Recruitment of Activated p56^{lck} on Endosomes of CD2-triggered T Cells, Colocalization with ZAP-70*

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Anne Marie-Cardine‡§, Siegmund Fischer‡, Jean-Pierre Gorvel†, and Isabelle Maridonneau-Parini‡

From INSERM U332, Institut Cochin de Génétique Moléculaire, 75014 Paris and Centre d’Immunologie INSERM/ CNRS, 13288 Marseille Luminy, France

We have previously established that upon CD2 activation of T cells, p56^{lck} showed a transient increase in its kinase activity and was partially internalized. Here we studied the possibility that p56^{lck} could retain its kinase activity in the endosomes of CD2-triggered cells. T cells were fractionated on a sucrose gradient, and the endosomal fraction was isolated. In CD2-triggered cells, part of Lck was internalized and presented a maximal kinase activity in the endosome-enriched fraction after 5 min, decreasing thereafter. In the endosomal fraction of activated cells, four tyrosine-phosphorylated proteins of apparent molecular masses of 30, 40, 56, and 70 kDa were detected. We demonstrated that the protein tyrosine kinase ZAP-70 was recruited to the endosomal fraction upon CD2 stimulation with kinetics similar to that of p56^{lck}, suggesting that recruitment of protein tyrosine kinases to endosomal vesicles could promote specific transduction signals at the intracellular level.

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p56^{lck} is a nonreceptor protein tyrosine kinase (PTK) of the Src family that is expressed primarily in lymphoid cells. The implication of p56^{lck} in T lymphocyte signal transduction has been first suggested by the observations that it is associated with either CD4 or CD8 coreceptors (Rudd et al., 1988; Veillette et al., 1988a) and that p56^{lck} kinase activity is increased in vitro in T cells stimulated with anti-CD4 mAbs (Veillette et al., 1988a). Similarly, incubation of T cells with anti-CD3 mAbs resulted in an increase in Lck kinase activity (Danielian et al., 1992) and in the appearance of a 62-kDa form of this protein (Danielian et al., 1989). The increase in apparent molecular mass of Lck was shown to be due to phosphorylation on serine and tyrosine residues and correlated with the return of tyrosine kinase activity to the basal level following activation (Veillette et al., 1988b; Danielian et al., 1989; Marth et al., 1989; Soula et al., 1994). Recently, we have shown that triggering T cells with a combination of anti-CD2 or anti-CD4 mAbs increased the kinase activity of p56^{lck} (Danielian et al., 1991; Marie-Cardine et al., 1992; Marie-Cardine et al., 1994).

It is well established that upon fixation of their ligand, receptor tyrosine kinases such as epidermal growth factor or platelet-derived growth factor receptor are dimerized, internalized, and processed within the endocytic pathway, where they become degraded (Carpenter and Cohen, 1976; Rosenfeld et al., 1984). Furthermore, a functional tyrosine kinase activity is necessary for the efficient degradation of the epidermal growth factor receptor after its internalization (Honegger et al., 1987; Glenney et al., 1988; Felder et al., 1990).

Although internalization of receptor tyrosine kinases from the plasma membrane to endosomes has been extensively studied, delocalization of Src family PTKs has been reported recently. In platelets, p60^{src} is mainly associated with the cytoplasmic face of the plasma membrane (Ferrell et al., 1990; Anand et al., 1993) but is also found in dense granules (Rendu et al., 1989) or in the cytosol (Dhar and Shukla, 1991). Additionally, c-src is particularly enriched in a population of late endosomes in fibroblasts (Kaplan et al., 1992). In activated platelets, p60^{src} was redistributed from the plasma membrane to the cytoskeleton fraction (Gordon et al., 1991; Horvath et al., 1992; Clark and Brugge, 1993; Pumiglia and Feinstein, 1993) or to the cytosol (Walker et al., 1993), and this correlated with enhanced kinase activity. Other PTKs of the Src family, p55^{hck} and p59^{hck}, were found to associate with secretory granules in resting neutrophils and translocate to the plasma membrane and the phagosomal membrane, respectively, upon cell activation (Gutkind and Robbins, 1989; Mohn et al., 1995). In T lymphocytes, we have described that a fraction of the plasma membrane-associated p56^{lck} was internalized upon cell stimulation via CD2 receptors (Marie-Cardine et al., 1992, 1994). This redistribution is specifically observed upon CD2 or CD45 triggering (Marie-Cardine et al., 1994) but not upon CD3 stimulation (Marie-Cardine et al., 1992; Ley et al., 1994), suggesting that internalization of Lck may be part of the process controlling the transduction of signals along these stimulation pathways.

Thus, we were interested in the characterization of the intracellular fraction of Lck and in its codistribution with potential substrates in CD2-triggered cells. To biochemically study the internalized fraction of Lck, a previously described preparation of endosomes was adapted to T lymphocytes (Goel et al., 1991). We report that Lck progressively accumulated in the endosome-enriched fraction of CD2-triggered Jurkat T cells, where its kinase activity was increased. Additionally, by Western blot analysis, we showed a parallel recruitment of the PTK ZAP-70 at the endosomal level in CD2-stimulated cells.

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EXPERIMENTAL PROCEDURES

Cells—Jurkat cells (clone J 77-68, CD4^-CD8^-TcR/CD3^-CD2^-CD45^-) were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (Boehringer Mannheim) and 2 mM l-glutamine, 100 μg/ml penicillin, and 100 units/ml streptomycin in a 5% CO2 humidified atmosphere at 37°C. Cell Stimulation and mAbs—Cells were cultured at a density of 4 ×
Association of Activated p56\(^{\text{ck}}\) with Endosomes

**RESULTS**

Preparation of an Endosome-enriched Fraction from J urkat T Cells—To test the kinase activity of p56\(^{\text{ck}}\) in the endocytic fraction of CD2-activated T cells, endosomes were isolated by cellular fractionation on sucrose gradient. Control J urkat cells, which had internalized the fluid phase marker HRP to label the endocytic compartment, were disrupted by cavitation in a nitrogen bomb. After centrifugation, the PNS was adjusted to 40.6% sucrose and placed at the bottom of a sucrose step gradient consisting of 2 ml of 35% sucrose and 1 ml of 8% sucrose homogenization buffer. After centrifugation, markers for the lysosomes (\(\beta\)-glucuronidase, Golgi apparatus (galactosyltransferase), and endosomes (HRP)) in each of the sucrose layers and interfaces (Fig. 1). We determined that the endocytic marker HRP was enriched at the 8/35% interface (Fig. 1A). The yield of endosomes, calculated as the percentage of the marker (HRP) present in the corresponding homogenate, is 15 ± 2% (\(n = 5\)). The lysosomal marker \(\beta\)-glucuronidase was predominantly localized in the 40.6% sucrose phase and in the 35/40.6% interface (Fig. 1B), whereas the Golgi marker galactosyltransferase was found throughout the gradient, with a peak in the 35% sucrose phase (Fig. 1C).

We have previously determined that a part of the plasma membrane-associated Lck was internalized. Therefore, it was necessary to ascertain that the endosome-enriched fraction was not contaminated by the plasma membrane. The protein tyrosine phosphatase CD45, which is located at the plasma membrane and, to a lesser extent, in the Golgi apparatus in control cells, remained associated with these compartments even after CD2 stimulation (Marie-Cardine et al., 1994). On the other hand, under the same conditions of stimulation, CD2 receptors were completely internalized (Marie-Cardine et al., 1992).

Thus, we examined by Western blot analysis the distribution of both glycoproteins throughout gradients obtained from control or CD2-stimulated cells (Fig. 1D). The partitioning of CD45 along the gradient did not change upon cell stimulation (Fig. 1D, upper panel); it was recovered under both conditions in the 40.6% sucrose phase and in the 35/40.6% interface. Part of CD45 was also detected in the 35% sucrose phase, which is probably due to the location of the Golgi in this fraction. Similarly, in control cells, CD2 was predominantly found in the 40.6% sucrose phase and in the 40.6/35% interface (Fig. 1D, lower panel), confirming that these fractions contained the plasma membrane. No CD2 was detected in the endosomal fraction from nonstimulated cells. Striking changes were observed after CD2 stimulation. Indeed, CD2 was no longer detected in the higher sucrose density fractions but was almost exclusively recovered in the endosomal fraction of activated cells, confirming the complete relocation of the protein from the plasma membrane to endosomal vesicles. Taken together, our results showed that we had obtained an endosome-enriched fraction that was not contaminated by the plasma membrane or by lysosomes and only minimally by the Golgi apparatus.

Association of p56\(^{\text{ck}}\) with Endosome-enriched Fraction in CD2-activated J urkat Cells—To determine if p56\(^{\text{ck}}\) was efficiently internalized upon CD2 activation, control cells or cells stimulated with the combination of anti-CD2 mAbs (T1\(_{12}\) + T1\(_{13}\)) were fractionated. The endosome-enriched fractions were collected in each tube and centrifuged at 200,000 \(\times\) g to remove the sucrose. The pellets were resuspended in sample buffer, and an equal amount of proteins from each sample was submitted to Western blot analysis using anti-p56\(^{\text{ck}}\) antibodies followed by 125\(\text{I}\)-protein A. The results presented in Fig. 2 show that a small amount of p56\(^{\text{ck}}\) was present in the endosomal fraction of control cells, consistent with recent data indicating that a fraction of Lck is present on vesicular structures surrounding the centrosome (Ley et al., 1994). After 1 min of stimulation, a slight increase in the amount of Lck in this intracellular compartment was observed. The maximum level of Lck in the endosomal fraction was observed after 5 min of stimulation, remaining constant at later time points of 30 min (Fig. 2) and 1 h (data not shown). These results are in agreement with our previous studies using indirect immunofluorescence in which intracellular staining of p56\(^{\text{ck}}\) was found to
persist for 1 h after activation of T cells via CD2 (Marie-Cardine et al., 1992). Additionally, the higher molecular mass form of Lck, migrating at approximately 62 kDa, appeared in the endosomal fraction between 1 and 5 min after stimulation (Fig. 2). The bands corresponding to Lck were cut out from the nitrocellulose, and the incorporated radioactivity was measured. By comparison with the total amount of p56\textsuperscript{Lck} present in the cells, we estimated that the fraction of Lck in the endosomes was 3.2 ± 0.5% in control cells (t = 0 min) and 12.8 ± 2.6% (n = 3) 5 min after CD2 activation. This indicates that roughly 10% of Lck was endocytosed in CD2-triggered T cells.

Determination of p56\textsuperscript{Lck} Kinase Activity in the Endosomal Fraction of CD2-stimulated Jurkat Cells—The kinase activity of p56\textsuperscript{Lck} was analyzed in parallel in PNS (Fig. 3A) and in endosome-enriched fractions (Fig. 3B) of control and CD2-stimulated cells. After addition of the mAbs to the cell suspensions, the kinase activity of immunoprecipitated p56\textsuperscript{Lck} from cell lysates (PNS) presented a transient increase as previously observed (Danielian et al., 1991; Marie-Cardine et al., 1992). After 1 min of stimulation (Fig. 3A), phosphorylation levels of p56\textsuperscript{Lck} and enolase reached a maximum value and then decreased (t = 5 min) and returned to basal level at 15 min. The higher molecular mass form of the protein appeared after 5 min of activation (Fig. 3A, open arrowhead) and is predominant at 30 min, thus correlating with the return of the kinase activity to basal level.

Next, the kinase activity of Lck was analyzed in the endosome-enriched fraction. As shown in Fig. 3B, p56\textsuperscript{Lck} presented a delayed activation in this fraction when compared to the PNS. In control cells (t = 0), almost no kinase activity was detected. Upon stimulation, autophosphorylation of Lck and phosphorylation of enolase reached a maximum value after 5 min of CD2 triggering. Between 15 and 30 min it began to decrease; at these time points, the higher molecular mass form of p56\textsuperscript{Lck} was visualized. An additional phosphorylated band migrating at 50 kDa was also detected, probably corresponding to the heavy chain of immunoglobulin.

The specific activity of p56\textsuperscript{Lck} was estimated in both PNS and endosomal fractions of CD2-triggered cells (Fig. 3C). The bands corresponding to enolase and to Lck (in immunoblots) were cut out from the gels and nitrocellulose sheets, respectively, and the incorporated radioactivity was counted (n = 3). After 1 min of cell stimulation, Lck specific activity presented a 2.7 ± 0.31-fold increase in the PNS, while, at the same time, it was not modified in the endosome-enriched fraction (0.95 ± 0.26).
This suggested that at this time point, the activated fraction of the kinase was still at the plasma membrane level. After 5 min of activation, the specific activity of p56\textsuperscript{Lck} in both the PNS and the endosomal fraction increased by 1.6-fold (1.6 ± 0.22 and 1.6 ± 0.12, respectively), indicating that an activated fraction of Lck was internalized upon cell triggering.

Colocalization of p56\textsuperscript{Lck} with Proteins Phosphorylated on Tyrosine Residues and with the PTK ZAP-70—Taken together, our results indicate that p56\textsuperscript{Lck} is transiently under its activated form in the endosomal fraction of CD2-stimulated T cells. Western blot analysis was then performed on endosomal proteins from CD2-stimulated cells (t = 0, 1, 5, 15, 30, and 60 min) using antiphosphotyrosine Abs (Fig. 4A). In control cells (t = 0 min), the antiphosphotyrosine antibodies reacted with two proteins of apparent molecular mass of approximately 56 and 70 kDa (p56 and p70, respectively). In cells triggered for 1 min with the combination of anti-CD2 mAbs, p56 and p70 presented increased labeling with antiphosphotyrosine antibodies that remained constant until 60 min. Additionally, two other proteins of approximately 40 and 30 kDa (p40 and p30) became detectable after 1 min. The phosphotyrosine staining of p40 decreased thereafter, whereas the reactivity of anti-phosphotyrosine antibodies toward p30 increased, reaching a maximum after 5 min of activation and decreasing thereafter. The same pattern of tyrosine-phosphorylated proteins was obtained after pre-clearing of the fractions with protein A-Sepharose before protein separation (data not shown), indicating that anti-CD2 mAbs did not interfere with the assay.

We found that a protein of 70 kDa was present in the endosome-enriched fraction. As a recent study reported that p56\textsuperscript{Lck} interacts via its src homology-2 (SH2) domain with ZAP-70 (approximately 62 kDa). C, incorporation of \textsuperscript{32}P in endolase from in vitro kinase assay and \textsuperscript{125}I-radiolabeled bands corresponding to immunoprecipitated Lck were counted at each time point in PNS and endosomal fractions; the specific activity of Lck was expressed in arbitrary units. The results shown are mean ± S.D. of three independent experiments.

\* p < 0.05 when compared with controls (0 min) calculated with paired Student's t test.
In the present report, we describe that stimulation of T cells via CD2 resulted in a time-dependent accumulation in the endosome-enriched fraction of an active population of p56lck, which colocalized with the PTK ZAP-70.

In Jurkat cells stimulated with an activating combination of anti-CD2 mAbs, we have previously shown, by indirect immunofluorescence, that Lck was partially relocated from the plasma membrane to endocytic vesicles (Marie-Cardine et al., 1993). To isolate the endosome-enriched fraction from T lymphocytes, we adapted a subcellular fractionation method previously applied to several cell types (Gorvel et al., 1991, 1995; Escola et al., 1995). After centrifugation of cell lysates on a discontinuous sucrose gradient, the partitioning of cellular organelles was tested using enzymatic markers. A fraction enriched in endosomes, separated from the plasma membrane, was obtained. This was an important point as it allowed us to clearly distinguish the endosome-associated p56lck from the pool that remained bound to the plasma membrane upon T cell stimulation via CD2.

Fractionation experiments showed that in Jurkat cells triggered via CD2, the internalized fraction of p56lck presented a 1.6-fold increase in its specific activity. The kinase activity of Lck recovered in the endosome-enriched fraction reached a maximum with delayed kinetics when compared to that of PNS. One min after activation, an increase in the amount of internalized Lck was detectable, whereas the activity of the enzyme was similar to that obtained in control cells. On the other hand, the kinase activity detected in the PNS at this time point was maximum, and the maximal kinase activity in the endosomal fraction was reached 5 min after the addition of the anti-CD2 mAbs. It has been estimated that in control Jurkat cells, 2% of p56lck interacts with CD2 (Bell et al., 1992; Carmo et al., 1993). In stimulated cells, we have estimated that about 13% of the Lck expressed in the cells was recovered in the endosome-enriched fraction. Therefore, changes in the degree of interaction between CD2 and p56lck could occur upon activation. As CD2 is devoid of enzymatic activity, it has been proposed that activation of Lck through CD2 involves other molecules. One appropriate candidate is the protein tyrosine phosphatase CD45, since it has been shown that its expression at the cell surface is necessary for Lck activation upon CD2 stimulation (Danielian et al., 1992) and that triggering of the cells with either anti-CD2 or anti-CD45 mAbs induced similar internalization of Lck and increase in its kinase activity (Marie-Cardine et al., 1994). Thus, it has been proposed that a functional complex between CD2, CD45, and p56lck is formed and involved in the activation of the kinase. It is possible that shortly after incubation with the anti-CD2 mAbs, pre-existing CD2/p56lck complexes (representing 2% of cellular p56lck) were endocytosed, leading to the presence of nonactivated Lck at the endosomal level after 1 min of stimulation. Simultaneously, at the cell surface, free molecules of CD2 could be triggered and functional complexes consisting of CD2, CD45, and Lck could be formed, which reflects the maximal activation of p56lck observed in the PNS 1 min after cell stimulation. This activated fraction of Lck could then be internalized, accounting for the peak of the kinase activity in the endosomal-enriched fraction at 5 min. At this point, CD2 receptors are endocytosed while CD45 phosphatase remains at the plasma membrane (Marie-Cardine et al., 1994), suggesting that the transduction complex is in part disrupted after enhancement of p56lck activity. Further identification of the molecular basis of the interaction between CD2, CD45, and p56lck will allow a better understanding of the mechanism of CD2-mediated signal transduction.

As Lck is a nonreceptor PTK, it is presumably associated with the inner face of the plasma membrane and the cytoplasmic face of endocytic vesicles. A consequence of the redistribution of Lck in CD2-stimulated cells may be the distinct accessibility of the kinase to regulatory proteins and substrates. We report here that the internalized fraction of p56lck colocalized with a few proteins phosphorylated on tyrosine residues. By Western blot analysis, four proteins of 70, 56, 40, and 30 kDa were detected. It is possible that the 56-kDa protein was p56lck itself. Indeed, p56lck can be phosphorylated on tyrosine residues independently of its activity. The negative regulation site, Tyr-505, is phosphorylated when the enzyme activity is repressed and its autophosphorylation site, Tyr-394, is generally phosphorylated when the kinase is activated. The tyrosine-phosphorylated protein p70 could be the PTK ZAP-70. In endosomal fractions from control cells, ZAP-70 was not detected, even after long time exposure of the blot, whereas the protein p70 was observed with the antiphosphotyrosine antibodies. This suggests that the bulk of the 70-kDa signal is probably not ZAP-70. A functional interaction between p56lck and tyrosine-phosphorylated ZAP-70 has been described previously (Duplay et al., 1994). Upon CD2 stimulation, the amounts of Lck and ZAP-70 in the endosomal fraction increased in parallel. Whether these two kinases interact at the endosomal level remains to be established. We also detected the presence of two tyrosine-phosphorylated proteins of 30 and 40 kDa in the endosomal fraction of CD2-activated cells. The level of p40 phosphotyrosine staining did not seem to be related to the activation state of p56lck, suggesting that Lck is not directly responsible for p40 phosphorylation. On the contrary, tyrosine phosphorylation detected on p30 was modulated according to the time of stimulation. Several proteins of similar molecular mass interacting with p56lck have been described, but none have been shown to be tyrosine phosphorylated in vivo upon CD2 stimulation (Schraenen et al., 1991, 1992; Ross et al., 1994). Identification of this protein will be of interest because it appeared in the endosomal-enriched fraction upon CD2 activation and was tyrosine phosphorylated in parallel to the activation of Lck. However, at present, we have been unable to detect any protein communoprecipitating with Lck, probably because of the low amount of material present in the endosomal fractions.

In conclusion, we showed that a fraction of p56lck is recruited at the endosomal level in CD2-triggered T cells. In the endosomes, the kinase activity of Lck and tyrosine phosphorylation of proteins increased in parallel. These observations might be critical in the transduction of CD2-mediated activation signals.

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