Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 328-334

EVIDENCE THAT A GTP BINDING PROTEIN REGULATES PHOSPHORYLATION OF THE CD3 ANTIGEN IN HUMAN T LYMPHOCYTES

Adelina A. Davies, J. Mark Hexham, Doreen A. Cantrell and Michael J. Crumpton

Cell Surface Biochemistry Laboratory,
Imperial Cancer Research Fund,
P.O. Box 123,
Lincolns Inn Fields,
London, WC2A 3PX, UK

Received August 26, 1988

Summary The role of guanine nucleotide binding regulatory proteins (G proteins) in the regulation of phosphorylation of the γ subunit of the CD3 antigen has been examined. CD3 γ chain phosphorylation in isolated T cell microsomes was stimulated by the G protein activator guanosine 5'-O thiotriphosphate (GTPγS), but cyclic adenosine monophosphate and guanosine 5'-diphosphate were ineffective at inducing γ chain phosphorylation. The effect of GTPγS was rapid and transient; a half maximal effect was observed with 50μM of the nucleotide. γ polypeptide phosphorylated in vitro in GTPγS stimulated microsomes incorporated phosphate on Serines 123 and 126. These data are consistent with the involvement of a G protein in the signalling mechanisms that regulate the phosphorylation of the CD3 γ chain.

T lymphocyte activation is regulated by the T cell antigen receptor/CD3 (Ti/CD3) complex (1). Ti has been identified as an idiotypic disulphide linked heterodimer comprising two glycosylated polypeptides (α and β) of Mr 50,000 and 43,000 respectively (1). The CD3 antigen which is invariant, is noncovalently associated with Ti and consists of 3 chains: two glycosylated polypeptides of Mr 26000 and 21000 (γ and δ respectively)

Abbreviations

Guanosine 5'-O thiotriphosphate: GTPγS, Guanosine 5' diphosphate: GDPβS

Cyclic adenosine monophosphate: cAMP, 4-(2-hydroxyethyl)-1

-piperazineethane sulphonic acid: Hepes, Phorbol 12,13 dibutyrate: Pdbu

0006-291X/88 $1.50

and one non-N-glycosylated polypeptide of Mr 19000 (ε) (2,3). Recently we have shown that an immediate consequence of antigen activation of T cells is a selective phosphorylation of the CD3 γ subunit (4-7). The proposed role for this phosphorylation is that it controls the cell surface expression and functions of the Ti/CD3 complex (7,8).

Two cell surface receptors are known to initiate the phosphorylation of CD3 γ chains in intact cells, namely the Ti/CD3 complex and CD2 antigen (4,9). There are also two candidates for the immune regulated kinase that mediates CD3 γ subunit phosphorylation, namely protein kinase C (pkC) and an unidentified calcium regulated kinase (7,10). The products of phosphoinositide metabolism are thought to act as intracellular signals coupling the Ti/CD3 complex and CD2 antigens to the CD3 γ chain kinase (11). Thus, triggering of both Ti/CD3 and CD2 molecules initiates an immediate breakdown of phosphatidylinositol biphosphate generating inositol phospholipids that regulate intracellular Ca^{2+} and diacylglycerols that collectively or separately control pkC and the other kinase. In T cells, as in many cells, the turnover of phosphoinositides is apparently controlled by guanine nucleotide binding (G) proteins (12-16). G proteins are activated when GTP is bound and inactivated when bound GTP is hydrolysed. Accordingly, non hydrolysable analogues of GTP can retain G proteins in a functional state and allow an assessment of the role of G proteins in a particular signal transduction pathway. In the present study we have explored the role of G proteins in the mechanisms that control the phosphorylation of the CD3 γ chain. We demonstrate that guanosine 5'-O-[3 thiotriphosphate] can modulate the phosphorylation of the CD3 γ subunit. This demonstration suggests that a G protein(s) is involved in the intracellular pathways that couple the CD3 γ chain to cellular kinases and/or phosphatases.

Materials and Methods

Reagents. Phorbol 12,13 dibutyrate (Pdbu) was purchased from Calbiochem (Behring Diagnostics), Guanosine and adenosine nucleotides were purchased from Boehringer Mannheim.

Phosphorylation. Human T lymphocyte microsomal membranes were prepared from exponentially growing T lymphocytes as described previously (10). Microsomal pellets were resuspended at 10mg protein/ml in Hepes buffer pH 7.5 using a Dounce homogeniser and stored in 1ml aliquots at -70°C. Microsomes (80-100μg) were phosphorylated at 30°C in a final volume of 50μl containing 10mM $MgCl_2$, 20mM Hepes buffer pH 7.5, 25 μM [γ-^{32}P] ATP (5μCi, Amersham) and guanosine nucleotides as indicated. The reaction was timed from the addition of the ATP and stopped with 500μl of ice cold NP40 lysis buffer containing 1mg/ml bovine serum albumin.

CD3 antigen was isolated by immunoprecipitation using the monoclonal antibody UCHT1 and the subunits were separated by SDS/PAGE under reducing conditions (7,10). ^{32}P-incorporation into the γ chain was quantitated by scanning autoradiographs densitometrically using a Joyce-Loebl Chromoscan 3. The relative intensities of the bands were compared on the basis of peak areas (arbitary units). Tryptic peptide analysis of the γ subunit for location of the phosphate group(s) by anion exchange was as described previously (10).

Results

GTPγS regulates CD3 γ chain phosphorylation. Microsomal membranes were incubated at 30°C with ^{32}P-labelled ATP in the presence or absence of 100μM GTPγS and immunoprecipitates of the CD3 antigen were subsequently analysed by SDS-PAGE. The data (Fig 1A) show that incorporation of ^{32}P into the Mr 26000 γ subunit was enhanced by the presence of GTPγS but not 100μM GDPβS in the incubation mixture. Fig 1B shows that 100μM cAMP did not stimulate CD3 γ chain phosphorylation. In 5 separate experiments the GTPγS enhancement of γ chain phosphorylation ranged from 4 to 10 fold. As shown in Fig. 1 three predominant phosphorylated bands were observed in CD3 immunoprecipitates prepared from microsomes phosphorylated in vitro. These include the Mr 26000 γ chain but also bands of Mr 55000 and 20000. The Mr 20000 band comprises an endoglycosidase F resistant component and a component that reduces to Mr 14000 after the removal of N-linked sugars (data not shown) and probably represents a mixture of phosphorylated CD3 ε and δ chains. The identity of the Mr 55000 polypeptide is, however, unknown. The effects of GTPγS on phosphorylation were rapid and transient, peaking within 30 seconds exposure to the guanine nucleotide and declining to baseline levels within 5 minutes (Fig 1C). A half maximal effect was observed with 50μM GTPγS (Fig 1D).

Tryptic peptide analysis of ^{32}P-labelled γ chain. Anion exchange chromatographic analyses of tryptic peptides of CD3 γ subunits phosphorylated in vivo, and of phosphorylated synthetic peptides corresponding to portions of the cytoplasmic domain of the γ chain, have identified two major phosphorylation sites in the CD3 α chain at serine residues number 123 and 126 (10), (Fig 2). The data in Fig 2 depict the anion exchange elution profile of the tryptic peptides derived from the γ polypeptide phosphorylated in vitro in GTPγS stimulated microsomes. The elution pattern comprises 3 peaks of radioactivity (A, B and C) and is consistent with multiple sites phosphorylation of the γ chain. Peaks B and C elute in identical positions with those expected for peptides phosphorylated on serine residues 123 and 126 respectively. The ratio of phosphorylation between serine residues 123 and 126 is approximately 1:1.

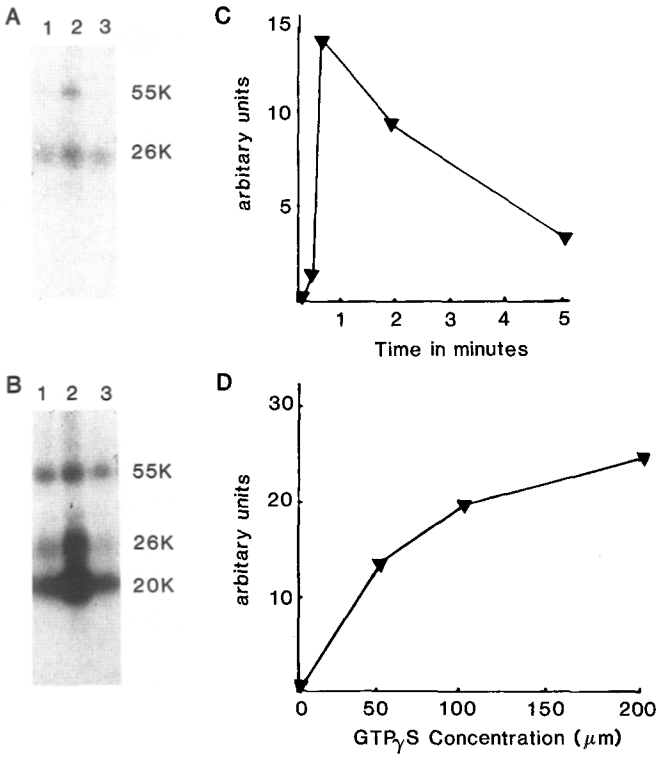

Fig 1. GTPγS regulates CD3 γ chain phosphorylation.
A),B) SDS/PAGE analyses of ^{32}P labelled CD3 antigen immunoprecipitates prepared from T cell microsomes which had been A) unstimulated (track 1), exposed to 100μM GTPγS (track 2) or 100μM GDPβS (track 3) for 2 minutes. B) unstimulated (track 1) or exposed to 100μM GTPγS (track 2) or 100μM cAMP (track 3) for 2 minutes.
C) Kinetics of GTPγS stimulated γ chain phosphorylation. CD3 immunoprecipitates were prepared from T cell microsomes phosphorylated in vitro in the presence of 100μM GTPγS for the indicated time.
D) Concentration dependence of GTPγS effects on γ chain phosphorylation. T cell microsomes were phosphorylated in vitro in the presence of the indicated concentration of GTPγS for 2 minutes. Data in C,D show ^{32}P incorporation into the γ chain as quantitated by densitometry (arbitary units) of autoradiographs of SDS/PAGE analyses of CD3 immunoprecipiates.

The identity of the more acidic peptide which elutes in peak D is unknown, although it could represent γ chains phosphorylated on both Ser 123 and 126.

The effect of phorbol esters on γ chain phosphorylation. GTPγS could effect γ chain phosphorylation in T cell microsomes via stimulation of pkC. The experiment shown in Fig 3 compares the effects of GTPγS and the pkC activator Pdbu on CD3 antigen phosphorylation. The data indicate that Pdbu enhanced γ chain phosphorylation, but its maximum effect was not as great as that induced by GTPγS.

A

Intra-cellular region of T3 γ chain

QDGVRQSRASDKQTLLPNDQLYQPLKDREDDQYSHLQGNQLRRN

123 126 150

membrane

|Tryptic cleavage sites

Peptides QS^{123}-PO_4R, QS^{123}-$PO_4AS^{126}DK$ elute in peak B.

Peptide AS^{126}-PO_4DK elutes in peak C.

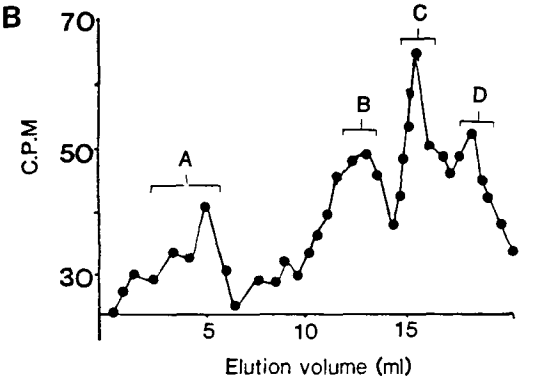

Fig 2. Anion exchange chromatographic analyses of tryptic peptides derived from in vitro ^{32}P labelled γ chain.

A) Amino acid sequence of the cytoplasmic domain of the CD3 γ subunit; vertical arrows indicate potential sites of tryptic cleavage.

B) Tryptic peptide analyses of the γ chain phosphorylated in vitro in T cell microsomes stimulated with GTPγS for 2 minutes. Previous studies have demonstrated that peak A represents radioactivity that does not bind to the column.

Peptides QS^{123}-PO_4R, QS^{123}-$PO_4AS^{126}DK$ elute in peak B.

Peptide AS^{126}-PO_4DK elutes in peak C.

The identity of peak D is unknown.

Discussion

The present data demonstrate that exposure of microsomes from T-lymphocytes to GTPγS leads to the phosphorylation of the γ subunit of the CD3 antigen, and that this effect is not mimiced by other nucleotides such as GDPβS and cAMP. GTPγS irreversibly activates G proteins and, thus, the present data provide indirect evidence for the involvement of a G protein(s) in the regulation of CD3 γ chain phosphorylation. This conclusion is compatible with the data of O'Shea et al who observed that aluminium fluoride complexes, which are thought to interact with G

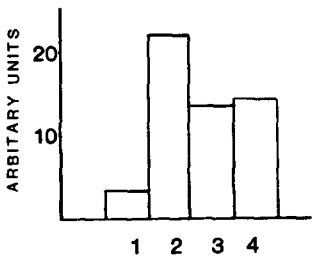

Fig 3. Effect of Pdbu on γ chain phosphorylation in T cell microsomes.

Data show ^{32}P incorporated into the γ chain, quantitated by densitometry (arbitary units) of autoradiographs of CD3 immunoprecipitates prepared from T cell microsomes phosphorylated in vitro for 2 minutes. Histograms 1-4 represent control, GTPγS (100μM), Pdbu (5ng/ml) Pdbu (50ng/ml) treated cells respectively.

proteins, modulated the phosphorylation of CD3 γ subunits (14). Protein phosphorylation is determined ultimately by a balance of kinase and phosphatase activities and, therefore, the enhancing effects of GTPγS can be explained in terms of the stimulation of kinase(s) and/or inhibition of phosphatase(s). One kinase that is known to regulate CD3 γ subunit phosphorylation in vivo is pkC (7). As GTPγS can activate phospholipase C in T lymphocytes (15,16), it is possible that GTPγS stimulates pkC in T cell microsomes via the breakdown of polyphosphoinositides and the subsequent generation of diacylglycerols. However, if the simple model of diacylglycerol activated pkC accounts for the enhancing effects of GTPγS on CD3 phosphorylation, then phorbol esters, which mimic the effect of diacylglycerols and activate pkC, should also enhance γ chain phosphorylation. Although Pdbu enhanced CD3 phosphorylation in T cell microsomes (Fig 3), it was much less effective than GTPγS, suggesting that GTPγS regulates CD3 γ chain phosphorylation via an alternative pathway or perhaps via a combination of pkC dependent and independent mechanisms. A further indication that pkC is not the only regulatory factor controlling the effects of GTPγS on CD3 phosphorylation in T cell microsomes, comes from the observation that in intact cells, serine residue 126 of the γ chain is the major target for pkC regulated phosphorylation, whereas GTPγS induced phosphorylation of residues 123 and 126. The latter pattern of phosphorylation is similar to that observed in intact cells following stimulation with ionomycin (10). As a result, it is possible that GTPγS modulates γ chain phosphorylation similarly to ionomycin via a calcium regulated kinase.

There are major differences between the pattern of phosphorylation of the CD3 antigen in T cell microsomes compared with intact cells. For example, in intact cells, the basal level of CD3 γ chain phosphorylation is

below the limits of experimental detection and induction of phosphorylation by external stimuli is both rapid and transient (7,8). In contrast, in T cell microsomes, phosphorylation of the γ chains was readily detected in the absence of known external stimuli. Another difference is that in intact cells the γ chain is the preferential target for phosphorylation, the δ chain is phosphorylated weakly and there is no detectable phosphorylation of the ε chain (7), whereas, in microsomes, there is marked phosphorylation of both the ε and δ chains. A possible explanation for the differences between the phosphorylation patterns in vivo and in vitro is that in intact cells the conformation of the CD3 antigen - T cell antigen receptor complex permits an interaction between a kinase and the γ chain, but prohibits a similar interaction with the δ and ε chains. It is also possible that in vitro, there is a breakdown in the restraints imposed by subcellular compartmentalisation on the localisation of the kinase with the result that potential phosphorylation sites in the extracellular domains of the δ and ε subunits become accessible to kinases.

References

1) Moller,G. (ed) (1984) Immunological Review 81
2) Borst,J., Alexander,S., Elder,J. and Terhorst,C. (1983) J.Biol.Chem. 258:5135-5141
3) Kanelopoulous,J.M., Wigglesworth,N.M., Owen,M.J. and Crumpton,M.J. (1983) EMBO J. 2:1807-1814
4) Cantrell,D.A., Davies,A.A., Londei,M., Feldman,M. and Crumpton,M.J. (1987) Nature 325:540-542
5) Samelson,L.E., Halford,J.B., Klausner,R.D. (1985) Cell 43:223-231
6) Samelson,L.E., Patel,M., Weissman,A., Harvard,J.B. and Klausner,R.D. (1986) Cell 46:1083-1090
7) Cantrell,D.A., Davies,A.A. and Crumpton,M.J. (1985) Proc.Nat.Acad.Sci.U.S.A 82:8158-8163
8) Krangel,M.J. (1987) J.Exp.Med. 165:1141-1159
9) Breightmeyer,J.B., Daly,J.F., Levine,H.B. and Schlossman,S.F. (1987) J.Immunol. 139:2899-2905
10) Davies,A.A., Cantrell,D.A., Hexham,J.M., Parker,P., Rothbard,J. and Crumpton,M.J. (1987) J.Biol.Chem. 262:10918-10921
11) Panteleo,G., Olive,D., Hogg,A., Kozumbo,W.J., Moretta,L., and Moretta,A. (1987) Eur.J.Immunol. 17:55-59
12) Zilberman,Y., Howe,L.R., Moore,J.P., Hesketh,T.R. and Metcalfe,S.C. (1987) Embo J. 6:957-962
13) Imboden,J., Shoebuck,D.M., Paterson,G. and Stobo,J.D. (1986) Proc.Nat.Acad.Sci.U.S.A 83:5673-5682
14) O'Shea,J.J., Urdahl,K.B., Luang,H.T., Chused,T.M., Samelson,L.E. and Klausner,R.D. (1987) J.Immunol. 139:3463-3469
15) Stryer,L. and Bourne,H.R. (1986) Annual Review of Cell Biology 2:391
16) Mire-Sluis,A.R., Hoffbrand,V., and Wickremasinghe,R.G. (1987) Biochemical and Biophysical Research Communications 148:1223-1231