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Stimulation of tyrosine phosphorylation without inositol lipid hydrolysis in human B lymphocytes on engaging CD72

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Occupancy of CD72 on resting tonsillar B cells by monoclonal antibodies (mAb) promotes entry into the G_1 phase of the cell cycle with an accompanying increase in MHC Class II expression and provides a co-stimulus to immobilized anti-μ for driving DNA synthesis. We now report that engagement of CD72 by mAb stimulates tyrosine phosphorylation in B cells with a peak of activity seen at 5–10 min. Two major substrates of 29 and 57 kDa showed a basal level of phosphorylation which increased with time, while a 40 kDa protein and several other minor components were phosphorylated de novo on the addition of mAb to CD72. Inositol lipid hydrolysis was found to be unperturbed, although a shallow rise in the basal level of intracellular free Ca^{2+} was provoked on engaging CD72. Receptor cross-linking was not a requirement for signaling human B cells through CD72: simple occupancy by univalent antibody was sufficient both to trigger the rise in basal $[Ca^{2+}]_i$ and to promote DNA synthesis

B lymphocyte; CD5; CD72; Lyb-2; Tyrosine kinase

1. INTRODUCTION

CD72 is a 45-kDa type II integral membrane protein of human B lymphocytes belonging to a family of Ca^{2+}-dependent (C-type) lectins [1]. Distributed throughout B cell maturation, it is the homolog to the Lyb-2 differentiation antigen of mouse [2]. The counter-structure to CD72 is the 67-kDa CD5 molecule expressed on T cells and a subset of B cells [3,4]. For both mouse and man, monoclonal antibodies (mAb) to CD72/Lyb-2 activate resting B cells [5–7], a small proportion of which progress directly to DNA synthesis while selective co-stimuli can augment this response [5–8]. For murine B cells, simple occupancy of Lyb-2 by univalent antibody appears sufficient to elicit change, indicating that an allosteric mechanism of signaling may be operative [9]. Analysis of signal transduction pathways stimulated in mouse B cells on engaging Lyb-2 has shown that hydrolysis of inositol lipid is accompanied by a rise in intracellular levels of free Ca^{2+} [10].

The signal transduction pathway operating in human B cells through CD72 is unknown. A summary from the Fourth International Workshop on Human Leucocyte Differentiation Antigens indicated that a small rise in basal intracellular free Ca^{2+} could be provoked through CD72, particularly in the presence of a second cross-linking antibody [11]. We now report that mAb to CD72 stimulates in resting human B cells tyrosine phosphorylation without measurable change in inositol lipid hydrolysis: the rise in basal $[Ca^{2+}]_i$ appears to result from the influx of extracellular ions.

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2. MATERIALS AND METHODS

2.1. *Reagents*

BU1 and BU40 are murine IgG_{2a} mAb specific for human IgM and CD72, respectively, and were purified using DEAE ion-exchange chromatography. Fab fragments of BU1 and BU40 were generated by enzymatic digestion of purified mAb with papain as described in detail elsewhere [12]; pepsin digestion was used to obtain $(Fab')_2$ fragments of BU40 [12]. Purity of antibody derivatives was assessed on 10% SDS-polyacrylamide gels followed by silver staining to reveal single bands of material migrating with the expected mobilities with no evidence of contamination. G28-5 (IgG_1) mAb to CD40 was a generous gift from J. Ledbetter (Bristol-Myers Squibb, Seattle, WA); rIL4 was kindly provided by S. Gillis (Immunex Corporation, Seattle, WA). Rabbit anti-mouse Ig and forskolin were from Sigma (Poole, Dorset, UK). Ionomycin was obtained from Calbiochem (La Jolla, CA). Purified and FITC-conjugated mAb to phosphotyrosine were both obtained from Upstate Biotechnology Inc. (Boston, MA). ^{125}I-Conjugated Protein A was purchased from Amersham Int.

2.2. *Measurement of DNA synthesis*

Resting (G_0) tonsil B cells were prepared by negative selections and density separations on Percoll (Pharmacia, Uppsala, Sweden) as described fully elsewhere [12]. Assessment of DNA synthesis was performed by culturing 10^5 cells in flat-bottom microwells (growth area = 0.32 cm^2) in 200 μl of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics at 37°C in a humidified 5% CO_2 atmosphere and pulsing with 0.5 μCi (18.5 kBq) of [^{3}H]thymidine (Tdr) in 50 μl of medium for the final 16 h. The amount of radioactivity incorporated into triplicate cultures was determined; individual determinations never varied by >10% and were usually within 5% of each other.

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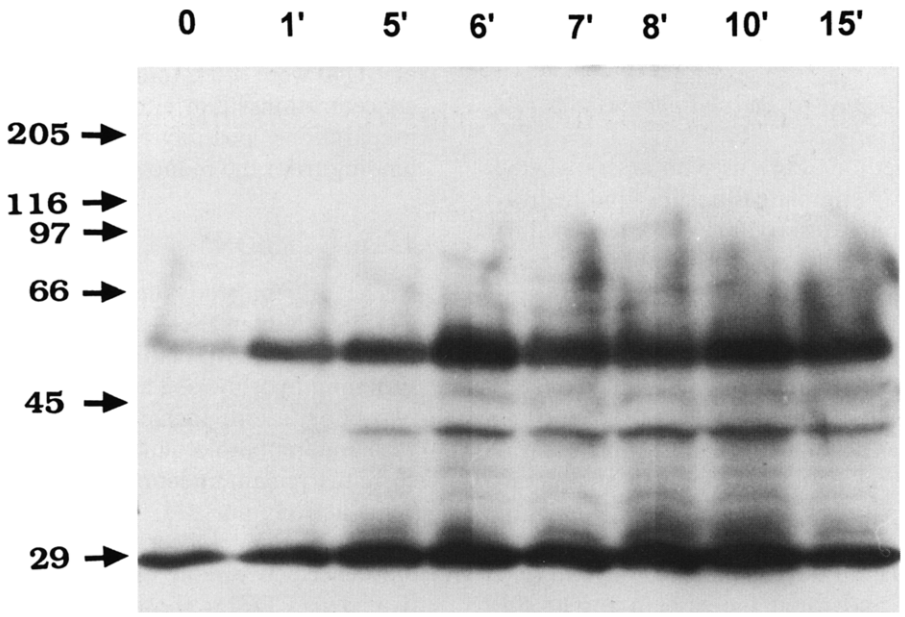

Fig. 1. Analysis of proteins phosphorylated on tyrosine. Resting B cells (10^7/ml/lane) were stimulated with BU40 (10 μg/ml) as indicated and proteins analyzed by Western blot with anti-phosphotyrosine mAb. The position of molecular weight markers in kDa are shown. Lane 0 are cells with no antibody added.

2.3. *Measurement of new tyrosine phosphorylation*

For analysis of protein substrates phosphorylated on tyrosine residues, resting B cells (10^7/ml/sample) were stimulated with BU40 (10 μg/ml) at 37°C and the reaction stopped by adding ice-cold phosphate buffered saline (PBS) containing 200 μM sodium orthovanadate. Cells were pelleted by spinning at 4°C and resuspended in 50 μl lysis buffer containing 1% Triton X-100 and 50 μl of 2 × Laemmli sample buffer with 200 μM sodium orthovanadate. Samples were then vortexed, boiled for 5 min, and sonicated for 15 s before running on a 10% SDS-PAGE resolving gel and being transferred electrophoretically onto a nitrocellulose membrane. The blots were incubated with mAb to phosphotyrosine (1 μg/ml in 3% skimmed milk) overnight at 4°C, washed with PBS containing 0.05% Tween 20 and then incubated with rabbit anti-mouse Ig (1: 2,000 dilution in 3% skimmed milk) for 2 h at room temperature. After washing, blots were developed with 1 μCi of ^{125}I-conjugated Protein A and labeled bands visualized by autoradiography.

2.4. *Measurement of inositol phosphate generation*

Resting B cells were cultured with additions indicated in the text at 37°C in a humidified 5% CO_2 atmosphere and the reaction stopped by addition of an equal volume of 20% $HClO_4$ (w/v) + 0.1% sodium phytate (w/v), vortexed briefly and stored on ice for 30 min. Cell debris was removed by centrifugation at 12,000 rpm and supernatant removed and titrated to neutrality by addition of 1 M KOH, 0.1 M 2-[*N*-morpholino]ethanesulphonic acid, 25 mM EDTA. Analysis of the inositol phosphates present in the resulting supernatant was made using two methods, each of which has been detailed elsewhere [13]: (i) radiolabeled inositol phosphates from 10^7 cells previously labeled with *myo*[2-^{3}H]inositol (10–20 Ci/mmol) added at 10 μCi/ml = 37 kBq/ml were analyzed by HPLC with reference to [^{3}H]inositol phosphate standards; (ii) the water soluble extract from 2×10^6 cells was measured for Ins(1,4,5)P_3 content using a competitive binding assay (Amersham Int.).

2.5. *Measurement of intracellular [Ca^{2+}]*

Resting B cells were suspended at 5×10^7 cells/ml in Hanks balanced salt solution (HBSS) + 0.5% bovine serum albumin + 20 mM HEPES (pH 7.2). The Ca^{2+}-dependent fluorescent indicator, Fura-2, was added to a final concentration of 1 μM and the cells were incubated at 37°C in a moist 5% CO_2 atmosphere for 45 min. The cells were then washed three times in HBSS + 10 mM HEPES (pH 7.2) + 10% FCS, resuspended at 5×10^6 cells/ml in HBSS + 10 mM HEPES (pH 7.2) and incubated at 37°C for 20 min before measuring fluorescence levels in either a Spex Fluorolog fluorimeter or the F2000 Luminescence Spectrometer. Fluorescence at 340 nm and 380 nm excitation wavelengths was monitored simultaneously to provide measurements of the Ca^{2+}-bound and Ca^{2+}- free forms of the dye and a ratio of these traces was used in the calculation of intracellular Ca^{2+} concentration using the on-board processing capability of the fluorimeter.

3. RESULTS

3.1. *Stimulation of tyrosine phosphorylation via CD72*

Analysis of protein substrates by Western blotting with specific anti-phosphotyrosine antibody revealed a number of targets for phosphorylation following stimulation of B cells through CD72. Two major substrates, of 29 and 57 kDa, showed variable degrees of basal phosphorylation among different B cell preparations; nevertheless, whatever the starting level of phosphorylation, a peak of stimulated activity in these two proteins was observed at 5–10 min following addition of BU40 to cells (Fig. 1). A third, relatively abundant substrate at 40 kDa was phosphorylated de novo on tyrosine following engagement of CD72, being first detected at around 5 min, reaching a maximum at 10 min, and then showing a fall-off in activity at later times. A series of minor substrates for CD72-stimulated tyrosine phosphorylation could be seen between 32 and 45 kDa, each

being phosphorylated de novo but reaching peak levels at differing times.

3.2. *CD72 is not coupled to the phosphoinositide (PI) hydrolysis pathway*

Two different methods were used to assess whether engagement of CD72 stimulated inositol lipid hydrolysis in resting B lymphocytes: (i) analysis of total cellular Ins(1,4,5)P_3 using a specific competitive binding assay; (ii) analysis of radiolabeled inositol phosphates by HPLC. Results shown in Fig. 2a detail the production of Ins(1,4,5)P_3 on stimulation by mAb. It can be seen that addition of the BU40 mAb to CD72 failed to stimulate any detectable increase in Ins(1,4,5)P_3 over 5 min of assay: results are shown as a mean of three separate experiments, where no significant change was obtained from any one preparation of B cells studied despite them responding well to the mAb by functional criteria [5]. By contrast, BU1 (an isotype-matched mAb to IgM) stimulated a large and rapid increase in Ins(1,4,5)P_3 levels which reached a peak at 15–30 s after addition. Analysis of inositol phosphates by HPLC revealed a similar outcome: anti-μ provoked an accumulation of label into inositol phosphates over time where no evidence for PI metabolism was evident in B cells stimulated through CD72 (Fig. 2b).

3.3. *Changes in intracellular [Ca^{2+}] on engaging CD72*

In seven different experiments, the addition of BU40 to resting B cells was found to promote a shallow rise in the basal level of free Ca^{2+}. Representative examples of this change are shown in Fig. 3 together with the rise in $[Ca^{2+}]_i$ which was registered on the addition of anti-μ: the latter showed the characteristic transient Ca^{2+} flux which is associated with signaling through surface membrane IgM (smIgM). The failure to elicit such a Ca^{2+} transient on the addition of mAb to CD72 is consistent with the inability of this route of triggering to provoke production of Ca^{2+}-mobilizing Ins(1,4,5)P_3. The small rise in basal $[Ca^{2+}]_i$ observed on engaging CD72 was inhibited by the presence of 5 mM EGTA in the extracellular medium: the initial peak response to anti-μ was unperturbed under these same conditions (results not detailed).

3.4. *Occupancy by univalent antibody is sufficient to signal B cells through CD72*

Murine B cells can be triggered through Lyb-2 by univalent fragments of antibody [9]. It can be seen from Fig. 3 that Fab fragments of BU40 provoke in resting human B lymphocytes the same shallow rise in $[Ca^{2+}]_i$ as intact antibody. Fab preparations from the isotype-matched anti-μ antibody (BU1) prepared in an identical way failed to trigger any elevation in $[Ca^{2+}]_i$ (data not detailed). Results presented in Table I show that univalent BU40 is able to provide a signal for stimulating resting B cells to DNA synthesis. The magnitude of the response obtained over several experiments was variable and two examples are illustrated. Fab fragments of BU40 were often found to be less effective at lower concentrations than either intact antibody or $(Fab')_2$ preparations, possibly reflecting a reduced avidity of binding from the monovalent ligand.

4. DISCUSSION

The present study demonstrates that triggering of CD72 on human B cells stimulates protein tyrosine kinase (PTK) activity without detectable increase in inositol lipid hydrolysis. The substrates for CD72-induced phosphorylation, including the three major proteins with apparent molecular weights of 29, 40 and 57 kDa, currently remain undefined but their characterization is being actively pursued.

An intriguing aspect of this study is the apparent difference between the signal transduction pathways activated via CD72 to those which have been described on stimulating Lyb-2, its murine equivalent. Antibodies to Lyb-2 generate PI turnover and trigger significant but

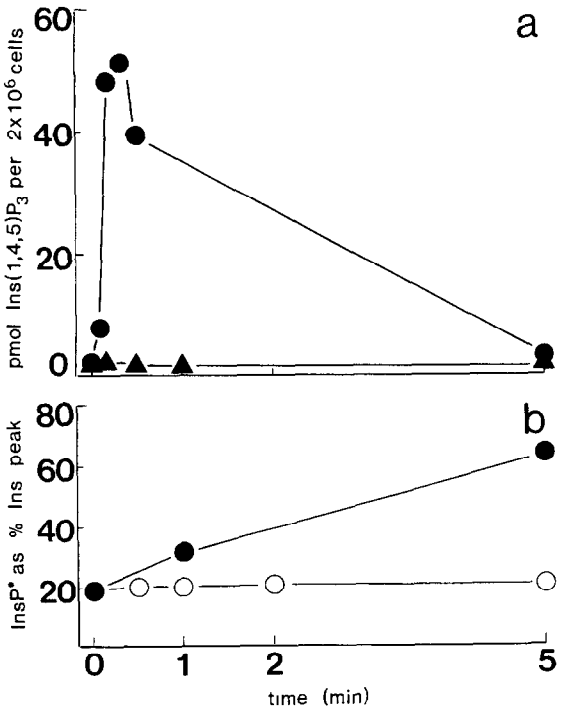

Fig. 2. Inositol lipid hydrolysis on engaging CD72. (a) Resting B cells (2×10^6 per sample) were stimulated with 20 μg/ml BU1 (●) or BU40 (▲) for the times indicated and Ins(1,4,5)P_3 levels measured in duplicate using a competitive binding assay: results from BU40-stimulated cells are represented as the mean of three different experiments with S.E.M. included but masked by the symbols; stimulation of Ins(1,4,5)P_3 by BU1 is represented by a typical result from at least 12 similar experiments. (b) Radiolabeled inositol phosphates from 10^7 B cells were analyzed by HPLC at the times shown following stimulation of duplicate samples with BU1 (●) or BU40 (○). Results are represented as the accumulated radioactivity in inositol mono- + bis- + tris-phosphates (InsP*) as a % radioactivity in inositol (Ins) peak.

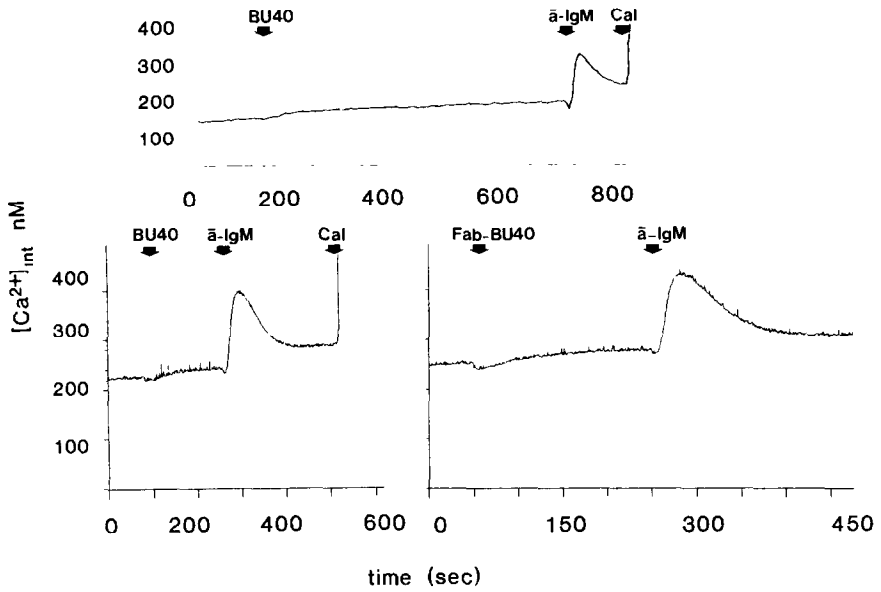

Fig. 3. Changes in $[Ca^{2+}]_i$ on engaging CD72. Resting B cells (2.5×10^6/ml) loaded with Fura-2 were placed in a cuvette and held in the fluorimeter at a constant temperature of 37°C before addition of mAb (20 μg/ml, ā-IgM = BU1; Fab-BU40 = Fab fragments of BU40) or ionomycin (CaI, 1 μg/ml) as indicated and changes in $[Ca^{2+}]_i$ followed. The top experiment is from measurements using the F2000 Fluorescence Spectrophotometer; the bottom experiments used the Spex Fluorolog fluorimeter.

transient elevation in cytoplasmic free $[Ca^{2+}]$ in mouse B cells [10]. In agreement with the preliminary report from the Fourth International Workshop on Human Leucocyte Differentiation Antigens [11], we found that mAb to CD72 promoted only a small shallow rise in the basal level of intracellular free $[Ca^{2+}]$. The increase corresponded to approximately 20% of the peak response achieved on ligating smIgM with a mAb of identical isotype and had none of the features associated with a transient release of Ca^{2+} from intracellular stores. Our inability to detect PI turnover on engaging CD72 is consistent with the absence of a Ca^{2+} transient: the blocking of the CD72-stimulated $[Ca^{2+}]_i$ rise on addition of EGTA is compatible with it having arisen from an influx of extracellular ions. Despite the differences observed in the signaling pathways stimulated, Lyb-2 in mouse and CD72 in man can both be activated by simple tethering of univalent antibody: receptor cross-linking is not required. Thus, whatever signal transduction pathways they are linked to, activation of this receptor in both species appears to be via an allosteric mechanism.

The discovery that CD5 is a counter-structure to CD72 [3] clearly provides a functional rationale to the behaviour of presumed ligand-mimetic mAb. Triggering through CD72 could thus be anticipated during T–B cell interactions and perhaps between B cells expressing CD5. In this regard it will be of interest to assess CD72-

Table I

Stimulation of resting B cells by univalent anti-CD72

Antibody fragment[b]	Radioactivity incorporated in DNA (cpm)[a]							
	Antibody concentration (μg/ml)							
	Experiment 1				Experiment 2			
	25	13	6	0	25	5	1	0
IgG	4,975	3,502	3,657	1,188	15,753	12,739	11,320	2,922
Fab	6,548	4,887	3,874	–	9,862	8,356	5,909	–
$(Fab')_2$	3,665	3,413	3,611	–	15,896	12,161	10,801	–

[a] B cells cultured at 10^5 per 200 μl medium containing 200 U of IL4; [^{3}H]Tdr incorporation was assessed between 48 and 64 h in triplicate cultures; mean determinations are given and replicates never varied by more than 10% of each other.

[b] BU40 was added to cultures either intact (IgG), or as Fab or $(Fab')_2$ derivatives at concentrations indicated.

dependent tyrosine phosphorylation during T–B conjugation and in CD5-positive B cells of normal and malignant origin. Given that activation through CD72 can be achieved with monovalent Fab fragments of mAb (this study) and that immobilized antibody is less effective than mAb in solution [5], the potential role of soluble CD5 in B cell regulation should be considered.

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