1 was not tyrosine phosphorylated in that experimental condition. In parallel experiments, PLCγ-1 immunoprecipitations were also performed after CD3 stimulation. As shown in Fig. 5 *a* the CD3-specific 66-kD tyrosine phosphorylated doublet was not detected in the anti-PLCγ-1 immunoprecipitate contrary to the PLCγ-1–associated tyrosine phosphorylated protein p35/36.

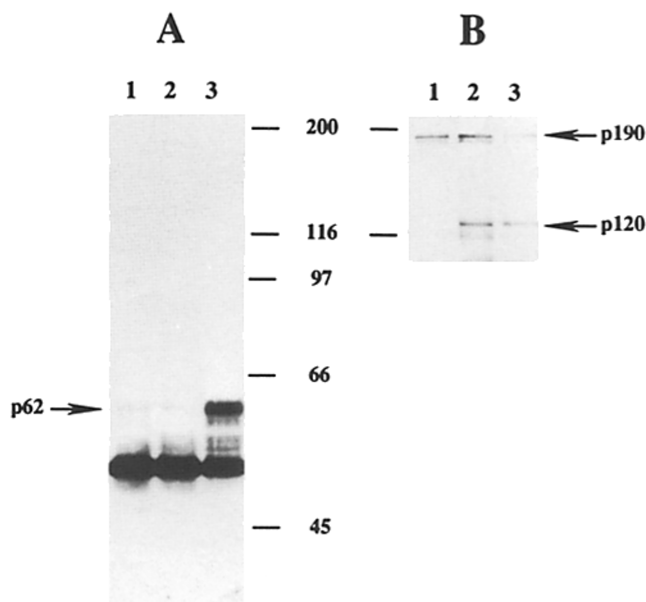

Figure 7. Only p62 is highly tyrosine phosphorylated in GAP immunoprecipitates after CD2 stimulation of Jurkat cells. (*A*) Jurkat cells (10^7 for each point) were left unstimulated (lane *1*), or activated with the CD3 mAb UCHT1 for 1 min (lane *2*), or the CD2 mAb pair X11+D66 for 5 min (lane *3*). Cells were then lysed in a lysis buffer containing 1% digitonin and lysates immunoprecipitated with anti-GAP mAb. Immune complexes were resolved by SDS-PAGE on a 8% acrylamide gel, and Western blot analysis using antiphosphotyrosine mAb 4G10 was performed. After ECL reaction, exposure of the film was 1 min. (*B*) The top of the same blot after a 5-min exposure of the film.

Since p63 tyrosine phosphorylation occurred specifically after CD2 triggering, one could expect some physical association with its corresponding surface receptor. However, we were repeatedly unable to coprecipitate p63 with the CD2 mAb X11 (data not shown). This probably indicates that p63 and CD2 did not associate or that their association was very weak, very unstable, or transient, since we have used digitonin as a detergent that allows mild lysis conditions and preserves protein interactions.

The appearance of slow migrating species of p56^{lck} after CD2 stimulation of Jurkat cells has been reported (16). Moreover, it has been demonstrated recently that the CD2 triggering could induce an increase in p56^{lck} activity in the 31.13 Jurkat cell variant (18). This suggested that p63 might be p56^{lck} itself. Therefore, p56^{lck} immunoprecipitation from the lysate of Jurkat cells stimulated with mAbs X11+D66 was performed and revealed using antiphosphotyrosine antibodies. As shown in Fig. 5 *b*, no tyrosine phosphorylated species of p56^{lck} were found to comigrate with p63 (compare lanes *2* and *3*). One has to notice that a few amounts of phosphotyrosine-labeled material appeared in the 56-kD region in the PLCγ-1 immunoprecipitate of CD2-stimulated cells as compared with the control (compare lanes *1* and *2* of Fig. 5 *b*). This could correspond to small amounts of tyrosine phosphorylated p56^{lck} coimmunoprecipitating with PLCγ-1, as recently shown in TCR-stimulated Jurkat cells (29). Similar conclusions arose from immunoprecipitation experiments using anti-p59^{fyn} antibodies since, once again, no

tyrosine phosphorylated form of $p59^{fyn}$ migrated as a 63-kD band after the stimulation of Jurkat cells with the couple of CD2 mAbs X11+D66 (Fig. 5 *b*).

The 62-kD Protein Coimmunoprecipitating with GAP Is Specifically Tyrosine Phosphorylated after CD2 Stimulation of Jurkat Cells. A 62-kD protein has been described to be highly tyrosine phosphorylated and to associate with GAP after signaling by tyrosine kinase growth factor receptor or in v-src tyrosine kinase–transformed cells (30). We therefore investigated whether p63 could correspond to this protein. Cell extracts from CD3 or CD2-stimulated Jurkat cells were immunoprecipitated in parallel with anti-PLCγ-1 or anti-GAP mAb, and the immune complexes probed in Western blot with GAP-specific mAb or antiphosphotyrosine mAb. As shown in Fig. 6 *A*, GAP mAb recognized in Jurkat cells a band with a molecular mass of 120 kD corresponding to one of the previously described forms of mammalian p21ras GAP (31). This band was only present in GAP immunoprecipitates. Importantly, we found in this experiment, as shown in Fig. 6 *B*, that a strong tyrosine phosphorylated 62-kD protein could be observed in anti-GAP immunoprecipitates from CD2-stimulated Jurkat cells (lane *6*). This band was only weakly labeled in control or CD3-stimulated cells (Fig. 6 *B*, lanes *4* and *5*). Clearly, this protein comigrated with the band that we found recognized by antiphosphotyrosine mAb in the PLCγ-1 immunoprecipitate of CD2-stimulated Jurkat cells (Fig. 6 *B*, lane *3*). Taken together with the previous data showing that p63 was strongly tyrosine phosphorylated only after CD2 stimulation, these results thus suggested that p63 might represent in fact the 62-kD GAP-associated protein. Besides p62, another tyrosine phosphorylated protein of 190 kD has been previously demonstrated to also associate with GAP (30, 32). Therefore, we investigated if p190 also could be differentially phosphorylated after CD2 and CD3 stimulation of Jurkat cells. As shown in Fig. 7 *A* whereas p62 was found heavily and specifically labeled in anti-GAP immunoprecipitates from CD2-stimulated cells, confirming the results of Fig. 6, only a very tenuous tyrosine phosphorylation of p190 was observed that needed overexposure of the membrane to be evidenced (Fig. 7 *B*). Moreover, it was found tyrosine phosphorylated even in unstimulated cells. One has to notice here that only a weak tyrosine phosphorylation of GAP could be observed in parallel, as also shown in Fig. 7 *B*. Interestingly, GAP appeared to be similarly phosphorylated in CD3- and CD2-stimulated cells. However, in contrast with p190, it was not tyrosine phosphorylated in unstimulated cells. These results ultimately suggest that in the so-called GAP complex, only the p62 protein is highly tyrosine phosphorylated upon CD2 stimulation of Jurkat T cells.

Discussion

The necessity of the CD2 molecule as an alternative activation pathway near the antigen-specific CD3-TCR complex, and its putative role, remain unknown. It has been shown that T cell activation through CD2 requires a functional CD3-TCR complex at the cell surface (5, 8, 23, 33), suggesting that signals triggered through these receptors were transduced by the same way. According to this idea, it has been reported that stimulating T cells with mAbs specific for CD3 or CD2 induces tyrosine phosphorylation of the same set of polypeptides (34). However, in contradiction to this, Jin et al. (35) demonstrated in Jurkat cells that CD3-TCR and CD2 pathways should be distinct since a 68-kD tyrosine phosphorylated protein was induced after CD3 but not CD2 stimulation. In the same way, recent studies in rat thymocytes revealed that different tyrosine phosphorylated proteins could be coimmunoprecipitated with CD3 or CD2 molecules (36). Here we provide evidence that different pathways could be activated depending on whether or not Jurkat T cells were stimulated through the CD3 or different epitopes of the CD2 molecule.

Numerous substrates are tyrosine phosphorylated after CD3 or CD2 stimulation of Jurkat T cells using a mitogenic pair of CD2 mAbs (28, 37). The identity of most of these tyrosine phosphorylated proteins is not yet known, except for some of them, such as PLCγ-1, the CD3ζ chain (38), the $p95^{vav}$ protooncogene (39), or the kinases $p56^{lck}$ (16, 18), $p59^{fyn}$ (19), and ZAP-70 (20). The tyrosine phosphorylation of many of these proteins, including PLCγ-1 and a 35/36-kD phosphoprotein previously shown to be associated in a stable complex (4), appeared to be common to both stimuli and was found to correlate with the rapid production of inositol phosphates leading in both cases to intracellular free calcium increases. This suggested that these substrates might be phosphorylated by the same tyrosine kinase, an assumption further strengthened by the finding that, as also shown in this report (Fig. 1), these proteins shared for a given stimulus an identical tyrosine phosphorylation kinetic. This PTK should be activated after CD3 or CD2 stimulation, and should therefore belong to a common signal transduction pathway.

Our results showed that besides this common pattern of tyrosine phosphorylated proteins, an additional 63-kD band could be induced specifically after CD2 triggering of Jurkat cells using different pairs of CD2 mAbs. Importantly, the tyrosine phosphorylation of p63 could be dissociated for the tyrosine phosphorylation of the PLCγ-1–p35/36 complex as evidenced when using a mAb directed against the D66 epitope of the CD2 molecule. Accordingly, no IP production or calcium response (data not shown) was induced in that case. This result suggested that tyrosine phosphorylation of p63 on the one hand, and of the PLCγ-1–p35/36 complex on the other, occurred through distinct PTKs activated after CD2 stimulation of Jurkat T cells. Interestingly, our results may lead to a similar conclusion for the CD3 pathway with regard to our finding of a 66-kD tyrosine phosphorylated doublet only after CD3 stimulation. Indeed, tyrosine phosphorylation of this doublet was not induced when using a comitogenic pair of CD2 mAbs, whereas as previously mentioned, the whole pattern was similar to the one obtained after CD3 activation. This 66-kD doublet could represent the CD5 surface antigen since CD5 appears as a 67–69-kD doublet which is tyrosine phosphorylated by CD3 in Jurkat T cells (40–42). Of course, it can be also hypothesized that the same kinase would be associated with CD3 and CD2 and would phosphorylate either p66 or p63 as a consequence

of the exclusive association of p66 with CD3 and p63 with CD2. However, our experiments with the $CD2^{+}CD3^{-}$ 31.13 Jurkat cell variant supported the idea of a distinct kinase activated after CD2 triggering.

As mentioned above, T cell activation through CD2 has been previously shown to require functional CD3-TCR complex at the cell surface, and especially functional CD3ζ chain (8), thus supporting the classical assumption of a common CD3-CD2 signaling pathway. Accordingly, tyrosine phosphorylation of the PLCγ-1–p35/36 complex could not have been induced in the ζ-negative 31.13 variant either with CD3 mAb or a mitogenic pair of CD2 mAbs. However, the isolate tyrosine phosphorylation of p63 was still observable after cell stimulation through the CD2 molecule, demonstrating that this CD2-dependent tyrosine kinase pathway was distinct from the tyrosine kinase pathway(s) linked to the CD3 molecular complex. We do not know which kinase is actually involved in the phosphorylation of p63. $p56^{lck}$ might be one candidate. Indeed, this kinase has been demonstrated to still be activated via CD2 in the 31.13 variant (18). However, if this is true, then p63 also should be present after CD3 stimulation in the Jurkat wild-type line since this stimulus has been reported, although this is a very controversial problem, to activate $p56^{lck}$ (18). Concerning the two other PTKs which are clearly involved in the human T cell activation process, $p59^{fyn}$ and ZAP-70, both have been demonstrated to be associated with the CD3 molecular complex and especially with its ζ chain (19, 43). Thus, according to the results obtained in the 31.13 variant, their contribution to p63 phosphorylation is unlikely.

Questions remained about p63 identity. We initially thought that it might be $p56^{lck}$ itself. Indeed, slow migrating species of the enzyme have been evidenced after CD2 stimulation in Jurkat cells. Moreover, as noted above, the CD2 triggering still induced an increase in $p56^{lck}$ kinase activity in the Jurkat cell variant 31.13 (18). However, using immunoprecipitation experiments, we found that in CD2-stimulated cells, the highest slow migrating tyrosine phosphorylated species of $p56^{lck}$ did not comigrate with p63, suggesting that this one might be a distinct protein. In the same way, the $p59^{fyn}$ kinase did not comigrate with p63, eliminating the possibility that they could be identical. This question about an identity between $p56^{lck}$ and p63 was reconsidered by our finding that, at least, PLCγ-1, p35/36, and p63 were found to be associated after CD2 stimulation with a mitogenic combination of mAbs. It has been shown recently using a PLCγ-1–containing fusion protein, that a small amount (<5%) of tyrosine phosphorylated $p56^{lck}$ could associate with PLCγ-1 after Jurkat cell stimulation with a TCR-specific mAb (29). Accordingly, it appeared from our experiments that small amounts of $p56^{lck}$ labeled by antiphosphotyrosine antibodies could be immunoprecipitated with PLCγ-1 after CD2 stimulation of Jurkat cells (Fig. 5 *b*, lane *2*; the $p56^{lck}$ comigrating doublet over the Ig band), a result further confirmed by in vitro kinase assays (data not shown). But no p63-comigrating forms could be again observed in this latter experiment. Interestingly, we found that immunoprecipitation of p63 by anti-PLCγ-1 also could be achieved in D66-stimulated Jurkat cells, in a stimulation system where no detectable tyrosine phosphorylated PLCγ-1 has been observed. This indicates that p63 associates at least in part with nonphosphorylated PLCγ-1, and could therefore represent a protein that regulates PLCγ-1 during the CD2 T cell activation process.

We have further hypothesized that p63 could represent the p62 GAP-associated protein. Our results demonstrate the presence of a highly tyrosine phosphorylated 62-kD protein coimmunoprecipitating with GAP in Jurkat T cell extracts after CD2 stimulation. At the same time, GAP was much less tyrosine phosphorylated. These findings were in complete agreement with the results recently obtained by Gold et al. (44) in B lymphoma cell lines after mIg cross-linking. p62 was first discovered in oncogene tyrosine kinase–transformed fibroblasts such as v-src or v-fps (30). In these cells, p62 was found to be constitutively and highly tyrosine phosphorylated without cell stimulation and to coimmunoprecipitate with GAP. Tyrosine phosphorylation of p62 and coimmunoprecipitation with GAP was also clearly evidenced in rat fibroblasts transfected with the human EGF receptor, but only after EGF stimulation (30). The same results were obtained in EBV immortalized B cells stimulated with an anti-IgM mAb (44). Taken together, these results suggest that p62 was a preferential substrate of transforming or mitogenic tyrosine kinases. This would probably explain why the tyrosine phosphorylation of p62 appeared to be so strong in Jurkat cells as compared with peripheral blood T cells. Indeed, in these cells, proteins migrating in the 66- and 63-kD regions were always very weakly labeled, and the imbalance between the CD3 and the CD2 activation pathway in stimulating the tyrosine phosphorylation of p63 was very difficult to evidence (data not shown). It is thus possible that this event represents in physiological T cells a phenomenon that is difficult to detect and that is greatly enhanced in Jurkat cells, presumably as a consequence of the malignant transformation and/or deregulated activities of the responsible kinases. This assumption would be in agreement with the fact that p62 was found to be highly phosphorylated only in transformed cells (45). Interestingly, we observed that tyrosine phosphorylated p62 could not be recovered from GAP immunoprecipitates performed on CD3-stimulated Jurkat cells. In addition to the fact that this is an additional argument for an identity of p62 with the PLCγ-1–associated 63-kD protein, this ultimately suggests that different regulations of the ras/GAP pathway between CD2 and CD3 may take place in Jurkat cells. Indeed, p62 binding to GAP protein is thought to inhibit its GTPase activity, thus favoring the maintenance of the activated form of p21ras (46). How p62 would associate with PLCγ-1 is an as yet unresolved question. However, tyrosine phosphorylated p62 has been demonstrated to associate with GAP through its SH2 domain (46). A similar mechanism therefore might be involved in its association with PLCγ-1, which also contains such domains.

A full activation process through the CD2 molecule in vitro needs the simultaneous use of two antibodies directed against distinct epitopes of the molecule. Thus far, it has been speculated that this peculiarity would reflect the existence

of two different physiological ligands for CD2. This is now confirmed by the findings that two different molecules, CD58 (LFA3) and CD59, could bind specifically to CD2. Indeed, whereas CD58 binds with a high affinity to the CD2 epitope recognized by a mAb such as X11, which defines an external epitope of the molecule, it is now suggested that the CD59 molecule would bind to another epitope on the CD2 receptor (3) and would have potentiating effects with CD58 on T cell activation (47). One intriguing problem, however, when discussing the possible function of CD2, is that it represents one of the earliest cell-surface markers to appear during ontogeny. Thus, early prothymocytes which further differentiate to mature T cells express CD2. However, they do not express CD3-TCR on their membrane. In these cells, although CD2 does not trigger intracellular calcium elevation or IL-2 synthesis, it induces nevertheless protein tyrosine phosphorylation, suggesting that CD2 would in addition to its coreceptor function, play its own role in T cell activation (48). Our results open new prospects concerning a putative CD2-dependent biochemical cascade involved in T cell differentiation. Alternatively, this may represent a specific CD2-linked regulation pathway of T cell activation, since p62 is believed to contribute to p21ras activation and since it was shown to associate with PLCγ-1 upon CD2 stimulation.

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