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Specific determination of tyrosine-phosphorylated proteins and peptides by differential iodination

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Abstract

A new method for the selective and quantitative determination of phosphotyrosine residues is presented using a differential iodination technique. Characterization of tyrosine-phosphorylated proteins was performed in a biological system using human U937 myeloid leukemia cells. The method is based on the saturation of free iodine binding sites using non-radioactive iodine. Samples are then treated with alkaline phosphatase. New iodine binding sites in dephosphorylated tyrosines are subsequently radio-iodinated, resulting in specific labeling of tyrosine phosphates. Separation is performed by RP-HPLC or sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Radiolabeled proteins are then identified using a radioactivity detector or autoradiography.

Keywords: Iodination; Phosphotyrosine residues; Proteins; Peptides; Tyrosine-phosphorylated proteins

1. Introduction

Different cell types require specific growth factors for their development to perform an appropriate function in the organism. Growth factor binding to the specific receptor stimulates tyrosine kinase activity and autophosphorylation on multiple tyrosine residues of the receptor (for review, see [1]). Subsequent phosphorylation of downstream adaptor molecules can recruit regulatory peptides from certain intracellular compartments to form short-lived complexes that relay signals throughout the cell (for review, see [2]). Thus, tyrosine phosphorylation is implicated in the control of elementary cellular pathways including cell growth, differentiation, and the regulation of the cell death program. Moreover, uncoupling of a phosphotyrosine-mediated trans-signalling cascade may significantly contribute to unregulated growth and consequently, the development of certain cancers (for review, see [3]).

Tyrosine phosphorylation represents one of the most frequent and important post-translational modifications of proteins and peptides to perform a key step in the regulation of several cellular functions [4,5]. Different methods allow the identification of phosphotyrosine-containing peptides under certain conditions. Traditional methods for direct mapping of phosphorylated tyrosines include biosynthetic radiolabeling procedures with [^{32}P]orthophosphate and subsequent isolation and cleavage of the protein of interest followed by peptide map analysis [6] or protein fragment analysis by mass spectrometry.

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Another specific method used affinity-purified antibody against phosphotyrosine for determination of phosphotyrosine-containing proteins and peptides [7]. In this context, we have previously shown that the method of differential iodination enables the selective and quantitative determination of tyrosine sulfate in peptides [8,9]. Using this differential iodination approach we demonstrate a new method for the selective and quantitative determination of phosphotyrosine residues in peptides and proteins. The method is based on the saturation of free iodine binding sites using non-radioactive iodine, followed by dephosphorylation of phosphotyrosines with alkaline phosphatase. Newly generated iodine binding sites in dephosphorylated tyrosines are subsequently radio-iodinated using [^{125}I]iodine. Peptides or proteins are separated by conventional RP-HPLC and incorporated labels are directly identified using a radioactivity monitor and a sodium dodecyl sulfate (SDS)–polyacrylamide gel (Fig. 1).

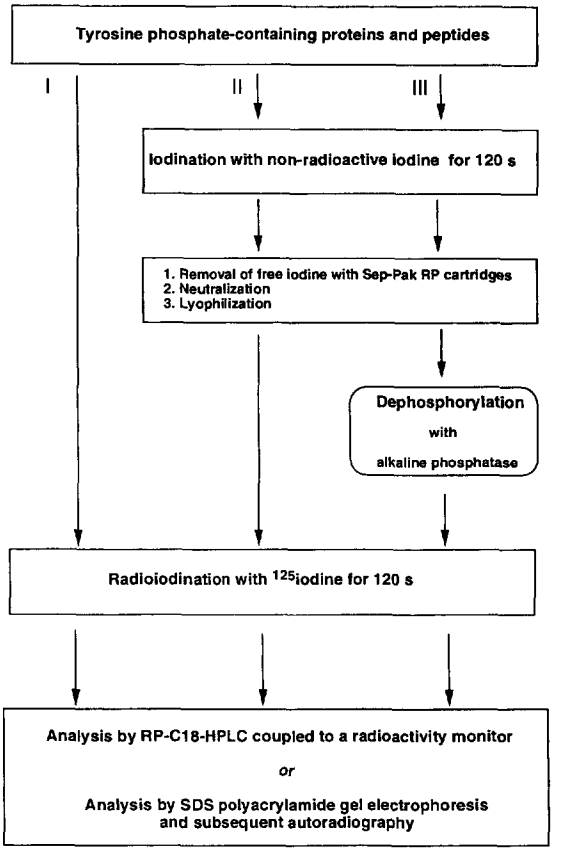

Fig. 1. Differential iodination protocol for determination of tyrosine-phosphorylated peptides and proteins. Control experiments were performed using direct radio-iodination of the sample (procedure I), saturation with non-radioactive iodine followed by direct radio-iodination with [^{125}I]iodine (procedure II). Procedure III represents the entire reaction sequence: saturation with non-radioactive iodine is followed by dephosphorylation with alkaline phosphatase. As a consequence, direct radio-iodination with [^{125}I]-iodine yields in specific labeling of tyrosine phosphorylated proteins and peptides which can be further analyzed by either C_{18} RP-HPLC coupled to a radioactivity monitor or separation by SDS–polyacrylamide gel electrophoresis and subsequent autoradiography.

The identification of phosphotyrosine-containing proteins and peptides requires two assay procedures. First, the degree of radio-iodination after non-radioactive iodination without dephosphorylation must be determined to obtain control values. Second, the sequence of non-radioactive iodination, dephosphorylation, and radio-iodination has to be applied. Data from both experiments may then be submitted to subtractive analysis thus reducing the background of radio-iodination due to incomplete saturation of iodine binding sites.

2. Experimental

2.1. Synthetic peptides

The method of differential iodination of phosphotyrosine-containing proteins and peptides was established in a mixture of two model peptides. A phosphorylated peptide TRD–P (TRDIY(P)ETD-OH) was synthesized as described by Austermann et al. [10]. A second synthetic peptide with a single tyrosine residue, Tyr–amid (KAEANleY-NH_2), served as control.

2.2. Cell culture and fractionation

In order to test this method in a biological system, human U937 myeloid leukemia cells were grown in RPMI 1640 medium containing 10% fetal bovine serum supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 m*M* L-glutamine [11]. $3 \cdot 10^8$ U937 cells were washed three times in phosphate-buffered saline (PBS) and resuspended in PBS containing 1 m*M* phenylmethylsulfonylfluoride.

The cells were homogenized 3×5 s in an Ultra-Turrax T25 (Janke and Kunkel, Staufen, Germany) at 13 500 rpm. The homogenate was centrifuged for 5 min at 14 000 *g* (4°C) to remove large particles and cell debris. Thereafter, the homogenate was ultracentrifuged for 30 min at 120 000 *g*. The resulting supernatant was used as cytosolic fraction and separated on a C_{18} RP-HPLC column. The gradient was developed using 10 m*M* HCl (mobile phase A) and 80% acetonitrile in 10 m*M* HCl (mobile phase B). Gradient for phosphorylated proteins in the cytosolic fractions: 0–100% mobile phase B in 30 min. Three fractions, A, B and C were selected and 50% of these samples were analyzed in the iodination assay followed by separation in a SDS–polyacrylamide gel, while the other 50% were analyzed by anti-phosphotyrosine Western blots.

2.3. Saturation of tyrosine with non-radioactive iodine

For iodination the chloramine-T method was used as previously described [8]. Briefly, 450 μl 0.25 *M* sodium phosphate buffer (pH 7.5), 100 μl sodium non-radioactive iodine (285 mg/ml in phosphate buffer) and 250 μl chloramine-T (10 mg/ml in phosphate buffer) were incubated with 100 μl of the protein or peptide sample in phosphate buffer at room temperature. After 120 s the reaction was stopped by addition of 100 μl $Na_2S_2O_5$ (315 mg/ml water).

2.4. Extraction with Sep-Pak cartridges

Sep-Pak C_{18} cartridges (Waters-Millipore, Eschborn, Germany) were equilibrated with 10 ml methanol followed by 20 ml of water. Following application of the non-radioactive iodinated sample the cartridge was rinsed with 10 ml water to remove unbound material. Elution was carried out in two steps using 1 ml 30% (v/v) isopropanol followed by 1 ml 50% (v/v) isopropanol. The eluate was immediately lyophilized.

2.5. Dephosphorylation

Dephosphorylation was performed with alkaline phosphatase from calf intestine, EC 3.1.3.1 (molecular biology grade; 1 U/μl, Boehringer Mannheim, Mannheim, Germany). In order to test the kinetics of alkaline phosphatase in this assay system, 10-μg samples of the phosphorylated synthetic peptide TRD–P were dissolved in 8 μl water and incubated with 1 μl alkaline phosphatase and 1 μl dephosphorylation buffer (10× concentrated: 0.5 *M* Tris–HCl, 1 m*M* EDTA, pH 8.5; Boehringer Mannheim) for different periods (0 to 60 min) at 37°C. Elution time of TRD and TRD–P in RP-HPLC were determined using synthetic TRD and TRD–P as standards. The peak areas were integrated and demonstrate the means±S.D. ($n=4$). The protein samples A, B and C of the cytosolic extract were dephosphorylated accordingly. Reaction was terminated by acidification with 10 m*M* HCl and immediately lyophilized.

2.6. Radio-iodination

Samples were dissolved in 50 μl 0.25 *M* sodium phosphate buffer (pH 7.5) and added to 15 μl 0.25 *M* sodium phosphate buffer (pH 7.5) with $1 \cdot 10^7$ cpm [^{125}I]iodine and 25 μl chloramine-T (10 mg/ml in phosphate buffer). After incubation for 120 s, the reaction was stopped by the addition of 10 μl $Na_2S_2O_5$ (315 mg/ml water). From the total assay volume of 100 μl, 50 μl were directly applied on a RP-HPLC column.

2.7. C_{18} RP-HPLC coupled to a radioactivity monitor

Separation of radio-iodinated samples was performed on a Kontron 322 HPLC system with a HPLC 360 autosampler and a HPLC detector 742 (Kontron, Neufahrn, Germany) connected to a radioactivity monitor LB 506 C-1 with a γ-mess cell J-1000 (volume: 100 μl, Berthold, Wildbad, Germany). Data acquisition was controlled by the LB 506 C-1-HPLC Data system. Separation of peaks was performed on a 250×4 mm I.D. RP C_{18} column (particle size 5 μm, 300 Å, Biotek, Östringen, Germany) at a flow-rate of 0.75 ml/min. The gradient was developed using 0.1% trifluoroacetic acid (mobile phase A) and 80% acetonitrile in 0.1% trifluoroacetic acid (mobile phase B): gradient for

peptides: 0–50% mobile phase B in 10 min; 50–100% mobile phase B in 20 min.

2.8. SDS–polyacrylamide gel electrophoresis of radio-iodinated proteins

Volumes of 50 μl of the 3 cytosolic fractions (A, B and C) of the C_{18} RP-HPLC were extracted by precipitation with 90% (v/v) acetone and dried under N_2. The dried samples were denatured and separated in a 10% SDS–polyacrylamide gel. The gel was dried with a gel dryer (Bio-Rad, München, Germany) for 3 h and exposed to an X-ray film (Kodak X-Omat) for up to 5 d.

2.9. Western blot analysis

Three fractions (A, B and C) of the C_{18}RP-HPLC of the cytosolic cell extract were separated in a 10% SDS–polyacrylamide gel and transferred to a PVDF membrane. After blocking nonspecific binding sites with 5% (v/v) heat-inactivated fetal bovine serum (FBS) in tris-buffered saline (TBS) for 2 h, the blots were incubated for 2 h with the monoclonal anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, NY, USA) diluted to 1 μg/ml in 0.25% FBS and 0.1% (v/v) Triton-X-100 in TBS (TBS–T). Following 1 h of incubation with rabbit anti-mouse IgG alkaline phosphatase conjugate (Sigma), the immune complexes were visualized by the 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium substrate (Sigma).

3. Results

3.1. Determination of a tyrosine-phosphorylated peptide

A peptide mixture containing a phosphorylated model peptide TRD–P and a control peptide, Tyr–amid, was used for the sequential iodination procedure. Iodination with non-radioactive iodine should result in coupling to all accessible iodine binding sites, predominantly tyrosine residues. Thereafter, dephosphorylation generates new iodine binding sites by removing phosphate groups from phosphotyrosines. These new iodine binding sites are then radio-iodinated with radiolabeled [^{125}I]iodine and analyzed on a HPLC system with radioactivity monitor (for reaction sequence see Fig. 1, procedures I, II, III).

Fig. 2a, b and c demonstrate the chromatograms of RP-HPLC separation of the peptide mixture with detection of incorporated [^{125}I]iodine. Several control experiments were performed to minimize and quantify the extent of non-specific labeling (the incorporation of [^{125}I]iodine into peptide residues other than tyrosine). Following radio-iodination (procedure I), only the control peptide (Tyr–amid) was radio-iodinated (Fig. 2a). Moreover, radio-iodination after cold iodination (procedure II), did not result in any detectable radio-iodinated peptides (Fig. 2b). The phosphotyrosine residue in TRD–P was inert to iodination by the chloramine-T method under these experimental conditions. TRD–P was radio-iodinated only after cold iodination and subsequent dephosphorylation (procedure III; Fig. 2c). Sensitivity of the method was assessed using repetitive determinations in several dilutions of TRD–P. Concentrations of 1–1000 p*M* revealed a linear range of the assay between about 1–100 p*M* and 100–1000 p*M*, respectively (Fig. 3). As demonstrated by RP-HPLC, specific radiolabeling of the tyrosine phosphate-containing peptide was achieved. Moreover, a control experiment evaluating the kinetics of alkaline phosphatase treatment in this assay demonstrated a plateau of dephosphorylated peptide after 10 min (Fig. 4). Thus, dephosphorylation of cytosolic fractions with alkaline phosphatase was performed within 15 min.

3.2. Determination of tyrosine-phosphorylated proteins in a cytosolic fraction of human leukemic U937 cells

While in the previous section (Section 3.1) we demonstrated incorporation of radioactive iodine into synthetic peptides containing phosphotyrosine, we were also interested to identify tyrosine-phosphorylated proteins in a complex biological system using the differential iodination assay. Fig. 5 shows the chromatogram of cytosolic proteins from U937 cells separated by RP-HPLC. Fractions A, B and C used for the differential iodination assay and Western blots against phosphotyrosine are indicated by the

three arrows (Fig. 5). Radiomonitored chromatograms of these three U937 fractions A, B and C after non-radioactive iodination for 120 s, followed by Sep-Pak extraction and a second radio-iodination for 120 s (procedure II) are demonstrated as controls in Fig. 6a, b and c, respectively. The quantification of these control experiments revealed in fraction A, 0.5% incorporated and 99.5% free radiolabeled [^{125}I]iodine, in fraction B, 0.6% incorporated and 99.4% free radiolabeled [^{125}I]iodine and in fraction C, 0.5% incorporated and 99.5% free radiolabeled [^{125}I]iodine. Dephosphorylation of these samples

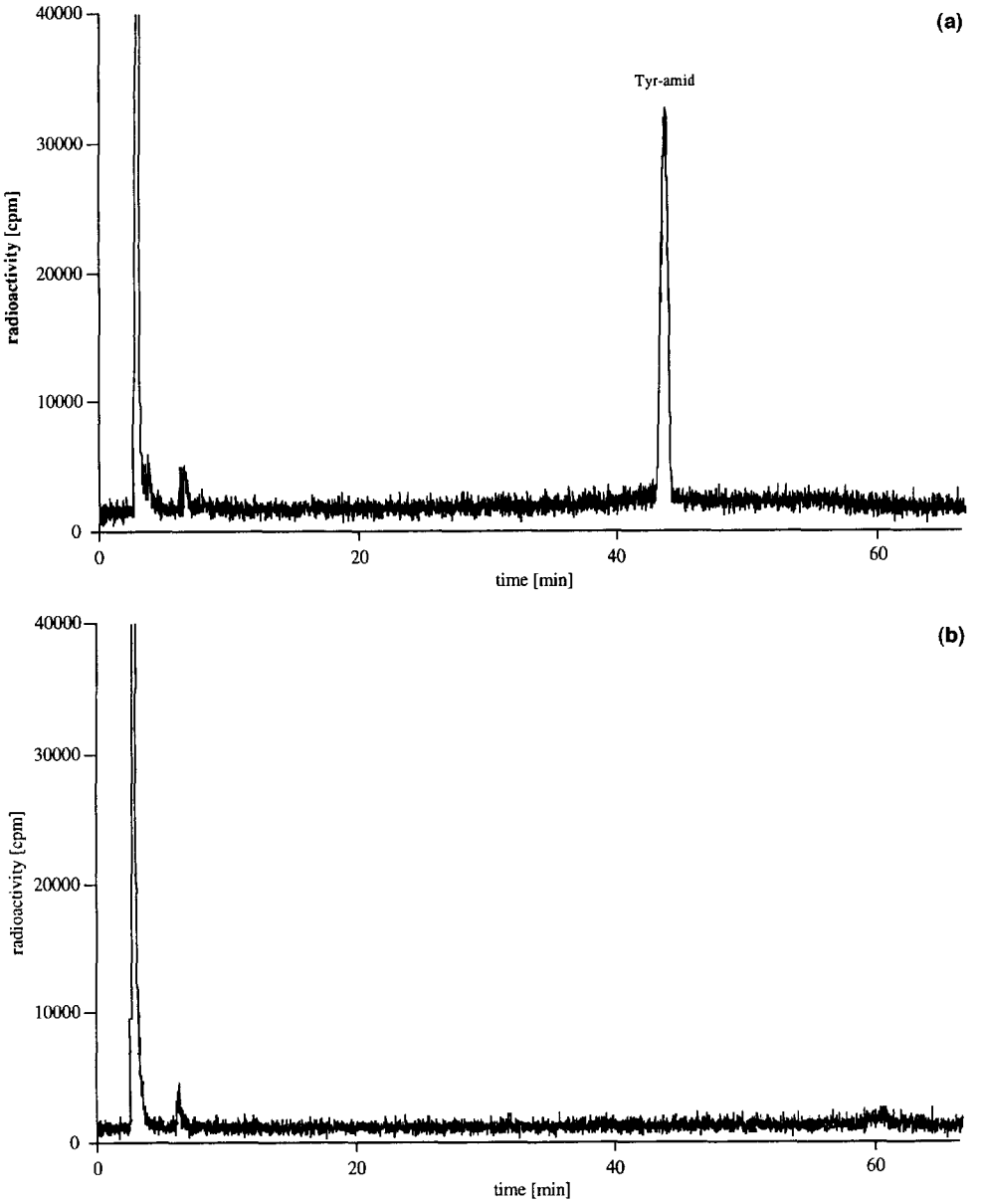

Fig. 2. RP-HPLC of a peptide mixture (Tyr–amid, TRD–P): (a) Direct radio-iodination of the mixture for 120 s. Peak: radio-iodinated Tyr–amid. (b) Radio-iodination for 120 s after iodination with non-radioactive iodine for 120 s. (c) Radio-iodination for 120 s after iodination with non-radioactive iodine for 120 s and dephosphorylation with alkaline phosphatase for 15 min. Peak: radio-iodinated TRD.

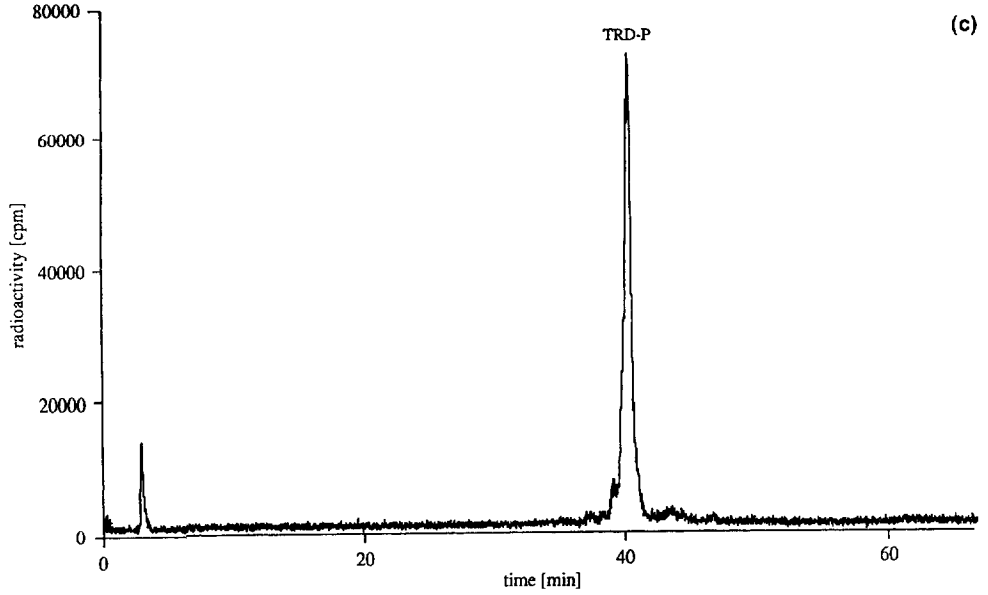

Fig. 2. (*continued*)

prior to radio-iodination (procedure III) resulted in fraction A in the appearance of one distinct major and one minor peak, in fraction B in one major double peak and at least 7 minor peaks with different intensities, and in fraction C in one major and two small peaks (Fig. 6a, b and c). The ratios of the radioiodination were 4.0% incorporated and 96.0% free radiolabeled [^{125}I]iodine in fraction A, while

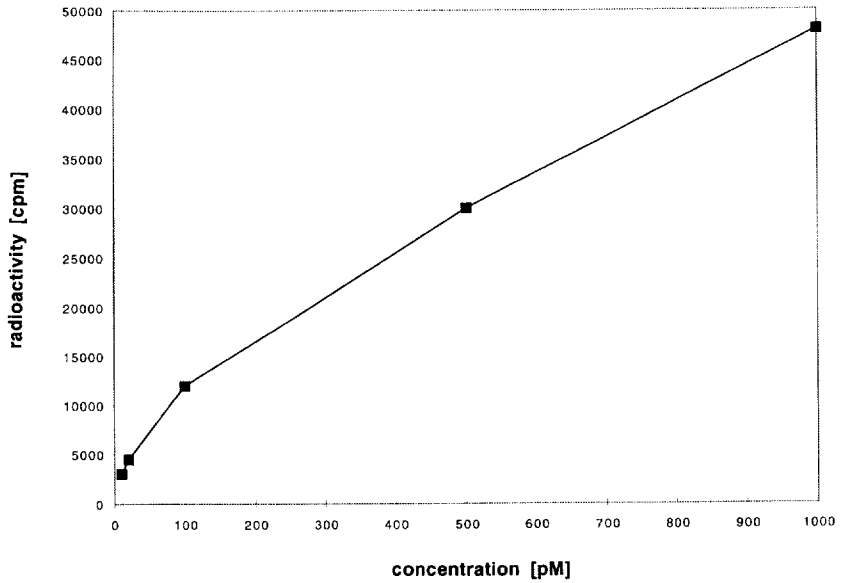

Fig. 3. Diagram of radioactivity of different concentrations of TRD–P after iodination with non-radioactive iodine, dephosphorylation and radio-iodination.

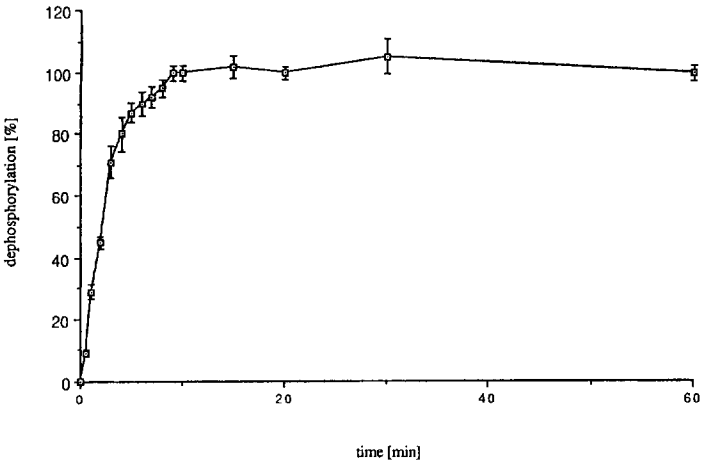

Fig. 4. Time course of dephosphorylation of 10 μg TRD–P by 1 μl alkaline phosphatase from calf intestine, EC 3.1.3.1 (1 U/μl) in dephosphorylation buffer. Data represent means±S.D. (n=4).

fraction B resulted in 11% incorporated and 89% free radiolabeled [^{125}I]iodine and 7% incorporated and 93% free radiolabeled [^{125}I]iodine in fraction C.

Following separation of the three radioiodinated fractions (A, B and C) in a 10% SDS–polyacrylamide gel and subsequent autoradiography, distinct labeled protein bands became visible (Fig. 7). Thus, fraction A revealed a major band at M_r≈ 75 000 and a diffuse high-molecular mass band. In fraction B a predominant protein band appeared at M_r≈75 000 probably overlayed by several distinct factors, and minor protein bands were detectable at M_r≈40 000 and ≈25 000. Fraction C demonstrated one distinct protein band at M_r≈75 000 (Fig. 7).

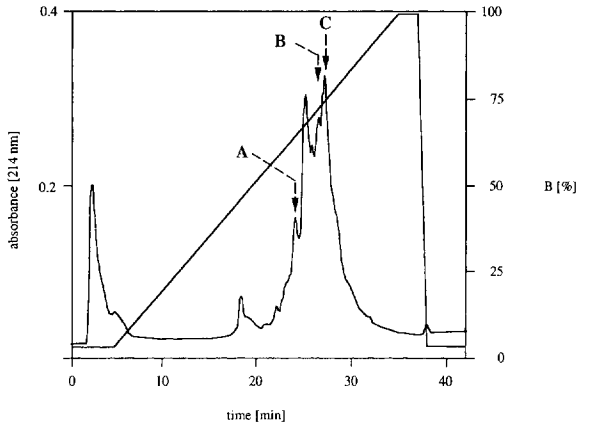

Fig. 5. Chromatogram of a RP-HPLC of cytosolic proteins from human U937 leukemia cells demonstrating the absorbance at 214 nm. The arrows labeled A, B, and C indicate the fractions used in the differential iodination assay and the anti-phosphotyrosine Western blot.

These data suggested, that the strong increase in radioactivity after dephosphorylation and the appearance of radiolabeled protein bands in the autoradiography represent tyrosine phosphate converted to tyrosine with subsequent iodination. Further support for this suggestion was obtained in a control experiment, where the separation of radioiodinated fractions A, B and C in a 10% SDS polyacrylamide gel without prior phosphatase treatment resulted in no detectable radiolabeled protein bands in either lane of the autoradiography (data not shown).

In order to evaluate these results by a standard procedure using a Western blot against phosphotyrosine, half of the two cytosolic fractions A, B and C as indicated by the arrows (Fig. 5) were separated in a 10% SDS–polyacrylamide gel, transferred to a PVDF membrane and visualized by an anti-phosphotyrosine antibody detection system (Fig. 8). All three lanes (A, B and C) demonstrated proteins recognized by the anti-phosphotyrosine antibody (Fig. 8) with a similar electrophoretic mobility when compared to the autoradiography of the same samples after the differential iodination assay and subsequent SDS–polyacrylamide gel separation (Fig. 7). However, all protein bands detected by the anti-phosphotyrosine antibody appeared less intense and

less sensitive compared to the autoradiography of the differential iodination assay (Figs. 7,8). In particular, a diffuse high-molecular-mass band in lane A of the autoradiography does not appear in the appropriate position of the Western blot (Figs. 7,8). Moreover, while a minor protein band of $M_r \approx 40\,000$ in lane B of the autoradiography parallels a similar weak band in lane B of the Western blot, another protein band of $M_r \approx 25\,000$ in the same lane is only detectable in the autoradiography but not in the Western blot (Figs. 7,8).

Taken together, these data indicate that several proteins of different cytosolic fractions from human U937 leukemia cells were distinguishable in the chromatograms of the differential iodination assay and a similar number of proteins were detectable by anti-phosphotyrosine Western blots.

4. Discussion

Specific determination of phosphotyrosines in peptides and proteins facilitates the analysis of important biological signal transduction mechanisms

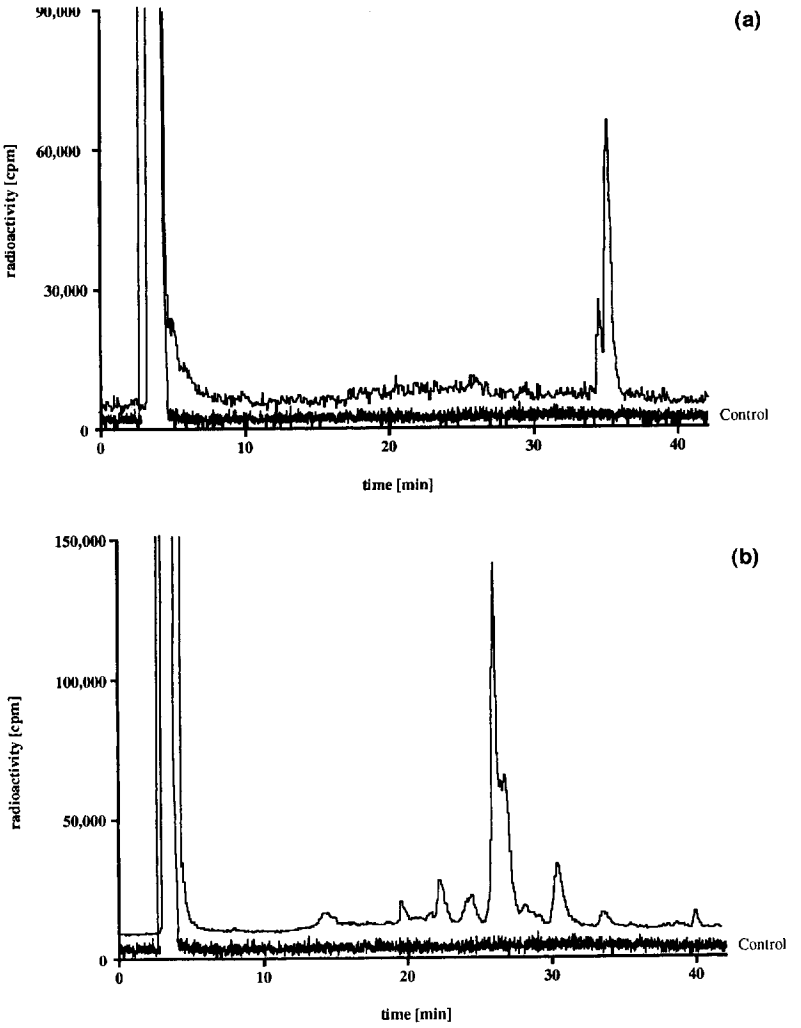

Fig. 6. RP-HPLC of the cytosolic U937 fractions A (a), B (b), and C (c) after radio-iodination for 120 s and iodination with non-radioactive iodine for 120 s (control) and after complete reaction sequence as detailed in Section 2.

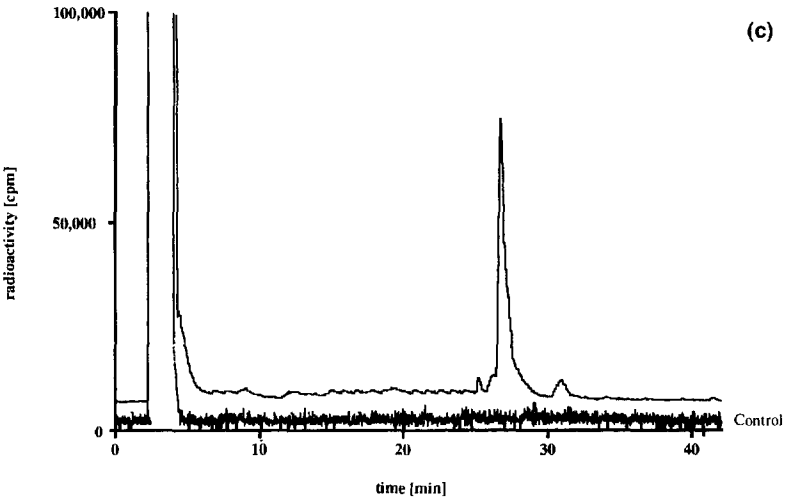

Fig. 6. (*continued*)

such as tyrosine kinases and their adaptor proteins. In order to understand the mechanism of the mitogenic or transforming tyrosine kinases the identification of tyrosine-phosphorylated substrate proteins and peptides is required.

Tyrosine-phosphorylated proteins are detected by autoradiography of gels using a traditional method of in vivo radiolabeling with [^{32}P]orthophosphate [12,13]. Here, serine and threonine residues also incorporate [^{32}P]orthophosphate resulting in high background levels. Since phosphotyrosine is more resistant to alkaline hydrolysis than phosphoserine or phosphothreonine, mild hydrolysis is performed prior to electrophoresis and autoradiography. However, this method is rather insensitive because not all phosphoserines or phosphothreonines are hydrolyzed and thus, the background radioactivity of the gel remains high. Moreover, in contrast to

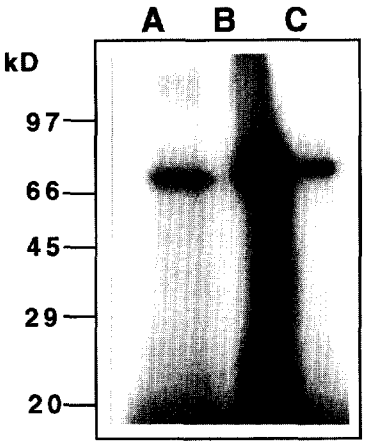

Fig. 7. Autoradiography of cytosolic fraction A (lane A), B (lane B), and C (lane C) after differential iodination assay and separation in a 10% SDS–polyacrylamide gel. Standards for molecular mass designations were: phosphorylase B (97 000), bovine serum albumine (66 000), egg albumin (45 000), carbonic anhydrase (29 000), trypsin inhibitor (20 000).

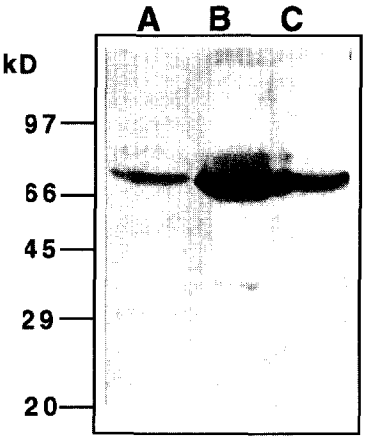

Fig. 8. Western blot analysis of cytosolic fraction A (lane A), B (lane B), and C (lane C) using a monoclonal anti-phosphotyrosine antibody and an alkaline phosphatase-conjugated rabbit anti-mouse IgG. Protein bands were visualized by the 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium substrates.

[^{32}P]orthophosphate labeling, the differential iodination method allows the quantitative determination of phosphotyrosines combined with the characteristic retention time of several distinct tyrosine-phosphorylated proteins and peptides. Phosphoserine or phosphothreonine are not labeled during differential iodination.

Another method for the detection of tyrosine-phosphorylated proteins and peptides represents the use of anti-phosphotyrosine antibodies. While phosphotyrosine antibodies have been shown to specifically react with tyrosine-phosphorylated proteins, they represent a useful tool to detect the phosphotyrosine content in known proteins or peptides [7]. However, a certain cross-reactivity with nucleotide monophosphates or phosphohistidine has been reported [7]. In this context, the identification of phosphotyrosine-containing proteins in crude protein mixtures using phosphotyrosine antibodies raises potential problems of cross-reactivities.

We therefore developed a new method for the detection of phosphotyrosine residues in peptides and proteins. As an alternative to phosphotyrosine antibodies, the differential iodination method provides a simple and specific way for mapping tyrosine phosphorylation sites in crude extracts of proteins and peptides, such as a cytosolic fraction. A certain limitation of this assay may arise from the possibility that in complex protein structures not all tyrosine residues will be dephosphorylated by alkaline phosphatase, however, the appearance of several iodinated peaks in the differential iodination assay corresponded to a similar number of protein bands detected by a phosphotyrosine antibody in a Western blot of the cytosolic fractions. Quantification of the peaks in the differential iodination assay may depend on different concentrations of the tyrosine-phosphorylated components or steric effects during iodination which may result from altered protein folding after dephosphorylation. Similarily, different intensities of the protein bands in the anti-phosphotyrosine Western blots may result from overlapping proteins, individual concentration of the factors, and/or different affinity of the antibody system to the phosphotyrosine containing proteins.

5. Conclusions

In conclusion, the differential iodination method is a fast and sensitive method for the identification and characterization of tyrosine-phosphorylated proteins and peptides. This method was successfully applied in complex biological systems, i.e. crude cytosolic cell extracts, enabling the specific determination of several distinct tyrosine-phosphorylated proteins. For this assay, less than 1/1000 of a cytosolic fraction from $3 \cdot 10^8$ cells was used.

In contrast to anti-phosphotyrosine Western blots, the differential iodination assay allows direct further analysis of samples with a variety of different methods such as chromatographic separation and subsequent quantification of fractions in a γ-counter or direct sequencing of the labeled protein peaks. In comparison to other methods, the differential iodination assay is also extremely sensitive with a detection limit of 1 pmol in a purified peptide assay. The differential iodination assay could significantly contribute to the investigation of signal transduction mechanisms involving tyrosine kinases and tyrosine phosphorylations in both, normal cells and their tumorigenic counterparts.

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